KIT Mutations in Australian Canine Mast Cell Tumours and Correlations with Patient Prognostic Factors

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Tamlin, V.S., Bottema, C. D. K., Peaston, A. E., Comparative aspects of mast cell neoplasia in animals and the role of KIT in prognosis and treatment. *Veterinary Medicine and Science.* [published online 24/10/2019]. doi: 10.1002/vms3.201.

Chapter 2 (Supplementary Material 2)

Tamlin, V. S., Dobson, E. C., Woolford, L. & Peaston, A. E., 2019, 'DNA purification increases PCR-amplifiable DNA extracted from formalin-fixed, paraffin-embedded canine mast cell tumors for routine KIT mutation detection', *Journal of Veterinary Diagnostic Investigation*, vol. 31, no. 5, pp. 756-760. doi: 10.1177/1040638719867743.

Abstract

Mast cell tumour (MCT) is the most common skin neoplasm in dogs. Accurately predicting MCT behaviour is essential for proper tumour management. Histological tumour grading is useful for canine prognosis and can be supplemented with the mutational evaluation of the *CD117 Proto-Oncogene Receptor Tyrosine Kinase* gene*, KIT*. Dogs with MCTs harbouring an internal tandem duplication (ITD) within exon 11 of the extracellular regulatory domain of *KIT* are more likely to respond to treatment with tyrosine kinase inhibitors. Conversely, MCTs with mutation of the intracellular tyrosine kinase domain are resistant to this class of drugs. *KIT* exon 11 ITDs are common in almost 50% of histologically high-grade cutaneous MCTs, whereas the prevalence of enzymatic pocket-type mutations was unknown. Therefore, the prevalence of canine MCT *KIT* gene mutations and their correlation with prognostic influencers were determined herein.

An exon 11 ITD mutation prevalence of 10% was determined in a cohort of 239 Australian dogs with cutaneous MCTs. An exon 11 ITD was detected in only one of 41 subcutaneous MCT. *KIT* mutation profiles were established using AmpliSeq™ Ion Torrent™ next-generation sequencing technology for 95 MCTs from 93 dogs. Non-synonymous *KIT* mutations and non-coding variants with a predicted gain-of-function effect on Kit protein activity were identified in 51.9% (n = 40/77) of cutaneous MCTs and 44.4% (n = 8/18) of subcutaneous MCTs. Enzymatic pocket-type mutations, predictably conferring tumour tyrosine kinase inhibitor resistance, were detected in 20.8% (n = 16/77) of the cutaneous MCTs.

A novel finding of this research is that mutation of the *KIT* enzymatic pocket domain statistically significantly predicts 12-month canine MCT-related death in multivariable analysis, independent of tumour histological grade. This finding may help identify potentially aggressive MCT cases which would have been otherwise overlooked by evaluation of histological grade alone. Conversely, exon 11 ITD status did not add any prognostically useful information in the multivariable analyses. However, the analyses revealed that Labrador dogs were at risk of developing high-grade MCTs at an old age (≥ 7 years).

In addition to dogs, over 30 other species of mammals, birds and reptiles have been documented with mast cell neoplasia. In a cohort of 20 domestic cats with cutaneous MCT, *KIT* mutations were detected in 60% of cases. *KIT*-mutant MCTs were not correlated with

tumour increased histological grade or mitotic index and hence, *KIT* mutation identification was not prognostically useful for cats with MCT. *KIT* mutations were discovered in the neoplastic DNA from two of four related cheetahs diagnosed with mast cell neoplasia. One of the cheetahs with abnormal *KIT* was euthanised as a result of visceral mastocytosis. The contribution of mutant-*KIT* to mast cell oncogenesis and disease malignancy is unclear in this case.

The implication of *KIT* in mast cell neoplasia in dogs, cats and other species is apparent. However, the mechanism of mutation and the contribution to mast cell pathogenesis and malignancy remains relatively obscure. Still, the findings herein will improve the use of *KIT* and genetic testing in canine MCT prognostication.

Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of Abbreviations

A, C, G, T	adenine, cytosine, guanine, thymine
AgNOR	argyrophilic nuclear organiser regions
AGRF	Australian Genome Research Facility
Akt	RAC-alpha serine/threonine-protein kinase
AML	acute myeloid leukaemia
ANKC	Australian National Kennel Council
ANOVA	analysis of variance
ATP	adenosine triphosphate
AU	Australia
BAM	binary sequence alignment map
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool (nucleotide database)
bp	base pair
CAHC	Companion Animal Health Centre, The University of Adelaide
cDNA	complementary DNA
CFA	*Canis lupus familiaris* chromosome
CI	confidence interval
CM	cutaneous mastocytosis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic Acid
Erk1/2	Extracellular signal-regulated kinases ½
ERV	endogenous retrovirus
EST	expressed sequencing tag
EU	Europe
FFPE	formalin-fixed, paraffin-embedded
Flt3	receptor-type tyrosine-protein kinase Flt3
FLT3	*Fms-Like Tyrosine Kinase 3*
GATK	Genome Alignment Tool Kit
gDNA	genomic DNA
GIST	gastrointestinal stromal tumour
GNAI2	*Guanine Nucleotide-Binding Protein G(I) Subunit Alpha-2*
GVCF	genomic variant call format
H&E	haematoxylin and eosin
HMC-1	human mast cell line possessing a Gly560Val *KIT* mutation
HPRT	*Hypoxanthine-Guanine Phosphoribosyltransferase*
HR	hazard ratio
IARC	International Agency for Research on Cancer
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin E
IGV	Integrative Genomics Viewer
Indel	insertion or deletion
ITD	internal tandem duplication
IUCN	International Union for Conservation of Nature
Jak	Tyrosine-protein kinase Jak
JM	juxtamembrane

KI	kinase insert
Kit	Mast/stem cell growth factor receptor Kit
KIT	*CD117 Proto-Oncogene Receptor Tyrosine Kinase*
LINE	long interspersed nuclear element
Mapk	Mitogen-activated protein kinase
MCT	mast cell tumour
MEGA	Molecular Evolutionary Genetics Analysis software
MEN 2	multiple endocrine neoplasia type 2
MFE	minimum free energy
mRNA	messenger RNA
MTC	medullary thyroid cancer
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
NSW	New South Wales
ORR	overall response rate
OS	overall survival
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGM	Personal Genome Machine
Pi3k	Phosphatidylinositol 3'-kinase
QLD	Queensland
Ras	GTPase Ras
Ret	Proto-oncogene tyrosine-protein kinase receptor Ret
RET	*RET Receptor Tyrosine Kinase Proto-Oncogene*
RNA	ribonucleic acid
SA	South Australia
SCF	stem cell factor
SD	standard deviation
SINE	short interspersed nuclear element
SM	systemic mastocytosis
SNP	single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
Stat3/Stat5	Signal transducer and activator of transcription 3/5
TAE	Tris Acetate EDTA
TET2	*Tet Methylcytosine Dioxygenase 2*
TKI	tyrosine kinase inhibitor
TK	tyrosine kinase
TM	transmembrane
TP53	*Cellular Tumour Antigen P53*
UCSC	University of California, Santa Cruz
USA	United States of America
UTR	untranslated region
VDL	veterinary diagnostic laboratory
VIC	Victoria
WA	Western Australia
WHO	World Health Organisation

Chapter 1: Review of the literature[1]

[1] This chapter has been published, in part, in *Veterinary Medicine and Science (Tamlin et al., 2019a)*. See Supplementary Material 1.

1.1 Mast cell function and malignancy

Mast cells are derived from pluripotent CD34+ haematopoietic progenitor cells and are normally found throughout the body in bone marrow, connective tissue, the skin, the gastrointestinal tract and the respiratory tract (Siebenhaar *et al.*, 2018). Mast cells are normal components in both innate and adaptive immune responses to bacterial and parasitic infections and they are notorious for the role they play in allergic reactions (Siebenhaar *et al.*, 2018). Allergic disorders such as atopic dermatitis, some food allergies, allergic rhinitis and allergic asthma are a consequence of hypersensitive mast cell reactions (Galli and Tsai, 2010, Oschatz *et al.*, 2011). IgE, secreted by plasma cells, has a high affinity for the FcεRI receptors located on mast cell surfaces (Galli and Tsai, 2012). IgE binding with FcεRI stabilises the receptor's expression, thereby fostering the development of many IgE-FcεRI aggregations on mast cell surfaces and sensitizing mast cell responses to specific antigens. Mast cells remain inactive until an antigen (e.g. an allergen) binds to the IgE antibodies coating the cell. This triggers mast cell degranulation, releasing various cytokines and chemokines into the blood, thereby facilitating host immune responses (Galli and Tsai, 2010, Galli and Tsai, 2012). Histamine and heparin release by mast cells increases vascular permeability and vasodilation, expediting the passage of leukocytes to tissues and having toxic effects against invading parasites (Oschatz *et al.*, 2011).

Mast cell neoplasia is characterised by abnormal mast cell proliferation and accumulation (Bodemer *et al.*, 2010, Siebenhaar *et al.*, 2018). Neoplastic mast cells masses can spontaneously degranulate, releasing biologically active molecules which can exert serious and potentially fatal effects elsewhere in the body. These are termed paraneoplastic disorders and include anaphylaxis, gastric and duodenal ulceration or perforation, glomerular disease and haemorrhage.

In dogs, mast cell tumours (MCTs) are common skin cancers and have extremely varied clinical appearance and biological behaviour. In other species, such as cats, MCTs are less common and have less variable biological behaviour. In humans, mast cell neoplasia, termed mastocytosis, often presents as a systemic disease rather than a defined mass and varies from benign disease in infants to aggressive malignancies in adults (Kiszewski *et al.*, 2004). Despite these distinct differences in behaviour and clinical manifestation, an underlying molecular abnormality shared by neoplastic mast cells in different species is mutation of the *CD117*

Proto-Oncogene Receptor Tyrosine Kinase gene*, KIT*.

1.2 The role of *KIT* in mast cell tumourigenesis

The *KIT* gene was first discovered in the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) (Besmer *et al.*, 1986). This acute transforming retrovirus causes fibrosarcomas in cats. The transforming activity of HZ4-FeSV is carried by the oncogene *V-KIT* which is thought to have arisen through truncation and transduction of feline *KIT* sequences in the feline leukaemia virus (Yarden *et al.*, 1987).

The *KIT* gene encodes Kit, a transmembrane tyrosine kinase receptor protein that is involved in the development, proliferation and function of mast cells, melanocytes, interstitial cells of Cajal and haematopoietic stem cells (Lennartsson *et al.*, 2005). Kit protein structure includes an extracellular region comprising five immunoglobulin-like domains, a transmembrane domain, and an intracellular region (**Figure 1.1**). The intracellular region includes the juxtamembrane domain (involved in autonomous inhibition of Kit activity) and two tyrosine kinase domains (ATP and phosphotransferase domains) separated by a kinase insert (Lennartsson *et al.*, 2005, Ma *et al.*, 1999). Kit protein activation by haematopoietic stem cell factor (SCF) causes Kit dimerization and subsequent phosphorylation, triggering distinct downstream signalling cascades within the cell inducing mast cell development, survival, proliferation, secretory function, and chemotaxis (**Figure 1.2**) (Lennartsson *et al.*, 2005, Letard *et al.*, 2008).

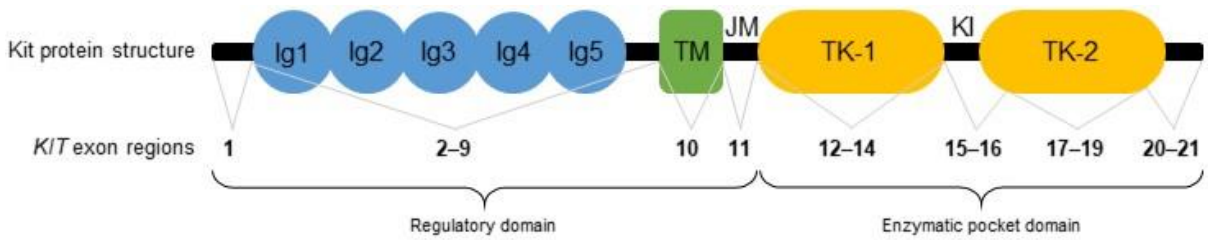

Figure 1.1. Structure of canine Kit protein and corresponding *KIT* gene exons. Noted is the regulatory domain comprised of the immunoglobulin-like domains (Ig1–5), transmembrane domain (TM) and juxtamembrane domain (JM), as well as the intracellular enzymatic pocket domain which comprises the tyrosine kinase domains (TK-1, TK-2) and the kinase insert (KI).

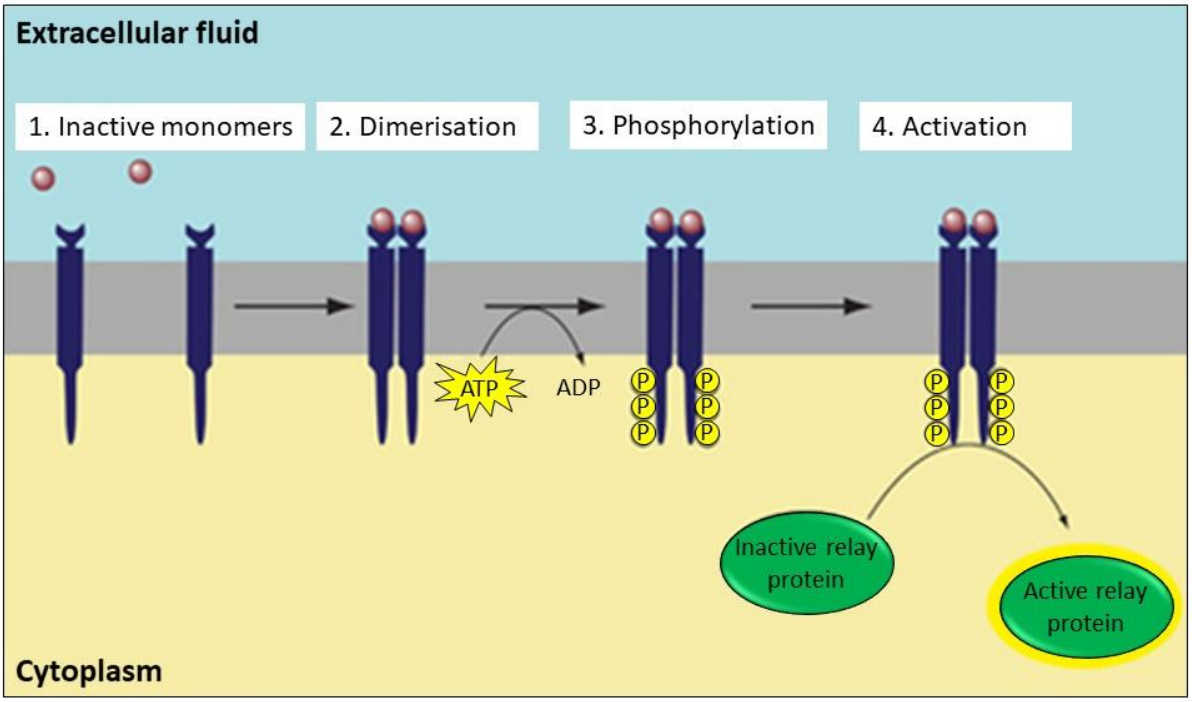

Figure 1.2. Kit tyrosine kinase protein dimerisation and internal substrate phosphorylation schematic. (1) Stem cell factor ligand (SCF) binds to the extracellular binding domain of each monomer. (2) Receptors dimerise and (3) transphosphorylate the neighbouring Kit monomer using ATP. (4) Phosphorylated Kit activates intracellular signalling proteins which trigger signalling cascades. Adapted from https://signaltransductionpathway.weebly.com/tyrosine-kinase.html.

Mutations within *KIT* are common in several human cancers including mastocytoma, gastrointestinal stromal tumours, melanoma and acute myeloid leukaemia (Longley *et al.*, 2001). Point mutations, initially discovered in HMC-1 cells derived from a human patient with mast cell leukaemia and then later in mouse and rat mastocytomas, suggested that mutations in *KIT* play a role in mast cell oncogenesis (Furitsu *et al.*, 1993, Tsujimura *et al.*, 1994, Tsujimura *et al.*, 1995). Comparison of the whole canine Kit protein sequence with the sequence from mice and humans shows 82% and 88% homology, respectively (London *et al.*, 1999, Ma *et al.*, 1999). High protein sequence conservation across species raised speculation about whether *KIT* mutations could be found also in MCTs of other species. Indeed, at least 51 unique gain-of-function mutations in the *KIT* gene have been identified in mast cell neoplasms of humans, cats and dogs (Downing *et al.*, 2002, Giantin *et al.*, 2012, Haenisch *et al.*, 2012, Hahn *et al.*, 2008, Isotani *et al.*, 2010, Kitayama *et al.*, 1995, Letard *et al.*, 2008, London *et al.*, 1999, Ma *et al.*, 1999, Marconato *et al.*, 2014, Nakano *et al.*, 2014, Riva *et al.*, 2005, Takeuchi *et al.*, 2013, Webster *et al.*, 2006b, Zemke *et al.*, 2002). Studies also document over 80 other nucleotide substitutions and frameshift mutations in neoplastic mast cells from these species, although

these have not been confirmed to be Kit activating. Whilst some identified nucleotide substitutions are silent or species-specific, many result in non-conservative amino acid changes and, hence, may contribute to mast cell malignancy (Giantin *et al.*, 2012, Haenisch *et al.*, 2012, Hahn *et al.*, 2008, Isotani *et al.*, 2006, Letard *et al.*, 2008, Marconato *et al.*, 2014, Nakano *et al.*, 2017, Sabattini *et al.*, 2017, Takeuchi *et al.*, 2013, Zemke *et al.*, 2002).

Gain-of-function mutations within the *KIT* gene disrupt normal Kit protein function, leading to constitutive Kit activation in the absence of ligand binding (Furitsu *et al.*, 1993, Letard *et al.*, 2008, Nakano *et al.*, 2017). These mutations can be classified into two groups according to their location within the gene: regulatory-type mutations or enzymatic pocket-type mutations (Longley *et al.*, 2001).

In humans, cats and dogs, Kit protein regulatory regions commonly affected by mutations include the extracellular ligand-binding fifth immunoglobulin-like domain, encoded by exons 8 and 9, and the juxtamembrane domain, encoded by exon 11, that regulates inhibition of Kit activation (Lennartsson *et al.*, 2005, Longley *et al.*, 2001). *KIT* mutations occurring in exons 13–21, encoding the intracellular kinase domains of the Kit protein, are termed enzymatic pocket-type mutations.

1.3 Tyrosine kinase inhibitors (TKIs)

Receptor tyrosine kinases, such as Kit, are candidates for molecular targeted therapy. Tyrosine kinase inhibitors (TKIs) are used in human and veterinary medicine to treat mast cell neoplasms harbouring *KIT* mutations. TKIs bind directly to the ATP-binding site in tyrosine kinase proteins, including Kit, blocking tyrosine kinase receptor autophosphorylation, preventing activation caused by regulatory-type mutations, and thereby preventing initiation of downstream signalling cascades (London, 2009). Consequently, neoplastic mast cell proliferation and tumour growth are inhibited.

The change in Kit protein structure resulting from mutations in the enzymatic pocket domain leads to the decreased affinity of TKIs to the binding site. Neoplastic mast cells harbouring this mutation type are resistant to most TKI therapeutics (Nakano *et al.*, 2017, Verstovsek *et al.*, 2006). Hence, the location of *KIT* mutations within the gene influences whether the tumour will be responsive or resistant to treatment with TKIs, and therefore, the mutation location is of prognostic importance.

1.4 Canine mast cell tumours

In dogs (*Canis lupus familiaris*), cutaneous MCTs have been reported to occur in 335 per 100,000 animals and account for 10–21% of all skin neoplasms (Leidinger *et al.*, 2014, Withrow *et al.*, 2013). Canine prognosis and treatment options are affected by patient signalment and clinical signs, and tumour anatomical location, growth rate, size, gross appearance (e.g. ulceration), metastasis, post-surgical recurrence, clinical stage and tumour histological grade (Blackwood *et al.*, 2012, Mullins *et al.*, 2006). The presence of multiple lesions should be considered in prognosis but does not necessarily indicate more aggressive disease. Unlike human patients, there is no unequivocal evidence of familial MCT inheritance in dogs, although boxers and other breeds of bulldog descent appear to be more predisposed to MCT development (Leidinger *et al.*, 2014, Mochizuki *et al.*, 2017a).

1.4.1 Canine MCT prognosis

Tumour histological grade is currently the single most powerful prognostic factor for dogs with cutaneous MCTs and has been described in depth (summarised in **Table 1.1**) (Blackwood *et al.*, 2012, Kiupel and Camus, 2019, Kiupel *et al.*, 2011, Patnaik *et al.*, 1984). The two established grading schemes, Patnaik and Kiupel, are not applicable to subcutaneous, visceral or mucosal MCTs or MCTs of other species due to differences in tumour biology and histological features.

Table 1.1. Canine mast cell tumour prognosis, incidence and *KIT* mutation prevalence according to the Patnaik and Kiupel histological grading schemes.

Histological grade	Histological grade frequency[a]	*KIT* mutation prevalence[ab]	Prognosis
Patnaik grade I	8–53%	0–8%	• Generally benign with a low chance of recurrence. • Surgery alone is often curative. • Predictably good long-term prognosis. • 12-month survival probability up to 100%.
Patnaik grade II	59–76%	6–35%	• Unpredictable biological behaviour. • Unclear treatment recommendations. • Discordance among pathologists when grading. • 12-month survival probability 87–92%.
Patnaik grade III	5–26%	33–71%	• Biologically aggressive with a high probability of local recurrence and metastasis. • Requires aggressive therapeutic management. • Poor long-term prognosis. • 12-month survival probability 16–46%.
Kiupel low-grade	59–89%	4–13%	• Good long-term prognosis. • Median survival times of more than 2 years. • 12-month survival probability 95%.
Kiupel high-grade	11–41%	14–52%	• Increased chance of metastasis or recurrence. • Poor long-term prognosis. • Median survival times of less than 4 months. • 12-month survival probability 24%.
References	(Giantin *et al.*, 2012, Kiupel *et al.*, 2011, Leidinger *et al.*, 2014, Mochizuki *et al.*, 2017a, Mochizuki *et al.*, 2017b, Murphy *et al.*, 2004, Patnaik *et al.*, 1984, Sabattini *et al.*, 2015, Tamlin *et al.*, 2017)	(Downing *et al.*, 2002, Giantin *et al.*, 2012, Mochizuki *et al.*, 2017b, Tamlin *et al.*, 2017, Webster *et al.*, 2006b, Zemke *et al.*, 2002)	(Kiupel *et al.*, 2011, Murphy *et al.*, 2004, Sabattini *et al.*, 2015)
ootnotes	a. Studies with less than 49 tumour samples were omitted from this Table as deemed too small to be an accurate representation of true grade incidence/mutation prevalence according to the sample size calculation equation described by Naing *et al.* (2006). A 95% level of confidence and precision of 10% was used in the calculations based on the prevalence determined by Webster *et al.* (2006b).		
	b. Prevalence includes mutations in *KIT* exons 8, 9 and 11.		

Clinical staging according to the World Health Organisation (WHO; **Appendix A**) clinical staging system for canine MCTs is regarded by some as an important prognostic indicator, with the lower clinical stages (stages 0 and I) having a better prognosis. However, the utility of stage III tumours, which includes cases of multiple masses, is compromised due to inconsistencies in the published literature regarding the associations between multiple masses and worse prognosis (Horta *et al.*, 2018b, Murphy *et al.*, 2006). In fact, it is well known that the tumours fitting the preceding stage (clinical stage II which includes cases of a single tumour with regional lymph node metastasis) are accompanied with short survival times of less than 1 year regardless of loco/loco-regional treatment, are generally of high histological grade and have a high risk for MCT progression and MCT-related death (Krick *et al.*, 2009, Marconato *et al.*, 2018). Further, in a retrospective study, the median survival time of eight dogs of with stage II disease was 431 days, whereas the median survival time for 50 dogs with stage III or stage I disease was not reached (Murphy *et al.*, 2006). It is thought, therefore, that this part of the staging system does not correlate with outcome. Amendments to the current WHO clinical staging criterion have been proposed (Horta *et al.*, 2018b).

Evaluation of cellular proliferation markers (AgNOR, Ki-67 and mitotic index) can be used to add prognostic value to histological grade by providing information about the speed of cell proliferation and subsequent inferred neoplastic biological behaviour of the tumour. Argyrophilic nuclear organiser regions (AgNORs) are regions of chromosomes that can be silver-stained and give an indirect measure of cellular proliferation (Derenzini and Trere, 1994). AgNOR count is often used in conjunction with the proliferation marker Ki-67. Ki-67 is a nuclear protein expressed in all active stages of the cell cycle (G_1, S, G_2 and mitosis), but is absent in cells at rest (G_0). The number of Ki-67 positive cells in a tumour sample is used to determine the proliferation index of the tumour (Abadie *et al.*, 1999). Together, AgNOR count and Ki-67 score assess tumour growth rate by measuring the number of cycling cells and the rate of cell cycle progression. Increased AgNOR histochemical staining and an increased Ki-67 score in canine MCT sections are statistically significantly associated with shorter disease-free intervals, higher tumour histological grade and increased likelihood of MCT-related mortality (Krick *et al.*, 2017, Scase *et al.*, 2006, Webster *et al.*, 2007, Webster *et al.*, 2008). Further, dogs with Patnaik grade II tumours and Ki-67 scores above 1.8 have substantially lower survival probabilities (Abadie *et al.*, 1999, Scase *et al.*, 2006). It has been suggested that Ki-67 score can be used to divide Patnaik grade II MCTs into two groups to help decrease the unpredictable, ambiguous behaviour of this class of tumour (Smith *et al.*, 2017).

As when calculating tumour's AgNOR count and Ki-67 score, tumour mitotic index should be determined by histologically assessing tumour areas with the highest proliferation activity (Kiupel and Camus, 2019). This can be challenging when evaluating highly granulated MCTs in haematoxylin and eosin (H&E)-stained sections and interobserver variation results in poor reproducibility. Furthermore, whilst a high mitotic index is indicative of increased proliferation and more malignant MCT phenotype, a low mitotic index is not an accurate determinant of benign behaviour, with 30% (n = 6/20) of Patnaik high-grade MCTs having a low mitotic index (≤ 5 mitotic figures per 10 high-power fields) (Kiupel *et al.*, 2011, van Lelyveld *et al.*, 2015). The Kiupel 2-tier grading schematic incorporates mitotic index into its diagnostic criteria but its sole use clearly cannot accurately determine prognosis.

Proliferating cell nuclear antigen (PCNA) has been also evaluated for its use in prognosis with conflicting outcomes between studies. PCNA is a DNA clamp which acts to increase the processing ability of DNA polymerase, ultimately increasing DNA synthesis and cellular division (Jaskulski *et al.*, 1988, Miyachi *et al.*, 1978). Previous studies found significant correlations between PCNA expression and patient survival times and tumour local recurrence and metastasis (Séguin *et al.*, 2006, Simoes *et al.*, 1994). More recent studies have found that PCNA is not significantly related to tumour behaviour, patient survival or histological grade (Kandefer-Gola *et al.*, 2015, Scase *et al.*, 2006, Webster *et al.*, 2007). PCNA gives an indication of how many cells are replicating but does not disclose the rate of proliferation. PCNA has been also found to be involved in DNA repair, and so, can be detected in post-mitotic cells (Shivji *et al.*, 1992). PCNA evaluation was not statistically significant in predicting local or distant tumour recurrence or MCT-related mortality in multivariable analyses and, therefore, is not reliable as an independent prognostic marker for MCT malignancy (Scase *et al.*, 2006, Webster *et al.*, 2007).

Immunohistochemical assessment of the Kit protein staining pattern is one other molecular marker often evaluated and used in canine MCT prognosis (Thamm *et al.*, 2019b). Kit protein normally resides within the peri-membranous region of the cell, and aberrant cytoplasmic Kit localisation may be correlated with malignant MCT phenotype. The majority of the literature demonstrates correlations between abnormal cytoplasmic Kit localisation and increased cellular proliferation (as measured by AgNOR count and Ki-67 score), increased rates of local recurrence and MCT-related mortality and high tumour histological grade (Kiupel *et al.*, 2004, Webster *et al.*, 2006b, Webster *et al.*, 2007). However, recent research reveals that

Kit protein staining pattern is not a significant predictor of overall canine survival in multivariable models, and thus, should not be used as an independent prognosticator (Horta *et al.*, 2018b, Thamm *et al.*, 2019b).

1.4.2 Canine KIT mutations

Between 8% and 29% of canine MCTs carry a regulatory-type mutation in exon 8, 9 or 11 (Giantin *et al.*, 2012, Hahn *et al.*, 2008, Horta *et al.*, 2018b, Letard *et al.*, 2008, Marconato *et al.*, 2014, Mochizuki *et al.*, 2017b). Exon 11 internal tandem duplications (ITDs) comprise 60–74% of these mutations and are prevalent in 18% of tumours (Giantin *et al.*, 2012, Hahn *et al.*, 2008, Letard *et al.*, 2008, Marconato *et al.*, 2014, Mochizuki *et al.*, 2017b, Webster *et al.*, 2006b). *KIT* mutation frequency increases with increasing tumour histological grade and exon 11 ITDs are associated with decreased survival times and the increased chance of tumour recurrence and metastasis (**Table 1.1**) (Downing *et al.*, 2002, Horta *et al.*, 2018a, Letard *et al.*, 2008, Tamlin *et al.*, 2017). Although less frequent, ITDs in exon 8 as well as insertions, deletions and nucleotide substitutions in exons 8, 9, 11, 14 and 17 are also documented (Giantin *et al.*, 2012, Hahn *et al.*, 2008, Letard *et al.*, 2008, Ma *et al.*, 1999, Marconato *et al.*, 2014, Mochizuki *et al.*, 2017b, Nakano *et al.*, 2017, Nakano *et al.*, 2014, Riva *et al.*, 2005, Takeuchi *et al.*, 2013, Zemke *et al.*, 2002). To date, all investigated missense point-mutations in exons 8, 9, 11, 14 and 17 have demonstrated constitutive Kit protein phosphorylation in the absence of normal ligand binding (Furitsu *et al.*, 1993, Kitayama *et al.*, 1995, Letard *et al.*, 2008, Nakano *et al.*, 2017).

In one study, the homozygous deletion of the entire intron 11 of canine *KIT* was detected in 49% of MCT samples compared to 13% in control, non-cancerous tissue (Reguera *et al.*, 2002). The intron 11 deletion was correlated with higher grade, more aggressive MCTs. However, this large deletion has not been reported in any other molecular studies evaluating genomic DNA. The veracity of this large deletion is questioned.

1.4.3 Other genetic mechanisms implicated in canine mast cell malignancy

Some genes known to be mutated in human mast cell diseases have been evaluated in MCTs from dogs (Zorzan *et al.*, 2015). Mutations in the *TET2* gene are present in up to 27% of all human systemic mastocytosis patients but only 2.7% of canine MCTs harbour *TET2* genetic aberrations (Damaj *et al.*, 2014, Zorzan *et al.*, 2015). No other genes known to be frequently mutated in human systemic mastocytosis cases have been shown to be mutated in canine

MCT samples (Zorzan *et al.*, 2015).

Missense mutations in exons 5–8 of the *TP53* tumour suppressor gene are detected in 7–15% of canine cutaneous MCTs (Vozdova *et al.*, 2019b, Vozdova *et al.*, 2019c). Two non-synonymous *TP53* mutations resulting in a predicted amino acid change (Ser203Arg and Ser229Phe) are analogous to known human *TP53* mutations (Ser215Arg and Ser241Phe). These variants are pathogenically associated with various human cancers including lung adenocarcinoma, breast cancer, ovarian cancer, pancreatic adenocarcinoma and squamous cell carcinoma (IARC *TP53* Database, version R20, July 2019) (Bouaoun *et al.*, 2016). In one study, *TP53* mutations were statistically associated with a high histopathological grade and short survival times in 27 dogs with cutaneous MCT (Vozdova *et al.*, 2019b). However, a different study including more animals (n = 41) by the same research group found no statistically significant associations between *TP53* mutation status and prognosis, despite both studies following the dogs for a median time of ≥ 13 months post-surgery (Vozdova *et al.*, 2019c). More research is warranted regarding the role of *TP53* in the development and pathogenicity of canine cutaneous MCTs.

Single nucleotide substitutions in the *GNAI2* gene and in various hyaluronidase genes on CFA14 and CFA20 are significantly associated with increased risk of MCT development in golden retriever dogs (Arendt *et al.*, 2015). Furthermore, the mean age of disease onset in dogs homozygous for the SNP in the *GNAI2* gene was significantly lower than that for heterozygotes (5.6 ± 0.4 years versus 7.6 ± 0.5 years, respectively), suggesting that this gene contributes to MCT pathogenesis. The *GNAI2* and hyaluronidase genes are normally involved in cellular signalling and cancer metastasis, respectively. Gain-of-function mutations are associated with various types of human cancers but the exact involvement of these genes in canine cancers, and more specifically in canine MCT pathogenesis, has not been investigated.

Comparative transcriptomic and proteomic analysis studies between low-grade MCTs with good patient prognosis and high-grade tumours with poor patient prognosis has identified a variety of differentially expressed genes and proteins potentially involved in mast cell aetiology and pathogenesis in dogs (Giantin *et al.*, 2016, Schlieben *et al.*, 2012). The roles of these various genes and proteins have not been further evaluated.

Global DNA methylation is a known heritable epigenetic modulator of gene expression. Hypomethylation of proto-oncogenes up-regulates gene expression, potentially favouring

carcinogenesis and a more aggressive tumour type. DNA hypomethylation predominates in poorly-differentiated, high-grade canine MCTs and may represent a novel target for epigenetic therapy (Morimoto *et al.*, 2017).

Chromosomal fragile site expression has been analysed in humans and linked to the predisposition of people to certain types of cancers (Ozeri-Galai *et al.*, 2012). Chromosomal fragile sites are heritable regions on the chromosome that are prone to breakage and consequent abnormal cell cycling. Chromosomal fragile sites on CFA3, CFA4 and CFA15 are identified from Boxers with MCTs but not observed in normal control Boxer mast cells (Stone *et al.*, 1991). When induced with aphidicolin, the number of cells from Dobermans, a breed not at risk of MCT development, expressing chromosomal fragile sites is significantly reduced when compared to Boxer dogs with or without MCT, 25% versus 67% or 72%, suggesting these sites might play a key role in canine MCT development. Unfortunately, this has not been explored further and the exact molecular events that contribute to the development of mutated mast cell cancers, in both dogs and humans, are unidentified.

1.4.4 Treatment of canine MCTs

Clinical treatment of MCTs is based on tumour size, location, histological grade and evidence of metastasis (Blackwood *et al.*, 2012). Small low-grade tumours are amenable to surgical excision from most anatomical sites without the need for further treatment and exhibit a low probability of recurrence or metastasis, regardless of histologically tumour-free margins (Donnelly *et al.*, 2015, Mullins *et al.*, 2006, Séguin *et al.*, 2006). Patnaik grade II tumours are locally non-recurrent in a significant proportion (77%) of cases following marginal excision (Séguin *et al.*, 2006, Weisse *et al.*, 2002). However, even with wide margins, there is a significant risk of recurrence and metastasis of Patnaik grade III or Kiupel high-grade tumours following surgical excision, and additional therapy is advised for these patients (Donnelly *et al.*, 2015). Common MCT treatment recommendations and conventional therapies have been outlined elsewhere (Blackwood *et al.*, 2012, Ilyinskaya *et al.*, 2018). The use of tyrosine kinase inhibitors (TKIs) is the subject of ongoing research.

Toceranib, the most commonly used TKI in veterinary medicine, is licensed to treat non-resectable or recurrent grade II and III canine cutaneous MCTs in the USA (U.S. Food and Drug Administration, 2009), EU (European Medicines Agency, 2017) and Australia (Agricultural and Veterinary Chemicals, 2011). Toceranib was originally developed as an antiangiogenic

agent but was later found to possess potent anti-tumour characteristics by inhibiting Kit autophosphorylation and mast cell proliferation (Halsey *et al.*, 2014). Early studies reported the successful use of toceranib in treatment of recurrent MCTs which failed standard therapies, with tumours more likely to respond if harbouring a *KIT* exon 11 ITD (London *et al.*, 2003, London *et al.*, 2009). Canine overall survival time, time to tumour progression and the duration of tumour response to toceranib were not influenced by tumour ITD mutation status (London *et al.*, 2003, London *et al.*, 2009). Further, in these studies, not all animals possessing an ITD responded to treatment. It is possible that unresponsive animals had MCTs which acquired a secondary mutation in the enzymatic domain of *KIT*, inducing tumour TKI resistance (Halsey *et al.*, 2014, Nakano *et al.*, 2017). In a more recent study, an objective response rate (ORR: complete response or partial response) of 46% to toceranib therapy was observed (Weishaar *et al.*, 2018), mimicking earlier observations (ORR = 37–50%) (London *et al.*, 2003, London *et al.*, 2009). The difference in ORR between dogs with ITD-mutant and non-ITD mutant MCTs was not statistically analysed in this study, although, overall canine survival was not influenced by tumour ITD status (Weishaar *et al.*, 2018). One study which did measure the response to tyrosine kinase inhibitors (masitinib or toceranib) taking into account tumour ITD mutation status found no statistically significant differences in measurable response to treatment or overall survival between 6 dogs with and 18 dogs without an ITD-mutant tumour (measurable response = 67% versus 44%, respectively, overall survival data not reported) (Horta *et al.*, 2018a). However, dogs were only followed for 2 weeks in this study and the number of dogs was restricted. In the studies by London and colleagues, progression-free survival times were significantly increased in dogs with non-mutant MCTs compared to dogs with ITD-mutant MCTs, however, this did not retain significance in a multivariable model (London *et al.*, 2003, London *et al.*, 2009). In the study by Weishaar and colleagues, a higher proportion of dogs with MCTs responded to treatment with toceranib than to treatment with vinblastine (46% versus 30%, respectively), but this difference was not statistically significant (Weishaar *et al.*, 2018). Given the greater expense and potentially more frequent and severe adverse events accompanying toceranib therapy, vinblastine is likely to remain a primary therapeutic option, regardless of tumour mutation status, with toceranib as a rescue therapy.

The TKI masitinib mesylate (Kinavet [USA], Masivet [EU]) is licensed for conditional use to treat non-resectable grade II and III cutaneous canine MCTs in the USA (Bataller, 2014) and may also be used in the EU (European Medicines Agency, 2013) provided the tumour is confirmed to harbour mutant *KIT*. It is not licensed for use in Australia. Masitinib significantly

improves overall survival of dogs with recurrent or non-resectable grade II or III MCTs harbouring a regulatory-type *KIT* mutation compared with results for placebo-treated dogs (Hahn *et al.*, 2010, Hahn *et al.*, 2008). Time to tumour progression is also increased in masitinib-treated dogs compared to placebo-treated animals, regardless of tumour mutation status (Hahn *et al.*, 2010, Hahn *et al.*, 2008). Tumour response to masitinib treatment was more pronounced in dogs with no prior chemotherapeutic treatment, suggesting that chemotherapy may select for growth of TKI-resistant neoplastic cells, limiting the effectiveness of masitinib treatment (Hahn *et al.*, 2008). Complete tumour response, partial tumour response or stable disease at six months after masitinib treatment initiation has a high predictive value for 12- and 24-month survival, whereas tumour response at six weeks does not provide predictive value (Hahn *et al.*, 2010). As the majority of studies evaluated patient response to TKIs for less than six months, the results must be interpreted carefully when estimating long-term health benefits for dogs receiving TKI therapeutics.

TKI-resistant tumours harbouring enzymatic-pocket type mutations may still be susceptible to combination therapy involving the use of TKIs with conventional chemotherapeutic agents. A phase I/II study evaluating combination therapy of toceranib with lomustine in 41 dogs determined the maximally tolerated dose of the drugs in a pulse delivery setting, and concluded that the combined therapy was well tolerated and had value in the treatment of high-grade unresectable or metastatic MCTs (Burton *et al.*, 2015). Tumour *KIT* mutation status did not influence response to treatment (Burton *et al.*, 2015). Complete response for more than 1 year was observed in two of ten dogs in a study using the same drug combination with a different administration schedule that was not well-tolerated by the dogs (Bavcar *et al.*, 2017).

In addition, toceranib in combination with vinblastine shows promise for dogs with grade II or III MCTs, with or without prior surgery whereby overall initial response rates (complete response and partial response) of 90% were observed (Olsen *et al.*, 2018). This combination chemotherapy is effective as a pre-operative treatment for local disease control and for facilitating surgery from difficult anatomical sites and demonstrates efficacy in dogs with gross metastatic spread (Olsen *et al.*, 2018).

Recently, TKI-independent means of canine MCT therapy have been investigated for use against MCTs resistant to TKIs and conventional chemotherapeutic agents. A pilot study investigating the use of Oncolytic Sendai Virus therapy against six cases of canine MCT cured

four dogs with cutaneous tumours and one of two dogs with subcutaneous tumour with no signs of relapse during the time of observation (1.5 to 3 years) (Ilyinskaya *et al.*, 2018). An *in vitro* study investigating the use of anthraquinone and an anthracene derivative on canine Kit expression found that these compounds decrease *KIT* mRNA and Kit protein expression by targeting DNA G-quadruplex regions on the *KIT* gene promoter (Zorzan *et al.*, 2018). A study targeting Kit with humanised anti-Kit monoclonal antibody demonstrates clinical benefit (partial response or stable disease) for mast cell tumour-bearing dogs, regardless of *KIT* mutation status (London *et al.*, 2017). These novel therapies may provide relief to canine patients with MCTs resistant to TKIs and conventional chemotherapeutic agents.

1.5 Feline mast cell tumours

The clinical manifestations of mast cell neoplasia in domestic cats (*Felis catus*) include visceral (splenic, intestinal) disease as well as cutaneous MCT. Siamese, Burmese, Russian Blue and Ragdoll cats are over-represented in some studies and hence, MCT development is suspected to be genetically inherited in these breeds (Blackwood, 2015, Melville *et al.*, 2015). Cutaneous MCTs are the second most common type of skin cancer in the cat, representing up to 21% of all cutaneous feline neoplasms in the USA (Miller *et al.*, 1991, Withrow *et al.*, 2013).

Clinical understanding of feline MCT biological behaviour is relatively poor and most prognostic markers have a relatively weak correlation with survival. Mitotic index is probably the strongest prognostic indicator for cats with cutaneous MCT, with high index associated with a worse clinical outcome although there is considerable variability (Blackwood, 2015, Sabattini *et al.*, 2013). Ki-67 score and staining of aberrant cytoplasmic Kit protein localisation are correlated with mitotic index but add no supplementary prognostic value to that achieved by mitotic index evaluation alone (Dobromylskyj *et al.*, 2015, Rodriguez-Carino *et al.*, 2009, Sabattini and Bettini, 2010). The Patnaik and Kiupel histologic grading systems provide no prognostically useful information for feline cutaneous MCTs. Instead, tumours have been histologically classified based on cellular and nuclear morphology as either atypical or mastocytic type tumours (Blackwood, 2015, Sabattini and Bettini, 2010). Mastocytic tumours can be further divided into well-differentiated or poorly-differentiated tumours, both sub-types of which can present with pleomorphic cells (Blackwood, 2015, Johnson *et al.*, 2002). However, there are no official guidelines for feline cutaneous MCT histological classification and, hence, there are inconsistencies in the definitions of histological sub-types between

published reports. These discrepancies in histological classifications and correlations with prognosis have been described previously (Blackwood, 2015). To overcome this, a recent study suggests a 2-tier histological classification of feline cutaneous MCTs (Sabattini and Bettini, 2019). High-grade tumours are categorised by the presence of > 5 mitotic figures per 10 high-power fields and at least two of the following three criteria: tumour diameter > 1.5 cm, irregular nuclear shape, and nucleolar prominence/chromatin clusters. According to this grading scheme, cats with high-grade tumours had a significantly shorter median overall survival (349 days) compared to cats with low-grade tumours (not reached, $P < 0.001$).

MCTs are the most common cause of splenic disease in cats. Affected cats are usually older than 10 or 11 years and there is no sex predilection (Evans *et al.*, 2018, Sabattini *et al.*, 2017). The disease frequently involves multiple other viscera and bone marrow. There is no consensus on which factors significantly influence prognosis (Evans *et al.*, 2018).

MCTs of the gastrointestinal tract are rare, but rank as the third most common intestinal tumour in cats, following lymphoma and adenocarcinoma, and have been previously viewed as an aggressive form of feline MCT disease (Barrett *et al.*, 2018). Intestinal MCT affects middle-aged to older cats, but no other predisposing factors are reported (Barrett *et al.*, 2018, Rissetto *et al.*, 2011, Sabattini *et al.*, 2016). Cats with poorly-differentiated intestinal MCTs, fitting a description of feline intestinal sclerosing MCT, survive 2–30 days compared with 28–538 days for cats with well- or moderately-differentiated tumours (Sabattini *et al.*, 2016). Feline intestinal sclerosing MCT is seldom reported, but the short survival for cats with this variant is agreed, although there is disagreement as to whether the tumours are characterised by a low or high mitotic index (Halsey *et al.*, 2010, Sabattini *et al.*, 2016). Intestinal sclerosing MCTs reportedly exhibit different histological appearance to gastrointestinal MCTs, and histological guidelines for diagnosis are yet to be developed (Halsey *et al.*, 2010, Sabattini *et al.*, 2016).

1.5.1 Feline KIT mutations

In cats, 56–68% of cutaneous and splenic MCTs harbour *KIT* mutations primarily in exons 8 and 9, but mutations in exons 6 and 11 also exist (Isotani *et al.*, 2006, Isotani *et al.*, 2010, Sabattini *et al.*, 2017, Sabattini *et al.*, 2013). ITDs in exon 8 are the most prevalent mutation type and are Kit protein activating (Hadzijusufovic *et al.*, 2009, Isotani *et al.*, 2010, Sabattini *et al.*, 2017). Cases of tumours with two simultaneous *KIT* mutations have been

recorded but significant correlations between mutation status and prognosis have not been documented (Isotani *et al.*, 2010, Sabattini *et al.*, 2017, Sabattini *et al.*, 2013). Gain-of-function mutations in feline intestinal MCTs have not been reported (Sabattini *et al.*, 2016).

1.5.2 Treatment of feline MCT

Treatment of feline MCTs has been reviewed recently (Blackwood, 2015, Blackwood *et al.*, 2012). Surgical excision is the standard of care for cutaneous atypical MCTs, which are generally thought to be benign (Blackwood, 2015, Litster and Sorenmo, 2006). Spontaneous tumour regression has been reported in younger cats, reminiscent of paediatric mastocytosis in humans (Chastain *et al.*, 1988, Wilcock *et al.*, 1986). However, over the last three decades, there have been no additional published cases and, hence, the existence of the spontaneously regressing variant in cats is uncertain. Since tumour recurrence and metastasis were not associated with incomplete excision, one group proposed wide surgical margins are not necessary (Litster and Sorenmo, 2006). Cats with pleomorphic mastocytic MCTs are at risk of an unfavourable outcome, hence, postoperative adjuvant radiation therapy and/or chemotherapy has been recommended for these cats (Blackwood, 2015, Sabattini and Bettini, 2010). Chemotherapeutic treatment with lomustine is well tolerated in cats and antitumor activity has been demonstrated (Rassnick *et al.*, 2008).

Splenectomy is the standard therapy for cats with splenic mastocytosis and provides longer disease-free survival than chemotherapy alone (856 days versus 342 days, respectively) (Evans *et al.*, 2018). Cats with intestinal MCTs historically carry a guarded prognosis and metastasis to mesenteric lymph nodes is common (Barrett *et al.*, 2018, Morrice *et al.*, 2019, Sabattini *et al.*, 2016). However, two recent case series reports suggest the disease may pursue an indolent course as demonstrated by the median survival time of 31 cats to be 531 days (95% CI 334–982 days) in one study (Barrett *et al.*, 2018), and 35% of cats (n = 6/17) surviving more than 1 year post-diagnosis in a different study which included a case where no treatment or surgery was received (Sabattini *et al.*, 2016).

Data using TKIs for treatment of MCTs in cats are scant, and prospective studies are lacking. An objective response was recorded in a cat with systemic and cutaneous MCT after treatment with imatinib mesylate (Gleevec) (Isotani *et al.*, 2006). A follow-up investigation demonstrated an objective response in five of eight cats with *KIT* mutated MCTs and in one of two cats without *KIT* mutations (Isotani *et al.*, 2010). Low animal numbers, previous

therapeutic drug administration and short follow-up times limit the interpretation of this study. Retrospectively, toceranib has been assessed as well tolerated by cats. Complete or partial responses were documented in 35 of 50 cats treated for cutaneous, visceral and gastrointestinal MCTs with toceranib alone or in combination with corticosteroids (Berger *et al.*, 2018). Other TKIs, including midostaurin, nilotinib and dasatinib, show dose-dependent growth inhibitory effects on exon 8 ITD *KIT*-mutant neoplastic feline mast cells *in vitro* (Hadzijusufovic *et al.*, 2009). Mutations in the enzymatic pocket domain of *KIT* have not been documented in feline MCTs making TKIs an attractive therapeutic option (Isotani *et al.*, 2010, Sabattini *et al.*, 2017, Sabattini *et al.*, 2016).

1.6 Mast cell neoplasia in other animals

Mast cell cancer is well documented in humans, cats and dogs but is also a frequent skin cancer in the ferret and has been documented in various other species including domesticated ungulates, non-human primates, birds and reptiles (**Table 1.2**). As with cats, the histological grading schemes that are useful for canine cutaneous MCTs cannot be applied to MCTs of other animals due to interspecies differences in clinical and histological disease manifestation.

1.6.1 Ferret (Mustela putorius furo)

MCTs represent between 16% and 44% of all cutaneous and subcutaneous neoplasms in ferrets (Avallone *et al.*, 2016, Miwa *et al.*, 2009). The tumours are typically benign and neither local recurrence after surgical excision nor metastatic disease has been reported (Vilalta *et al.*, 2016). Histologically, most ferret MCTs consist of well-differentiated mast cells where mitotic figures are rare and, similar to cats, few eosinophils are present (Vilalta *et al.*, 2016). In one study of 15 tumours from 10 ferrets, neither H&E nor Toluidine Blue staining detected mast cell granules in histologic sections (Vilalta *et al.*, 2016). However, in cytologic preparations of 12 tumours stained with Toluidine Blue or Wright's-Giemsa stains, metachromatic granules were visualised in all cases, but not in any case stained with Modified Wright's stain (Vilalta *et al.*, 2016). The discrepancy in Toluidine Blue staining between cytology and histology in ferret MCTs is confusing and could lead to misdiagnosis. In the same study, all tumours had either cytoplasmic or membrane immunostaining with Kit (Vilalta *et al.*, 2016). As would be expected from the lack of reported tumour recurrence or metastasis, there was no relationship between Kit immunostaining and prognosis (Vilalta *et al.*, 2016).

1.6.2 Horse (Equus ferus caballus)

MCTs comprise 3.4% of all cutaneous equine tumours, at least in the Pacific Northwest of the USA (Valentine, 2006). Equine MCTs appear most frequently on the head as a single nodule, although, multiple lesions are not uncommon and are indicative of malignant disease (Ressel *et al.*, 2015). Arabian horses appear to be more at risk compared to other breeds but no sex predilection has been described (Mair and Krudewig, 2008, Ressel *et al.*, 2015). Similar to MCTs in ferrets, equine MCTs follow a benign disease course and appear histologically well-differentiated with a low mitotic rate. Aberrant cytoplasmic Kit staining is detected by immunohistochemistry in 15% of cases but is not correlated to malignant disease or worse prognosis, albeit numbers were low in this study (n = 9) (Ressel *et al.*, 2015). Metastatic MCT behaviour and MCT-related death in horses is seldom documented and the necessity for treatment is questionable, although some owners opt for therapy for the cosmetic appearance of the horse. Surgery alone is curative in the majority of cases and other therapies including surgical laser ablation and intralesional injection with methylprednisone acetate appear to be effective, non-invasive treatment options (Mair and Krudewig, 2008).

1.6.3 Cow (Bos taurus)

In cattle, MCTs comprise less than 1% of all bovine neoplasms (Hill *et al.*, 1991). Reports range from multiple MCTs randomly distributed over the entire body with or without organ involvement to single visceral lesions (Hill *et al.*, 1991, Khodakaram-Tafti *et al.*, 2015, Perez *et al.*, 1999). Published data predominantly describe MCTs affecting Holstein cattle, however, statistical evidence of breed predisposition does not exist (Hill *et al.*, 1991, Khodakaram-Tafti *et al.*, 2015, Perez *et al.*, 1999). Curiously, neoplastic mast cells appear well-differentiated in cattle, even in cases with metastasis (Hill *et al.*, 1991). Whether the tumours progress to lethal disease is unknown because individuals are often euthanised due to poor carcass quality. The most recent record of a bovine MCT was of a four-year-old female Holstein with multiple cutaneous lesions (Khodakaram-Tafti *et al.*, 2015). The authors were able to demonstrate Kit protein immunohistochemistry in a cow for the first time (Khodakaram-Tafti *et al.*, 2015).

1.6.4 Pig (Sus scrofa domesticus)

Mast cell neoplasia in pigs is rarely reported and can range from well-differentiated, benign lesions to malignant and metastatic cancer (Bundza and Dukes, 1982, Newman and Rohrbach, 2012). In one study, multiple cutaneous and visceral MCTs were reported in three

pigs, two of which were simultaneously diagnosed with *Eperythrozoonosis* (Bundza and Dukes, 1982). In a recent case report, staining with Toluidine Blue or Giemsa was inconclusive and the diagnosis of MCT was based on positive Kit and tryptase immunohistochemistry (Williams *et al.*, 2018). Rare cases of mast cell leukaemia in miniature pigs have been reported with a poor outcome (Bean-Knudsen *et al.*, 1989, Sipos *et al.*, 2010).

1.6.5 Other species

Mast cell neoplasia is reported in a variety of species and ranges from solitary, benign tumours to malignant, systemic disease. Case reports of visceral MCT have been documented in a number of the big cat species, perhaps resembling the disease in the domestic cat and reflecting the close genetic relation between these species (**Table 1.2**).

MCT is occasionally reported in non-human primates (**Table 1.2**). Three macaques and a baboon were observed with benign MCT with similar histological appearance to that of indolent systemic mastocytosis in humans (Colgin and Moeller, 1996, Jones *et al.*, 1974, Seibold and Wolf, 1973, Zoller and Kaspareit, 2010). A fourth macaque harboured subcutaneous MCT metastatic to the local lymph nodes and internal organs (Tsugo *et al.*, 2017). Immunohistochemical staining of membranous Kit protein was comparable between humans and this macaque case (Tsugo *et al.*, 2017).

Table 1.2. Documented cases of vertebrates infrequently diagnosed with mast cell neoplasia. Male (M), female (F), unknown (U).

Species	Age (years)	Sex	MCT Type	Affected organs	Outcome	Reference
Big Cats						
Cheetah (*Acinonyx jubatus)*	13	F	Visceral (spleen)	Metastatic (larynx)	Non MCT-related death	(Owston *et al.*, 2008)
Cougar (*Puma concolor*)	9 months	M	Visceral	Stomach	Alive at end of study	(Martin *et al.*, 1985)
Indian lion (*Panthera leo*)	16	F	Cutaneous	Multiple skin tumours (>20)	Euthanised	(Stolte and Welle, 1995)
Jaguar (*Panthera onca*)	26	F	Visceral (jejunum)	Metastatic (liver, kidneys)	Died during anaesthesia	(de Castro *et al.*, 2003)
Tiger (*Panthera tigris*)	6	M	Visceral (spleen)	Metastatic (liver, lymphoid, kidney, pulmonary organs)	Died during anaesthesia	(Graille *et al.*, 2013)
	14	M	Visceral	Metastatic (LN, lung, liver)	Euthanised due to other causes	(Owston *et al.*, 2008)
Miscellaneous mammals						
African hedgehog (*Atelerix albiventris*)	~1	F	Subcutaneous	Metastatic (lymph node)	Euthanised	(Raymond *et al.*, 1997)
	>1	F	Cutaneous	Confined to skin	U	(Raymond and Garner, 2001)
	>1	U	Cutaneous	Confined to skin	U	(Raymond and Garner, 2001)
	3	U	Cutaneous	Metastatic (lymph)	U	(Raymond and Garner, 2001)
Llama (*Lama glama*)	9	F	Cutaneous	Multiple cutaneous tumours	Alive at end of study	(Lin *et al.*, 2010)
Nubian goat (*Capra aegagrus hircus*)	4	F	Systemic mastocytosis	Heart, lung, liver, spleen, lymph, bone marrow	MCT-related death	(Khan *et al.*, 1995)
	6 weeks	F	Cutaneous (ear)	Confined to skin	Alive at end of study	(Allison and Fritz, 2001)
Pacific Walrus (*Odobenus rosmarus divergens*)	>15	F	Visceral (lung)	Non-metastatic	Discovered at slaughter	(Seguel *et al.*, 2016)
Richardson's ground	4	F	Cutaneous	Metastatic (lymph)	U	(He *et al.*, 2009)

squirrel (*Spermophilus richardsonii*)						
Sheep (*Ovis aries*)	U	U	Visceral (liver)	Metastasis (lymph)	Discovered at slaughter	(Johnstone, 1972)
	U	U	Visceral (liver)	Metastasis (hepatic)	Discovered at slaughter	(Johnstone, 1972)
Non-human primates						
Baboon (*Papio sp.*)	Young	U	Cutaneous (neck)	Confined to skin	Discovered at necropsy	(Jones *et al.*, 1974)
Cynomolgus macaque (*Macaca fascicularis*)	3	M	Systemic mastocytosis	Liver, cecum	Non-MCT-related euthanasia	(Zoller and Kaspareit, 2010)
Japanese macaque (*Macaca fuscata)*	19	F	Subcutaneous	Metastatic (lymph, kidney, peritoneum, mammary lobule)	MCT-related death	(Tsugo *et al.*, 2017)
Rhesus macaque (*Macaca mulatta*)	7	M	Cutaneous (thorax)	Confined to skin	Alive at end of study	(Colgin and Moeller, 1996)
	Adult	F	Subcutaneous (thigh)	-	U	(Seibold and Wolf, 1973)
Birds						
Burrowing owl (*Speotyto cunicularia*)	U	U	Cutaneous (oral)	Confined to skin	Released, lost to follow-up	(Schmidt and Okimoto, 1992)
Chicken (*Gallus gallus domesticus*)	5	F	Cutaneous (multiple)	Metastasis (lung)	Discovered at slaughter	(Hafner and Latimer, 1997)
	1.5	M	Cutaneous (eyelid)	Confined to skin	Alive at end of study	(Patnaik and Mohanty, 1970)
	Adult	F	Multiple, cutaneous	Confined to skin	Discovered at slaughter	(Hall *et al.*, 1994)
Great horned owl (*Bubo virginianus*)	Adult	M	Cutaneous	Multiple (eye, ear)	Dead	(Swayne and Weisbrode, 1990)
Lovebird (*Agapornis personata*)	12	F	Cutaneous	Metastatic (kidney, liver, spleen, periovarian, bone marrow)	MCT-related death	(Dallwig *et al.*, 2012)
Pueo (*Asio flammeus sandwichensis*)	U	U	Cutaneous (upper eyelid)	Confined to skin	MCT-related euthanasia	(Schmidt and Okimoto, 1992)

Reptiles						
African fat-tail gecko (*Hemitheconyx caudicinctus*)	3	F	Systemic	Liver, kidneys, skeletal muscle, bones, spleen, uterus, ovaries and lungs	MCT-related death	(Irizarry Rovira *et al.*, 2014)
Boa constrictor (*Boa constrictor constrictor*)	U	U	Malignant	Cutaneous	U	(Frye, 1994)
Desert tortoise (Xerobates agassizii)	U	U	Cutaneous	Cutaneous	U	(Frye, 1994)
Eastern kingsnake (*Lampropeltis getulus getulus*)	16	M	Cutaneous	Metastatic (liver, heart, lung, kidney, spleen)	MCT-related death	(Schumacher *et al.*, 1998)
Giant Galapagos tortoise (*Geochelone nigra vicina)*	Subadult	F	Cutaneous	Confined to skin	Healthy 11 months post-surgery	(Santoro *et al.*, 2008)
Green iguana (*Iguana iguana*)	Adult	F	Cutaneous	Mastocytosis of periphery	Euthanised	(Reavill *et al.*, 2000)
Amphibians						
Axolotl (*Ambystoma mexicanum*)	11-17 (18 animals)	U	Cutaneous	Single or multiple lesions, some metastatic (skeletal muscle)	Dead	(Harshbarger *et al.*, 1999)
Tiger salamander (*Ambystoma tigrinum*)	Neotenic (6 animals)	U	Cutaneous	Single or multiple lesions, some metastatic (skeletal muscle)	Dead	(Harshbarger *et al.*, 1999)

1.7 Mastocytosis in humans

Similar to the disease in dogs and cats, mastocytosis in humans can range from a spontaneously regressing skin lesion to a highly aggressive, multisystem malignancy. Mastocytosis is divided into two main groups based on the presence or absence of extracutaneous organ involvement: cutaneous mastocytosis (CM) and systemic mastocytosis (SM). Whilst CM and SM present differently, the disorders exhibit common genetic abnormalities in the *KIT* gene (Bodemer *et al.*, 2010, Valent *et al.*, 2017).

1.7.1 Human cutaneous mastocytosis (CM)

Cutaneous mastocytosis (CM), also referred to as urticaria pigmentosa, is isolated to the skin, with no other organ involvement and represents 80% of all mastocytosis cases in humans (Siebenhaar *et al.*, 2018). CM is characterised by brown-red pigmented cutaneous lesions which urticate when touched. This is due to accumulated hyperactive mast cells within the lesions and is known as Darier's sign (Kiszewski *et al.*, 2004). CM is more common in infants but can occur at any age and is more frequent in males than in females with a ratio of up to 1.8:1 (Bodemer *et al.*, 2010, Kiszewski *et al.*, 2004). Paediatric CM describes the disease in infants and has a favourable prognosis, often spontaneously regressing once the individual reaches adolescence (Kiszewski *et al.*, 2004). In a minority of cases, paediatric CM progresses into mast cell activation syndrome, a multisystem inflammatory disorder of chronic mast cell hyper-reactivity, or systemic mastocytosis, a proliferative and accumulative neoplastic mast cell disease of one or multiple organs (Valent *et al.*, 2017). Both are malignant forms of the disease.

1.7.2 Human systemic mastocytosis (SM)

The classification of systemic mastocytosis (SM) is based on mast cell morphology and immunohistochemistry and includes five sub-variants according to the World Health Organisation (WHO) classification system (Valent *et al.*, 2017). With increasing severity, these sub-classifications are: indolent systemic mastocytosis, smouldering systemic mastocytosis, systemic mastocytosis with associated haematologic non-mast cell lineage disease neoplasm, aggressive systemic mastocytosis and mast cell leukaemia. All sub-variants are characterised by extracutaneous organ involvement and gain-of-function mutations in the *KIT* gene.

Patients with SM may exhibit pruritic, pigmented skin lesions, as manifested in patients with CM, but with additional concurrent spleen, liver or gastrointestinal tract involvement. Demarcated mast cell deposits are almost always found in SM patients bone marrow, and thus, bone marrow biopsies are used in diagnosis (Jaffe *et al.*, 2001, Valent *et al.*, 2017).

1.7.3 Familial mastocytosis

Familial predisposition to mastocytosis is evident in up to 13% of human cases but the exact mode of inheritance is unclear (Bodemer *et al.*, 2010). Mastocytosis can occur in the presence or absence of *KIT* mutations and there is no apparent relationship between a patient's genotype and familial or spontaneous disease development (Bodemer *et al.*, 2010). Familial mastocytosis is known to pass through generations from parent to offspring, although it appears that *KIT* mutations are not inherited (Wohrl *et al.*, 2013). Instead, the mutations arise somatically, potentially as a secondary event resulting from a common germline abnormality predisposing individuals to familial disease (Bodemer *et al.*, 2010, Jawhar *et al.*, 2015).

1.7.4 KIT mutations in human patients

In contrast to dogs and cats, mutations are more frequent in the enzymatic domain of *KIT* in human SM patients. Approximately 44% of paediatric mastocytosis patients and less than 5% of adult SM patients harbour a regulatory-type *KIT* mutation, in either exons 8, 9 or 11 (Bodemer *et al.*, 2010, Haenisch *et al.*, 2012). Comparatively, enzymatic-type *KIT* mutations are detected in mastocytoma or bone marrow biopsies in over 80% of adult and paediatric human patients with mastocytosis (Bodemer *et al.*, 2010, DeAngelo *et al.*, 2017). The commonest *KIT* mutation is a nucleotide substitution occurring in exon 17 at codon 816 where aspartic acid is replaced with valine (Asp816Val or D816V). Unlike in dogs, tumour *KIT* mutation status in humans is not correlated with survival or disease progression (Bodemer *et al.*, 2010, Verzijl *et al.*, 2007). However, mutation status is a minor criterion for mastocytosis diagnosis and is clinically relevant when deciding optimal treatment regimens for mastocytosis patients.

1.7.5 Other genetic mutations involved in human mastocytosis

Mutation of the *TET2* gene is present in almost 30% of all SM patients (Damaj *et al.*, 2014) and in almost 50% of SM patients harbouring the D816V *KIT* mutation (Jawhar *et al.*,

2016b). The data do not provide a clear conclusion regarding the prognostic value of *TET2* mutations for survival (Damaj *et al.*, 2014, Jawhar *et al.*, 2016b, Soucie *et al.*, 2012, Traina *et al.*, 2012).

Mutations in various genes encoding splicing factors *SRSF2, SF3B1* and *U2AF1,* epigenetic regulators *ASXL1, DNMT3A* and *TET2,* the transcription factor *RUNX1* and signalling molecules *JAK2*, *CBL, KRAS* and *NRAS* are frequently identified and co-exist with *KIT* mutations in SM patients (Damaj *et al.*, 2014, DeAngelo *et al.*, 2017, Jawhar *et al.*, 2016a, Jawhar *et al.*, 2016b, Traina *et al.*, 2012). Mutations in *ASXL1, DNMT3A, RUNX1* and *SRSF2* are predictive of overall patient survival and aberrations in other genes predict response to particular treatment types (Jawhar *et al.*, 2016b, Traina *et al.*, 2012). Genetic annotation for SM patients is crucial for targeted therapy and accurate prognostication. Further, characterising patients' genetic status following treatment may accurately assess therapeutic success. Mutations in these genes have not been investigated in other species in relation to MCTs.

1.7.6 Treatment of mastocytosis

Therapy is seldom sought for human patients with CM due to its benign clinical course, however, some CM cases may require antihistamines and corticosteroids to stabilise symptoms (Kiszewski *et al.*, 2004). Conversely, patients with mast cell leukaemia experience average survival times of less than 12 months even with the best treatment available (Valent *et al.*, 2017).

Various TKIs have been investigated for therapeutic use against mastocytosis. Imatinib and masitinib show efficacy for patients with wild-type *KIT* mast cell neoplasia (Alvarez-Twose *et al.*, 2017, Lortholary *et al.*, 2017). However, they are not a suitable treatment option for many patients due to the high prevalence of the D816V mutation which confers drug resistance (Siebenhaar *et al.*, 2018, Valent *et al.*, 2017).

The TKI nilotinib (AMN107, Tasigna) was initially designed against the BCR-ABL fusion protein, the Philadelphia chromosome hybrid protein product, to treat patients with imatinib-resistant chronic myelogenous leukaemia. Nilotinib exhibits potent growth-inhibitory and pro-apoptotic activity against mast cells harbouring the regulatory-type mutation D560G (Gleixner *et al.*, 2006, Verstovsek *et al.*, 2006). There is conflicting evidence regarding the benefits of nilotinib against wild-type and D816V mutant mast cells. In some studies, nilotinib was found to exert no significant cytotoxic or growth inhibitory effects against wild-type or D816V

mutant mast cells (Hochhaus *et al.*, 2015, Verstovsek *et al.*, 2006). In other studies, nilotinib possessed potent anti-proliferative activity against wild-type and D816V mutant mast cells both *in vitro* and *in vivo* (Gleixner *et al.*, 2006, Hochhaus *et al.*, 2015, von Bubnoff *et al.*, 2005).

TKIs that demonstrate growth inhibitory activity against both wild-type and *KIT* D816V mutant neoplastic mast cells include dasatinib (Sprycel), midostaurin (Rydapt) and ponatinib (Iclusig) (Gleixner *et al.*, 2007, Gleixner *et al.*, 2013). Combination therapies evaluating *in vitro* cooperative antineoplastic effects of dasatinib with cladribine, midostaurin and ponatinib show synergistic, anti-proliferative and apoptotic effects against D816V neoplastic mast cells (Gleixner *et al.*, 2007, Gleixner *et al.*, 2013). Ponatinib also down-regulates phosphorylation of the tyrosine kinase protein Lyn and the transcription factor Stat5, suggesting that ponatinib may contribute to neoplastic mast cell growth inhibition in a Kit-independent manner (Gleixner *et al.*, 2013). Ponatinib and midostaurin also demonstrate IgE-blocking properties by inhibiting histamine release by mast cells and basophils to provide relief to SM patients (Kiwanuka *et al.*, 2015, Peter *et al.*, 2016).

A compelling new therapy for SM patients with D816V mutation is the use of BLU-285, a potent and selective inhibitor of the Kit activation loop. *In vitro,* BLU-285 blocks autophosphorylation of D816V mutant Kit as well as phosphorylation of downstream Kit signalling proteins (Akt and Stat3), thereby inducing cellular apoptosis (DeAngelo *et al.*, 2017, Drummond *et al.*, 2016, Jawhar *et al.*, 2016a). In phase I human clinical trials, BLU-285 was well tolerated by patients and mast cell burden in peripheral blood, bone marrow and organs decreased (DeAngelo *et al.*, 2017, Drummond *et al.*, 2016). Moreover, BLU-285 shows efficacy in D816V SM patients resistant to the TKI midostaurin (Drummond *et al.*, 2016, Jawhar *et al.*, 2016a). Such therapies may be of value in the veterinary setting as well.

Other tyrosine kinase inhibitor-independent therapies including interferon-alpha, cladribine, cytosine arabinoside, rapamycin, tanespimycin, nuclear factor-κB inhibitor IMD-0354 and hematopoietic stem cell transplantation are used with some success in mastocytosis patients (Testa, 2008, Willmann *et al.*, 2019).

1.8 Gaps in the literature and study rationale

Over the last decade, substantial progress has been made in understanding the pathogenesis and complexity of MCTs in dogs. Novel diagnostic and prognostic parameters

have been discovered and validated for their use in clinical practice. Mutational analysis of the *KIT* gene supplements traditional histopathological tumour assessment in canine prognosis and tumour management decisions. International veterinary diagnostic laboratories routinely evaluate high-grade cutaneous MCTs for *KIT* exon 8 and exon 11 ITDs. Dogs bearing high-grade unresectable or recurrent exon 11 ITD-mutant tumours are candidates for treatment with tyrosine kinase inhibitors. Unfortunately, genetic testing can be an expensive addition to the costs already associated with the surgery. Further, testing is currently not available in Australia, although plans to implement testing are in progress. Hence, many veterinary oncologists rely on data available in the literature to predict patient response to therapy.

Tyrosine kinase inhibitors such as toceranib, imatinib and masitinib show efficacy in decreasing the tumour burden in dogs with ITD-mutant and wild-type *KIT* tumours. Similarly, response to tyrosine kinases inhibitors is seen in cats with MCT and humans with mastocytosis, advocating the use of these small molecule inhibitors for the management of mast cell cancers. However, a minority of animal and human patients with mast cell neoplasia do not respond to these tyrosine kinase inhibitors most likely as a result of mutation of the intracellular enzymatic pocket domain of *KIT*. Enzymatic pocket-type mutations alter the structure of the intracellular region of the Kit protein, decreasing the affinity of tyrosine kinase drugs to the binding site and subsequent tumour resistance. In dogs, enzymatic pocket-type mutations are extremely infrequently reported. This is most likely a result of few studies having evaluated only portions of this domain. The place of tyrosine kinase inhibitors in canine MCT treatment is not entirely clear but they appear to provide equivalent health benefits for patients when compared to traditional remedies.

The aim of this work was to establish a *KIT* mutation profile using the DNA extracted from cutaneous and subcutaneous MCTs from Australian dogs and correlate mutational data to canine prognostic indices. The first objective in achieving this aim was to develop a DNA purification protocol optimising MCT-extracted DNA amplification by PCR. Utilising this method, MCT DNA samples were screened for the most common canine *KIT* mutation, the exon 11 ITD, to determine the prevalence of this mutation type in a cohort of canine MCTs seen in general practice throughout southern-eastern Australia. Following this, a randomly selected subset of these MCT DNA samples was used to create a canine MCT *KIT* mutation profile using AmpliSeq™ Ion Torrent™ Personal Genome Machine next-generation sequencing technology. The aim of the AmpliSeq™ project was to evaluate the *KIT* gene coding sequence

in its entirety to identify mutations outside *KIT* gene mutation hotspots which may highlight additional regions of *KIT* involved in canine MCT pathogenesis. Next, tumour *KIT* mutation status was correlated with known canine prognostic indicators in order to predict the value of *KIT* mutation status in canine prognosis. Finally, the canine *KIT* gene sequence and mutational data were compared with that from domestic feline MCTs and a case series of cheetah MCTs to determine whether the *KIT* gene plays a similar role in MCTs from cats. Overall, it is hoped that this work increases the understanding of the involvement of *KIT* in neoplastic mast cell biology and provides the basis for establishing individualised treatment plans for dogs with MCTs.

Chapter 2: DNA purification increases PCR amplifiable DNA extracted from formalin-fixed, paraffin-embedded canine mast cell tumours for routine *KIT* mutation detection[1]

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[1]This chapter has been accepted for publication in the *Journal of Veterinary Diagnostic Investigation* (Tamlin *et al.*, 2019b). See Supplementary Material 2.

2.1 Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissue archives are a valuable resource for retrospective studies of a plethora of diseases due to the economic and space-efficient storage requirements of the blocks in comparison to frozen materials. However, using polymerase chain reaction (PCR) to amplify DNA extracted from FFPE tissues can be problematic due to the variable and often high level of DNA degradation induced by formalin fixation. Further, the type of fixative, length of fixation, extraction method, length of enzymatic tissue digestion and age of block impact the quality of the expected DNA and all have been targets for optimisation (Abed and Dark, 2016, Granato *et al.*, 2014, Jelassi *et al.*, 2017, Sato *et al.*, 2001). This limits amplification for most FFPE samples to small fragments of less than 250 base pairs (bp) (Abed and Dark, 2016, Dedhia *et al.*, 2007). Various techniques have been investigated to optimise extracted DNA integrity and quality for downstream molecular analyses. The extraction of high-quality DNA from either FFPE or fresh-frozen tissue samples has been reported as peculiarly problematic for mast cell tumours (MCTs) in comparison to other tumour types and normal tissues, most likely due to the co-extraction of unknown neoplastic mast cell-specific PCR inhibitors (Tamlin *et al.*, 2017, Weiss *et al.*, 2011).

Veterinary diagnostic laboratories in the USA, Europe and elsewhere use PCR and gel electrophoresis to test MCT DNA for exon 11 internal tandem duplications (ITDs) in the *KIT Proto-Oncogene*. Some diagnostic laboratories also offer *KIT* exon 8 ITD mutation screening. Dogs who test positive for a *KIT* exon 8 or 11 ITD in their MCT, and are unresponsive to conventional chemotherapeutic treatments, are suitable candidates for treatment with tyrosine kinase inhibitors. However, *KIT* gene amplification by PCR fails in 9–62% of cases (Sabattini *et al.*, 2017, Tamlin *et al.*, 2017, Thompson, 2012, Weiss *et al.*, 2011). In one study, PCR amplification of FFPE-extracted DNA from feline lymphoma, feline myocardium and canine cutaneous histiocytoma samples was successful in 100% of cases for amplicons up to 245 nucleotides in length (Weiss *et al.*, 2011). Comparatively, FFPE-extracted MCT DNA produced PCR amplicons of 80, 161 and 245 nucleotides in only 60%, 38% and 45% of cases. Further, fresh-frozen genomic MCT DNA amplification failed in 43% of cases. Similarity, 40% (n = 49/123) of cutaneous MCT DNA samples failed PCR amplification in a preliminary investigation in our laboratory (Tamlin *et al.*, 2017). Further, a proportion of MCTs (11%, n = 3/27) were not able to be amplified by *KIT* or *β-Actin* PCR assays in a cohort of cutaneous and

subcutaneous MCTs (Thompson, 2012), and *KIT* amplification failed in 9% (n = 2/22) of feline splenic MCT DNA samples (Sabattini *et al.*, 2017). This illuminates a MCT-specific enigma.

The primary aim of this study was to develop a method for the successful PCR amplification of FFPE-extracted canine MCT DNA samples in 100% of cases. It was hypothesised that high-grade tumours would be less likely to produce a PCR amplicon when compared to low-grade tumours, as a result of increased genetic changes and a harsher cellular environment induced by a rapidly growing tumour.

2.2 Methods

2.2.1 Tissue collection

Canine MCT specimens that had been submitted for standard histological diagnosis and assessment to the Veterinary Diagnostic Laboratory at the University of Adelaide (Roseworthy, SA, AU) and to Gribbles Veterinary Pathology commercial laboratories in Melbourne (Clayton, VIC, AU) and Adelaide (Glenside, SA, AU) were collected between 2011 and 2017. The laboratories provided the MCT specimens in the form of FFPE tissue blocks.

Each MCT block was assigned an independent alpha-numeric identifier and information detailing that sample was stored in an Excel (2013) spreadsheet. The samples collected from the University of Adelaide were labelled “U1, U2, etc.” to denote being the first/second/etc. sample sourced from the University. Samples sourced from Gribbles Veterinary Pathology Adelaide were labelled “A”, and samples collected from Gribbles Veterinary Pathology Melbourne assigned the letter “M” as an identifier.

2.2.2 Genomic DNA extraction

A 20-µm thick section was cut from each FFPE MCT block and placed into a sterile 1.5 mL Eppendorf tube using sterile forceps. To avoid DNA cross-contamination, a new disposable microtome blade was used to process each sample and the microtome cutting area and forceps were cleaned with 70% ethanol followed by a rinse with nuclease-free water.

The QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), which has been previously determined to yield good quality FFPE-extracted DNA in comparison to other techniques, was used to extract DNA from each MCT section (Janecka *et al.*, 2015). Xylene was used for

deparaffinization of FFPE tissue sections and DNA was extracted according to manufacturer's instructions, eluted in 60 µL of a 0.1 mM EDTA buffer supplied in the kit (Buffer ATE, **Appendix B**) and stored at 4°C for later use. A commercial Qiagen kit (QIAmp AllPrep FFPE Tissue Kit) was used to extract DNA from the fresh-frozen testes of a healthy dog which were discarded after routine surgical neutering procedures at the University of Adelaide's Companion Animal Health Centre (CAHC). This DNA served as a positive control for PCR amplification and represented the normal template of the canine *KIT* gene sequence. This was verified by Sanger sequencing and alignment to the *KIT* reference gene sequence and ensembl.org BLAST (described in Chapter 3).

MCT DNA quantity (ng/µL) and quality (A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios) were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was considered to be of good quality with an A_{260}/A_{280} absorbance ratio of 1.8. Readings lower than 1.8 were considered to be contaminated with proteins and other impurities and readings greater than 1.8 were considered to be contaminated with RNA.

2.2.3 MCT DNA amplification and purification

Each sample underwent PCR amplification using two different primer sets (**Table 2.1**). Primers for the housekeeping gene H*ypoxanthine-guanine Phosphoribosyltransferase* (*HPRT*) were used to determine whether the DNA was of an adequate quality to generate a small PCR product. Previously published primers were used for identifying ITDs in exon 11 of the *KIT* gene (Downing *et al.*, 2002). PCR conditions were identical for both sets of primers. PCR reactions were prepared to final concentrations of 250 nmol/L of each primer, 33.3 units (0.15 µL of 5 U/µL) of ThermoPrime *Taq* DNA Polymerase (Thermo Fisher Scientific), 250 µmol/L of dNTP mix (Fisher Biotech Australia, Wembley, WA, Australia), 1.5 mM $MgCl_2$ and 2 µL of 10× Reaction Buffer IV (Thermo Fisher Scientific). Approximately 10–50 ng of sample DNA (in a 1 µL volume) was added to each reaction and made up to a total volume of 20 µL with Molecular Biology Grade H_2O (DNase and RNase free; Sigma Aldrich, St. Louis, MO, USA). The PCR cycling conditions were 2 min at 95°C; 35 cycles of 15 sec at 95°C, 10 sec at 60°C and 1 min at 72°C; and a final extension of 10 min at 72°C in the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Normal canine testis DNA and water were used as positive and negative controls, respectively, for PCR amplification. Resulting PCR products were analysed on a 2% agarose gel by electrophoresis in 1X TAE buffer.

Table 2.1. Nucleotide sequences of *HPRT* and *KIT* gene primers used in PCR assays.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
HPRT	HPRT2 FRD2 GCG AGA GAG AAC CTT GTG TG	HPRT2 REV2 GGG ACT TTG GGG AAC TGA C	163
KIT* exon 11	PE1 CCC ATG TAT GAA GTA CAG TGG AAG	PE2 GTT CCC TAA AGT CAT TGT TAC ACG	Wild-type: 190 ITD mutant: >190

bp: base pair.
*primers from Downing *et al.* (2002).

DNA that failed to amplify by PCR using either one or both primer sets underwent a "washing" step using the DNA Clean and Concentrator Kit (Integrated Sciences, Zymo Research, Irvine, CA, USA), as per manufacturer's instructions. In brief, DNA samples were mixed with DNA Binding Buffer at a ratio of 5:1 and then transferred to a Zymo-Spin Column in a collection tube, before centrifugation at 13,000 x g for 30 seconds. The columns were washed twice with 200 µL of DNA Wash Buffer, and each wash was followed by 13,000 x g centrifugation for 30 seconds. DNA was eluted in 40 µL of the 0.1 mM EDTA elution buffer supplied in the Zymo Kit (DNA Elution Buffer, **Appendix B**) and the concentration measured on the NanoDrop Spectrophotometer. The samples then underwent a second PCR amplification test. Samples that still failed to produce a PCR amplicon underwent a second DNA washing procedure, a direct repeat of the first wash, followed by a third PCR test.

2.2.4 MCT histological classification

At least one H&E-stained section of each tumour was graded independently by board-certified veterinary pathologists (Drs Allan Kessell, Elizabeth Dobson and Lucy Woolford). Tumours were diagnosed as cutaneous or subcutaneous and cutaneous tumours were further divided using two systems widely used in veterinary pathology: the Patnaik scheme and the Kiupel scheme (Kiupel *et al.*, 2011, Patnaik *et al.*, 1984). The diagnosis of a subcutaneous MCT was based on the tumour being located completely within the subcutis, with no invasion of the dermis or epidermis. Pathologists were blinded to the results of the molecular analysis and to the other pathologists' grades. Where the grades were discordant, the results were unblinded and the pathologists re-examined and discussed the same sections until a unanimous decision was made. If an agreement could still not be made, the highest

histological grade was assigned to the tumour in order to account for the worst-case scenario.

2.2.5 Statistical analyses

Statistical analyses were performed in IBM SPSS statistical software (version 25, Armonk, NY, USA). A Pearson chi-square test was used to investigate the relationship of washing requirements between *HPRT* and *KIT* primers and the degree of association was determined by the phi coefficient. Pearson chi-square test was used to determine the relationship between the age of the block (in years) and washing requirements using either primer pair.

The ability of sample DNA concentration and the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios to predict the success of PCR amplification using both primer sets was investigated using binary logistic regression analysis fitted with a Hosmer-Lemeshow model. Two-sample t-tests determined the significance of DNA concentration and absorbance ratios for samples successfully amplified by *HPRT* primers in the first instance, but which required a wash for successful PCR amplification using *KIT* primers.

A generalized linear logistic model with binary logistic regression fitted with a Wald chi-square analysis was used to test for significance between washing requirements for *HPRT* and *KIT* primers with tumour type. For the purposes of statistical analysis, tumour type was defined as subcutaneous MCT, cutaneous Kiupel low-grade MCT or cutaneous Kiupel high-grade MCT as determined by histology. A pairwise comparison was used to determine differences in washing requirements between the tumour types.

2.3 Results

2.3.1 Evaluable cases

A total of 280 FFPE canine MCT blocks from 248 dogs were sourced from the Veterinary Diagnostic Laboratory at the University of Adelaide (n = 16) and from Gribbles Veterinary Pathology commercial laboratories in Clayton (n = 175) and Glenside (n = 89). At the time of DNA extraction, 68 blocks were ≤ 1 year old, 189 blocks ranged from 1–2 years old and 23 blocks were 2–4 years old. Sample fixation times are unknown, but the large majority of samples are predicted to have been fixed for a maximum of 1–2 days.

2.3.2 PCR amplification and DNA purification

Using the *HPRT* primers, 179/280 (63.9%) samples were successfully amplified directly after DNA extraction. Eighty-six (30.7%) samples required a single wash and 15 (5.36%) samples required two washes before amplifiable DNA was obtained. Using the *KIT* primers, 127/280 (45.4%) samples required no washing, 130 (46.4%) samples required a single wash and 23 (8.21%) samples required two washes for amplifiable DNA to be obtained. Overall, 153 of the 280 (54.6%, 95% CI: 48.8–60.5%) MCT DNA samples required at least one wash for successful PCR using both primer sets (**Table 2.2**).

Table 2.2. The number of mast cell tumour DNA samples that required a wash to obtain a successful PCR amplicon when using the *HPRT* and *KIT* gene primers.

		***HPRT* primers**		
		No wash required	Wash required	**Total**
***KIT* primers**	No wash required	127	0	127
	Wash required	52	101	153
Total		179	101	280

As determined by Pearson chi-square test, MCT DNA samples that required washing for successful PCR amplification by one primer set were more likely to require washing for PCR amplification success when using the other primer set, χ^2 (1, n = 280) = 131, P < 0.001. The association of *HPRT* with *KIT* primer pairs for washing requirements was strong, ϕ = 0.684. There was no significant relationship between age of block and washing requirements when using either the *HPRT* primers (P = 0.262) or *KIT* primers (P = 0.074) as determined by the Pearson chi-square test.

DNA samples varied from being more dilute to more concentrated after washing than the original, unwashed sample, although, the overall median DNA concentration for the samples tended to decrease after each washing step (**Figure 2.1A**). No obvious trend was seen in the DNA A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios for samples requiring no wash, one wash or two washes (**Figure 2.1B–C**). DNA quantity (concentration) and quality (absorbance ratios) either pre- or post-washing were not statistically significant in predicting successful DNA amplification using both the *HPRT* and *KIT* primer sets (P = 0.700).

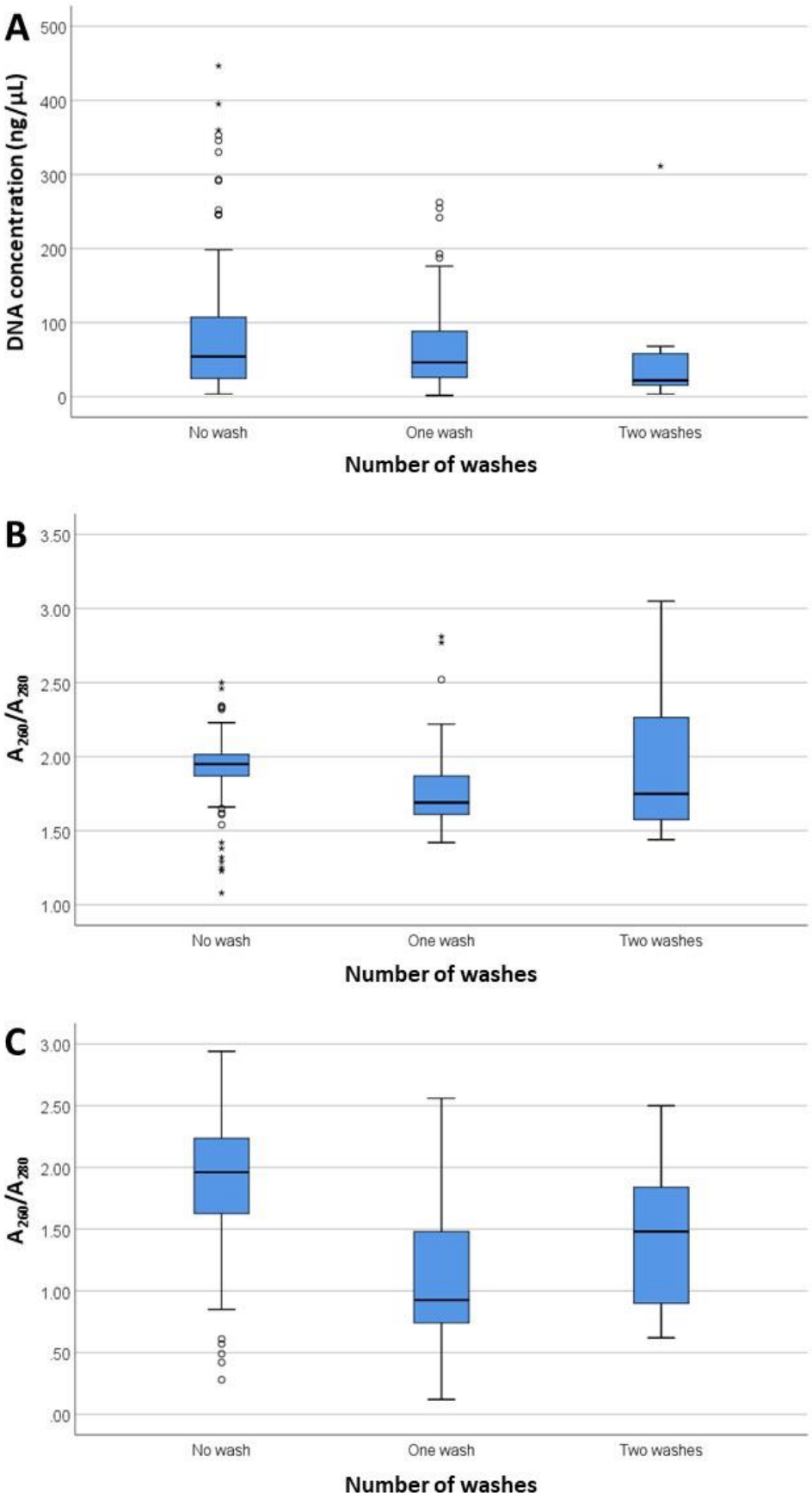

Figure 2.1. Box and whisker plots summarising the (A) concentration and the (B) A_{260}/A_{280} and (C) A_{260}/A_{230} absorbance ratios for canine mast cell tumour DNA samples directly after extraction (no wash; n = 127) and after one (n = 130) or two washes (n = 23). An open circle represents an outlier. A star (*) represents an extreme outlier.

To further investigate the cause of PCR failure, we conducted *HPRT* PCR assays on MCT DNA that required at least one wash for successful PCR amplification. Two assays were prepared from a single Mastermix solution. The first assay was conducted according to the standard *HPRT* protocol whereby 1 µL of 10–50 ng of MCT DNA was added to each reaction. The second assay was identical to the first, but each reaction was "spiked" with 1 µL of canine control DNA (concentration of 12 ng/µL) in addition to the 1 µL of MCT sample DNA. PCR products were analysed on a 2% agarose gel by electrophoresis (**Figure 2.2**). The canine control DNA in the "spiked" assay was not amplified in samples that failed to amplify in the standard PCR but was amplified in both control reactions.

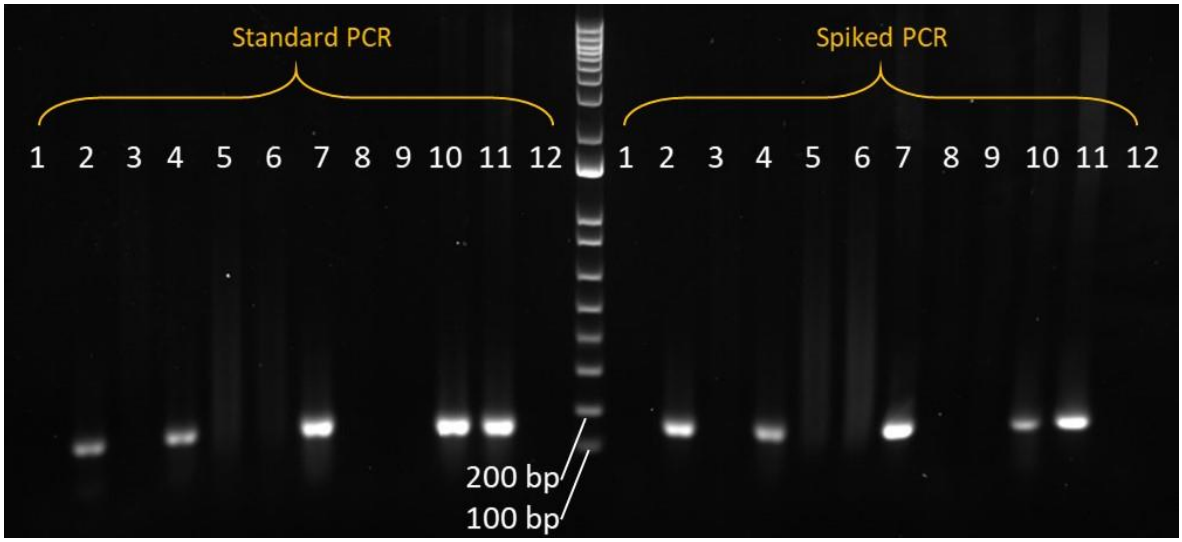

Figure 2.2. Agarose gel electrophoresis of genomic DNA extracted from four formalin-fixed, paraffin-embedded canine mast cell tumour blocks, amplified by PCR using *HPRT* housekeeping gene primers and run against a 1 Kb DNA ladder marker. Expected product size is 163 base pairs (bp). Two PCR assays were performed from a single Mastermix solution. The first assay amplified canine mast cell tumour DNA according to the "standard" PCR protocol. The second assay was the same as the first except that each reaction, excluding the negative control, was "spiked" with wild-type canine testis positive control DNA in addition to the tumour sample DNA. Lanes 1 and 2: sample A86 with no wash and after one wash, respectively. Lanes 3 and 4: sample M186 with no wash and after one wash, respectively. Lanes 5, 6 and 7: sample M127 with no wash, after one wash and two washes, respectively. Lanes 8, 9 and 10: sample U47 with no wash, after one wash and two washes, respectively. Lane 11: positive control (wild-type canine testis DNA). Lane 12: negative control (water).

Of the 179 samples successfully amplified by *HPRT* primers directly after DNA extraction, 52 (29.1%) required washing to obtain a *KIT* amplicon. Two-sample t-tests determined significant decreases in the mean DNA concentration (60.5 versus 87.5; $P = 0.016$) and the A_{260}/A_{280} (1.75 versus 1.91; $P < 0.001$) and A_{260}/A_{230} (1.19 versus 1.87; $P < 0.001$) absorbance ratios for these 52 samples compared to the remaining samples.

2.3.3 Tumour histological classification

MCTs were histologically classified as cutaneous MCT (n = 239/280, 85.4%) or subcutaneous MCT (n = 41/280, 14.6%). The cutaneous cohort was represented by 13 grade I (5.44%), 181 grade II (75.7%) and 45 grade III (18.8%) tumours according to the Patnaik scheme. Kiupel low-grade and high-grade tumours comprised 77.0% (184/239) and 23.0% (55/239) of tumours, respectively. All Patnaik grade I tumours were classified as Kiupel low-grade and all Patnaik grade III tumours were classified as Kiupel high-grade. Patnaik grade II MCTs included 171 Kiupel low-grade and 10 Kiupel high-grade tumours. Due to low numbers representing the Patnaik grade I class, only the Kiupel grading scheme was investigated in the subsequent statistical analyses.

2.3.4 Histological classification and washing requirements

When using *HPRT* primers, 17/41 (41.5%) subcutaneous MCTs, 71/184 (38.6%) cutaneous Kiupel low-grade MCTs and 13/55 (23.6%) cutaneous Kiupel high-grade MCTs required at least one wash to obtain amplifiable DNA. A generalized linear logistic model with binary logistic regression fitted with a Wald chi-square analysis suggested a potential relationship between washing requirements for *HPRT* primers with tumour type (P = 0.060). For *KIT* primer pair, 27/41 (65.9%) subcutaneous MCTs, 105/184 (57.1%) cutaneous low-grade MCTs and 20/55 (36.4%) cutaneous high-grade MCTs required at least one wash to obtain amplifiable DNA. Pairwise comparison showed MCTs that were cutaneous low-grade (P = 0.005) or subcutaneous (P = 0.001) were significantly more likely than cutaneous high-grade MCTs to require at least one wash to obtain amplifiable DNA when using *KIT* primer pair. Washing requirements for cutaneous low-grade MCTs and subcutaneous MCTs were not significantly different from each other (P = 0.167).

2.4 Discussion

The primary aim of this study was to develop a method allowing for successful PCR amplification in 100% of canine FFPE-extracted MCT DNA samples. Two primer sets for two independent genes were investigated: the *HPRT* housekeeping gene and the *KIT* proto-oncogene. PCR amplification failed for at least one primer set in 54.6% of cases. Spiking MCT DNA *HPRT* PCR assays which failed PCR amplification with positive control canine testis DNA

still failed to produce a PCR amplicon directly after DNA extraction and without any washing steps (**Figure 2.2**). DNA quantity, DNA quality and age of FFPE block were not associated with the success of PCR amplification, supporting the conclusion that tissue-specific contaminants are responsible for PCR inhibition. Collagen, melanin, myoglobin, haemoglobin, heparin and IgG are among some known PCR inhibitors that could have been present in FFPE MCT specimens and subsequently co-extracted with MCT DNA (Bessetti, 2007). The actual contaminants in the MCT DNA solutions are unknown but can be removed using the DNA purification by washing method described here.

The disparity in MCT DNA amplification when using *HPRT* primers compared to *KIT* primers is unclear. Almost a third of samples successfully amplified by *HPRT* primers directly after DNA extraction required washing to obtain a *KIT* amplicon. It is possible that in these cases, primer annealing properties or primary DNA structural properties together with PCR inhibitors may have contributed to the finding. For samples successfully amplified by *HPRT* primers directly after DNA extraction, the DNA concentrations and absorbance ratios were significantly different for samples requiring and not requiring a wash for successful *KIT* primer amplification. It is unclear whether post-washing DNA concentrations were the sole contributor to successful PCR amplification in these cases, or if removing inhibitors also influenced the outcome.

High-grade cutaneous MCTs are notoriously malignant and commonly affected by local inflammation, ulceration and tissue necrosis associated with spontaneous degranulation by tumour cells of bioactive molecules (Blackwood *et al.*, 2012). It was initially hypothesised that high-grade MCTs would experience greater PCR inhibition as a result of these factors (Bessetti, 2007). However, our results did not support this hypothesis and instead showed that the DNA from the more benign MCTs, cutaneous low-grade MCTs and subcutaneous MCTs, was more likely to require washing to enable successful *KIT* PCR amplification. One possible explanation is related to the copy number of the *KIT* gene in high-grade tumours. MCTs with *KIT* mutations have reportedly increased copies of the *KIT* locus on CFA13 (Mochizuki *et al.*, 2017b). An increased quantity of initial DNA template might offset the effect of tissue inhibitors in some PCR reactions. This is speculative and needs further clarification.

A second possible explanation for the poor amplification of low-grade MCT DNA may be related to heparin concentrations in low-grade versus high-grade MCTs. Heparin inhibits PCR amplification of DNA by directly interacting with purified DNA, thereby blocking PCR

activity (Ghadessy *et al.*, 2001). It is conceivable that heparin concentrations might be higher in well-differentiated, highly-granulated neoplastic mast cells compared to the heparin concentrations in poorly-differentiated mast cells. If so, PCR amplification of DNA extracted from low-grade MCTs would be more inhibited than the DNA from higher-grade tumours. However, no research has been conducted investigating whether heparin concentrations differ between the histological grades. This is an interesting avenue to explore.

This research is limited by a lack of data regarding tissue sample processing. It is predicted that veterinary clinics would have sent the biopsy to the pathology laboratories on the day or day after the surgery and that samples would be processed at the pathology laboratory upon receipt. However, the time delay between tumour surgical excision and placement in formalin was ultimately unknown and standard operating procedures regarding tissue processing likely vary between veterinary pathology laboratories, technicians and tumour cases. Discrepancies in these processes may have affected downstream molecular analyses. The thickness of FFPE sections taken for DNA extraction may have also contributed to the co-extraction of PCR inhibitors, although this has not been properly researched. Nonetheless, the washing method described here is effective in resolving this issue.

2.5 Conclusion

FFPE-extracted MCT DNA amplification by PCR failed in 54.6% of cases of cutaneous and subcutaneous MCTs, presumably due to the combined effects of DNA copy numbers and the co-extraction of tissue-specific contaminants. This problem can be solved readily by the DNA purification by washing method described here. The exact biological contaminants interfering with PCR amplification remain unknown, but our results suggest that they are more prominent in cutaneous low-grade MCTs and subcutaneous MCTs.

Chapter 3: Prevalence of *KIT* exon 11 internal tandem duplications in 239 cutaneous and 41 subcutaneous mast cell tumours excised from Australian dogs[1]

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[1]This chapter is supported by a preliminary Honours investigation but which is not part of the assessment for this thesis (Tamlin *et al.*, 2017).

3.1 Introduction

Cutaneous mast cell tumours (MCTs) represent the most common malignant skin cancer in dogs. Approximately 50% of high-grade MCTs harbour an internal tandem duplication (ITD) in exon 11 of the *KIT Proto-Oncogene* (Downing *et al.*, 2002, Tamlin *et al.*, 2017, Webster *et al.*, 2006b, Zemke *et al.*, 2002). Exon 11 ITDs cause constitutive activity of the mast/stem cell growth factor receptor tyrosine kinase Kit protein which contributes to mast cell malignancy and disease severity (Letard *et al.*, 2008). Veterinary diagnostic laboratories use PCR and gel electrophoresis to test DNA from high-grade MCTs for exon 11 ITDs. Dogs who harbour a *KIT* exon 11 ITD in their MCT and which are unresponsive to conventional chemotherapeutic treatments are suitable candidates for treatment with tyrosine kinase inhibitors. Tyrosine kinase inhibitors block the abnormal autophosphorylating activity of the Kit protein caused by ITDs in exon 11 of *KIT* (London *et al.*, 2009, Pryer *et al.*, 2003).

Worldwide data report the prevalence of exon 11 ITDs to range from 0–33% in cutaneous canine MCTs (**Appendix C**). However, there are bias and sample size issues with a number of these studies, confounding their results. Unbiased international reports from the USA and Japan elucidate an exon 11 ITD prevalence of approximately 14–17% (Takeuchi *et al.*, 2013, Webster *et al.*, 2006b, Webster *et al.*, 2007, Webster *et al.*, 2008). Comparatively, four unbiased studies from Europe report a significantly lower prevalence of 0–10%, perhaps elucidating differences in the prevalence of exon 11 ITDs between distinct canine populations (Giantin *et al.*, 2012, Marconato *et al.*, 2014, Reguera *et al.*, 2002, Riva *et al.*, 2005). The differences in the genomic status of dogs with cutaneous MCTs from different countries is also evident in a genome-wide association study whereby germ-line risk factors predisposing dogs in the USA to cutaneous MCT development were different to the germ-line risk factors for dogs in Europe (Arendt *et al.*, 2015).

A preliminary investigation conducted in our laboratory determined the prevalence of exon 11 ITDs in Australian canine cutaneous MCTs s to be 17.6% (Tamlin *et al.*, 2017). Whilst this parallels data from the USA and Japan, it is now apparent that this might be an overestimation of the true prevalence in Australian dogs. As described in Chapter 2, DNA extracted from low-grade MCTs is more likely to require additional sample processing for

successful PCR amplification compared to high-grade MCT-extracted DNA. In the preliminary investigation, 49/123 (39.8%) samples failed PCR amplification and were excluded from further analyses (Tamlin *et al.*, 2017). It is likely that these were low-grade tumours and, therefore, that the study was biased towards including high-grade MCTs. Exon 11 ITDs are commonest in high-grade MCTs, and hence, the over-representation of this class of tumour may have skewed the data to a higher ITD prevalence.

The aim of this study was to determine the prevalence of ITDs in *KIT* exon 11 in an unbiased cohort of canine cutaneous MCTs submitted to veterinary pathology laboratories in South Australia and Victoria (Australia).

3.2 Methods

3.2.1 Sample selection

The MCT DNA samples evaluated for PCR amplification in Chapter 2 were used in the present chapter to determine the prevalence of *KIT* exon 11 ITD mutations. ITD mutations were detected by the PCR and gel electrophoresis methods for the *KIT* gene as described in Chapter 2.

Tumour histological grading was performed by three board-certified veterinary pathologists as described in Chapter 2.

3.2.2 Sanger sequencing for mutation validation

The normal canine testis control sample and the samples positive for exon 11 ITD mutations, as determined by the presence of a larger, upper band in agarose gels after *KIT* primer amplification, were amplified by a follow-up *KIT* PCR in three separate 20 µL PCR reactions prepared from the same Mastermix solution. The reactions were pooled and then run on a 2.75% agarose gel for 3 hours at 70 volts. A large amount of DNA was necessary to obtain a significant quantity of DNA for gel extraction and Sanger sequencing. A long running time of the gel at a low voltage was essential for the proper separation of the ITD-mutant sequence from the wild-type DNA sequence. The upper mutant bands and the canine testis control band were excised from the gel and then purified using a QIAquick Gel Extraction Kit (Qiagen), according to manufacturer's instructions. To confirm sequence identity, 10 ng of the

purified bands were submitted to the Australian Genome Research Facility (AGRF; Urrbrae, SA, Australia) for Sanger sequencing in the forward and reverse directions using the *KIT* primers.

Sanger sequence results were aligned the canine *KIT* reference gene sequence (ENSCAFG00000002065) using ensembl.org Basic Local Alignment Search Tool (BLAST; https://asia.ensembl.org/Canis_familiaris/Tools/Blast?db=core, accessed 03/09/2018) sequence database program to determine exon 11 ITD positions. Sequence chromatograms were visually reviewed in Chromas (version 2.4.3, Technelysium Pty Ltd, QLD, Australia) for ITD and SNP confirmation.

3.3 Results

3.3.1 Histological classification and pathologist concordance

The initial study was designed to only investigate cutaneous MCTs. At the time of DNA extraction and PCR testing, tumour histological review was not complete. Histological evaluation revealed that 239/280 (85.4%) MCT specimens were cutaneous tumours, and the remaining 41 (14.6%) were subcutaneous MCTs. Little is known about the involvement of *KIT* in subcutaneous MCT malignancy. One recent study reports an exon 8 mutation in a single subcutaneous MCT but prognostic data are not available (Vozdova *et al.*, 2019b). Rather than discarding the subcutaneous tumour DNA samples in the current investigation, it was decided that they should remain in the study for *KIT* mutation testing and prognostic evaluation.

The same two board-certified veterinary pathologists independently graded all 280 tumours. Twenty-four of the 41 subcutaneous MCTs (58.5%) were initially miss-classified as cutaneous MCT by a pathologist; 14 tumours as Patnaik grade II Kiupel low-grade, one as Patnaik grade II Kiupel high-grade and three tumours as Patnaik grade III Kiupel high-grade. After review of each case, it was agreed that these 41 tumours resided completely in the subcutis, with no dermal or epidermal involvement. Of the remaining 239 cutaneous MCTs, histological grading according to the Patnaik and Kiupel grading schemes were 83.7% (n = 200/239) and 92.5% (n = 221/239) concordant between the two pathologists, respectively. A subset of cutaneous tumours (n = 74) was independently graded by a third board-certified veterinary pathologist. There was 75.7% (n = 56/74) and 91.9% (n = 68/74) agreement

between the three pathologists when using the Patnaik and Kiupel grading schemes, respectively (**Table 3.1**).

Table 3.1. Initial concordance between three board-certified veterinary pathologists when using the 3-tier Patnaik and 2-tier Kiupel histopathological grading schematics for cutaneous canine mast cell tumours.

Schematic	Grade	Grade concordance (%)	Schematic concordance (%)
Patnaik *et al.* (1984)	Grade I	4/6 (66.7)	56/74 (75.7)
	Grade II	36/50 (72.0)	
	Grade III	16/18 (88.9)	
Kiupel *et al.* (2011)	Low-grade	49/52 (94.2)	68/74 (91.9)
	High-grade	19/22 (86.4)	

Pathologists became unblinded to the results of the other pathologists' grades. Where the grades were discordant, the pathologists re-examined and discussed the same sections until a unanimous decision was made. Three and one cases could not be agreed upon after tumour re-evaluation using the Patnaik and Kiupel grading schemes, respectively. In these instances, the highest histological grade was recorded to account for the worst-case scenario.

The cutaneous cohort was represented by 184 and 55 Kiupel low-grade and Kiupel high-grade MCTs, respectively. When using the Patnaik schematic, 13, 181 and 45 tumours were respectively classified as grade I, II and III. All Patnaik grade I tumours were classified as Kiupel low-grade and all Patnaik grade III tumours were classified as Kiupel high-grade. Patnaik grade II MCTs included 171 Kiupel low-grade and 10 Kiupel high-grade tumours.

3.3.2 Normal canine testis control DNA sequence

The normal canine testis control DNA sequence was an exact match to the canine *KIT* gene reference sequence.

3.3.3 KIT mutations

A *KIT* exon 11 ITD was observed in 22/55 (40.0%) high-grade tumours and 2/184 (1.09%) low-grade tumours, with an overall prevalence in cutaneous MCTs of 10.0% (24/239; 95% CI: 6.23–13.9%). When using the Patnaik scheme, 0/6 grade I (0%), 5/181 (2.76%) grade II and 19/45 (42.2%) grade III tumours carried an exon 11 ITD. One cutaneous low-grade

(Patnaik grade II) tumour harboured a 21-bp exon 11 deletion (**Figure 3.1**) and one of the 41 subcutaneous MCTs carried an ITD.

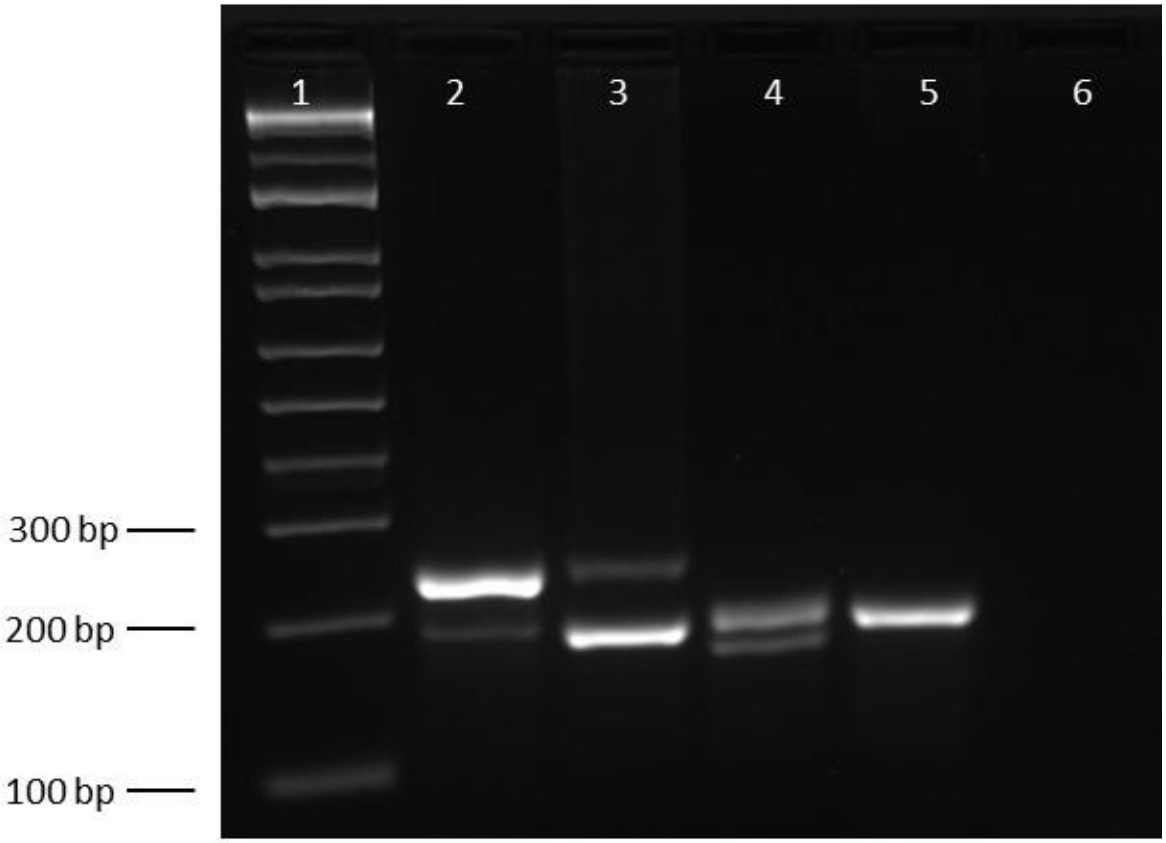

Figure 3.1. Agarose gel electrophoresis of genomic DNA extracted from three formalin-fixed, paraffin-embedded canine mast cell tumour blocks amplified by PCR using exon 11 *KIT* gene primers. Wild-type PCR product is 190 base pairs (bp), internal tandem duplication PCR products are larger (represented by the upper bands in lanes 2 and 3) and the deletion mutant is smaller (represented by the lower band in lane 4). Lane 1: 1 Kb DNA ladder marker. Lane 2: sample A11; 48-bp ITD. Lane 3: sample A91; 60-bp ITD. Lane 4: sample M29; 21-bp deletion. Lane 5: positive canine testis control DNA; wild-type *KIT*. Lane 9: negative control (water).

The length of the ITDs ranged from 25- to 60-bps and resided closer to the 3' end of exon 11. Nine ITDs extended into intron 11 (**Figure 3.2**). The ITD extending the furthest into intron 11 finished 35 nucleotide bases from the 5' end of the reverse primer. The genomic location and the sequences of the 24 ITDs and the single 21-bp deletion can be found in **Appendix D** and **Appendix E**, respectively.

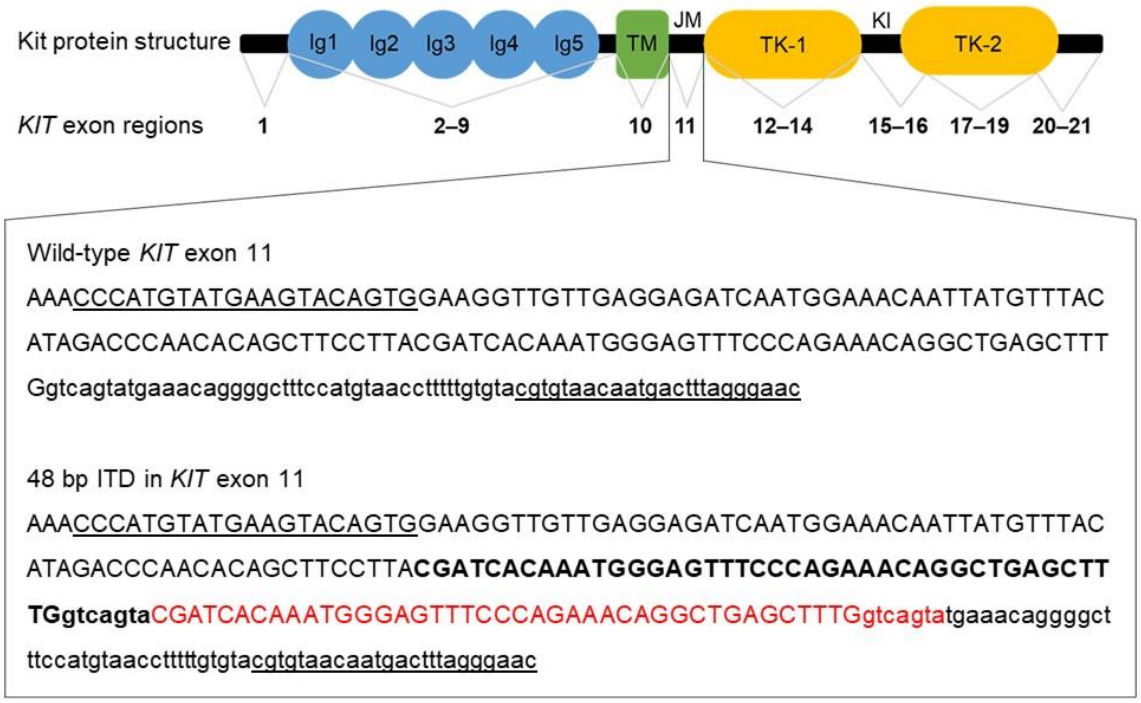

Figure 3.2. Structure of Kit protein and corresponding *KIT* gene exon regions. Noted are the immunoglobulin-like domains (Ig1-5), transmembrane domain (TM), juxtamembrane domain (JM), tyrosine kinase domains (TK-1, TK-2) and the kinase insert (KI). The textbox represents the wild-type DNA sequence of exon 11 (uppercase text) and part of intron 11 (lowercase text) of the canine *KIT* gene. Primer sequences are underlined. Also shown is the sequence of a 48-base pair (bp) internal tandem duplication (ITD) detected in a mast cell tumour. The region of the *KIT* sequence that is duplicated is in bold text and the duplication is in red lettering.

Sequence chromatograms were reviewed in Chromas for exon 11 ITD sequence confirmation (**Figure 3.3**). ITD sequences were confirmed in the forward and reverse directions. A C>T SNP was identified in eight MCT DNA samples; gDNA position 47,178,598, mRNA position 1731, amino acid position 577. Five samples had a heterozygous C/T genotype and three had a homozygous T/T genotype, resulting in a T allele frequency of 22.9%. This SNP results in a codon change from TAC to TAT but the corresponding amino acid, tyrosine, is conserved. The remaining samples were homozygous for the reference allele (C/C).

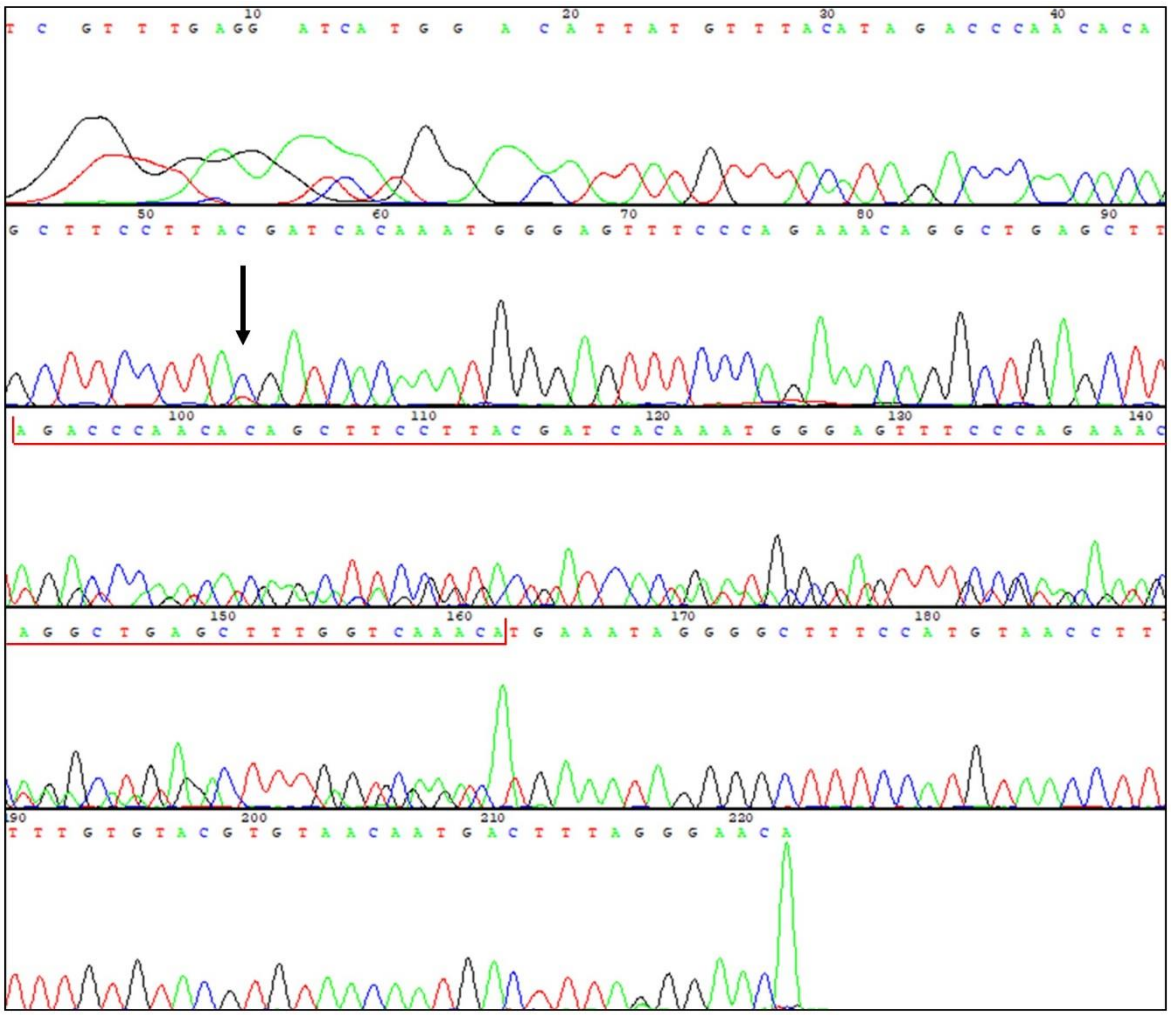

Figure 3.3. Chromatogram visualised in Chromas (version 2.4.3, Technelysium Pty Ltd, QLD, Australia) of a 60-base pair internal tandem duplication (ITD) in exon 11 of *KIT* from a cutaneous canine mast cell tumour. The arrow shows heterozygous C/T genotype for the synonymous single nucleotide polymorphism rs853024368 (ensembl.org). The double peaks observed from the nucleotide bases underlined in red indicate an ITD.

3.4 Discussion

KIT exon 11 ITD mutations were detected in 10% (n = 24/239) of cutaneous MCTs. One low-grade (Patnaik grade II) MCT harboured a 21-bp deletion and one subcutaneous MCT harboured a 54-bp ITD. This is the first report of an exon 11 ITD in a subcutaneous MCT, although one recent report describes a 12-bp exon 8 ITD in one of 11 dogs with subcutaneous MCT (Vozdova *et al.*, 2019b).

The 280 MCTs were sourced from 248 dogs. Of the dogs with multiple tumours, one dog with four cutaneous lesions possessed a 42-bp ITD in one tumour, but only wild-type exon 11 *KIT* DNA was detected in the other three tumours. Two different dogs, who both had two

tumours, harboured an ITD in one lesion but possessed only wild-type DNA in their other tumour. Another dog with two tumours possessed an identical 48-bp ITD in both lesions. In the latter case, the tumours may have developed as a result of metastatic spread, hence both lesions harbouring the same ITD. Comparatively, dogs with multiple tumours of different *KIT* mutation status may have grown independently with the mutations arising somatically. However, this cannot be guaranteed from the available data. Regardless, in the case of multiple MCT, these data advocate genetic testing of each tumour to optimise prognostication and for predicting treatment response.

Reflecting earlier research, exon 11 ITDs were more frequently detected in Kiupel high-grade tumours when compared to low-grade tumours (40% versus 1.09%, respectively). No mutations were detected in Patnaik grade I tumours, whereas 2.76% and 42.2% of Patnaik grade II and III tumours, respectively, harboured an ITD. The grade I class from the Patnaik schematic may be useful for identifying MCTs from Australian dogs that are unlikely to harbour an exon 11 ITD without the need for molecular testing. However, only six grade I tumours were included in the current study reducing the strength of this interpretation. Further, international studies have reported *KIT* mutations in Patnaik grade I MCT DNA, including an exon 11 ITD from one MCT in an American study (Downing *et al.*, 2002, Giantin *et al.*, 2012, Mochizuki *et al.*, 2017b). Utilising the Patnaik schematic for *KIT* mutation prediction is an interesting concept that requires exploration in a larger tumour cohort.

In the current study, the most common ITD was a 45-bp duplication and was observed in three tumours (**Appendix D**). The ITD extending the furthest into intron 11 extended 35 nucleotide bases 5' of the reverse primer (**Figure 3.2**). This distance between the end of the duplication and the beginning of the primer binding site is large enough such that the ITD mutation should not significantly influence primer ability to bind and amplify *KIT* DNA and impede MCT DNA PCR amplification.

In instances where ≥ 2 nucleotide bases are detected at the same position in a DNA sequence, Sanger sequencing technology calls the most dominant allele. Minor allele variants are not reported in the resulting DNA sequence file. Minor alleles can be observed by manually reviewing the sequence chromatograms (**Figure 3.3**). Using this method, a synonymous c.1731C>T SNP was observed with an allele frequency of 22.9% in the 24 ITD sequences. The genotype at this position for the tumour with the 21-bp deletion was not determined because this variant falls within the deleted region. This variant has previously been reported at a

similar frequency (24.6%) in other canine *KIT* MCT research investigations (Riva *et al.*, 2005, Zemke *et al.*, 2002) and is registered on the ensembl.org database with an 18% frequency (http://asia.ensembl.org/Canis_familiaris/Variation/Explore?r=13:47178098-47179098;v=rs853024368;vdb=variation;vf=6113378, accessed 23/04/2019). This C>T SNP is also documented in a non-MCT related multi-breed canine variant discovery research project evaluating 213 genotypes in 12 dog breeds and four wolf populations (UniBE - Institute of Genetics, 2012). It is for this reason that this SNP is assumed to be a species-specific variant and not associated with MCT.

The current finding of a 10% *KIT* exon 11 ITD prevalence in cutaneous MCTs is lower than the 17.6% ITD prevalence established in a smaller, preliminary Australian investigation, however, remains within the 95% confidence interval range (8.9–26.2%) (Tamlin *et al.*, 2017). The current investigation is presumed to be a more accurate representation of the true exon 11 ITD prevalence in MCTs from Australian dogs due to a considerably larger sample size and the inclusion of more low-grade MCTs which more accurately reflects the proportion of tumour types seen in general practice. Low-grade MCTs represented 70.3% and 77.0% of the tumours in the preliminary study and current study, respectively. The increased frequency of low-grade MCTs in the present study explains the decrease in overall exon 11 ITD prevalence in the present findings. It is possible that the overall ITD prevalence in MCTs from isolated Australian canine populations more closely resembles the prevalence seen in European populations and is indeed lower than that documented in dogs from the USA and Japan (**Appendix C**). Notably, the majority of international investigations sourced their MCTs from University veterinary diagnostic laboratories, teaching hospitals or referral hospitals, all of which are intrinsically biased towards more aggressive cases. Hence, it is not unreasonable to speculate that the reported exon 11 ITD prevalences are an overestimation of the true population prevalence. Further research needs to be conducted to confirm the propositions of geographical differences and biased population sampling.

Ultimately, the overall prevalence of exon 11 ITD mutations in any canine MCT research investigation would depend on histological grade distributions. Inability to amplify low-grade MCT DNA would skew the data to report a higher prevalence. Further, general practices may not see as many instances low-grade MCTs due to the benign nature of some cases. These lumps may go unnoticed by dog owners or not be of enough concern for a visit to the veterinary clinic. Hence, these tumours may go undiagnosed, skewing prevalence

studies to report an increased frequency of high-grade MCTs and a corresponding proportional increase in exon 11 ITD prevalence. The distributions of histological tumour grades seen at primary veterinary practices may vary between economic, social and cultural regions, between rural and urban districts and potentially between countries, which may influence prevalence.

Cutaneous MCTs have a highly variable prognosis with high-grade tumours presenting the greatest risk for patient MCT-related mortality (Horta *et al.*, 2018b). However, it is the intermediate-grade tumours which pose the greatest difficulties to veterinary oncologists and pathologists due to their unpredictable biological behaviour and accompanying unclear treatment recommendations. Agreeing with previous research, Patnaik grade II MCTs accounted for the majority of cutaneous tumours in this study (75.7%, n = 181/239) and a high degree of inter-observer variation existed between the pathologists when grading these tumours (Kiupel *et al.*, 2011, Mochizuki *et al.*, 2017a, Sabattini *et al.*, 2015, Takeuchi *et al.*, 2013, Tamlin *et al.*, 2017). Histological grading concordance between the three pathologists was 75.7% (n = 56/74) and 91.9% (n = 68/74) when using the Patnaik and Kiupel grading schemes, respectively. Consistent with previous data, use of the Kiupel grading scheme provided higher consistency and less ambiguity than when applying the 3-tier Patnaik system (Kiupel *et al.*, 2011, Sabattini *et al.*, 2015). The greatest variation surrounded the use of Patnaik's grade I and II classes (**Table 3.1**). Ambiguous histopathology may result in some tumours falling into grade II category to account for the worst-case scenario. This can lead to the unnecessary treatment of benign tumours in some instances. It is for these reasons that the Kiupel grading system is preferred by many veterinarian oncologists and pathologists. Nonetheless, pathologists typically grade each tumour according to both schematics.

3.5 Conclusion

KIT exon 11 ITDs are prevalent in 10% of cutaneous MCTs from Australian dogs. This is lower than the 17% prevalence reported in the preliminary investigation, however, can be attributed to the inclusion of a higher proportion of low-grade MCTs. An increased frequency of ITDs detected in high-grade when compared to low-grade MCTs is consistent with international data. An exon 11 ITD was detected, for the first time, in a subcutaneous MCT.

Chapter 4: Establishing a *KIT* mutation profile in 77 cutaneous and 18 subcutaneous canine mast cell tumours

4.1 Terminology for the current chapter

3′ untranslated region (3′ UTR): the section of the mRNA that immediately follows the translation termination codon and which is not translated to protein.

5′ untranslated region (5′ UTR): the section of the mRNA that immediately precedes the translation initiation codon and which is not translated to protein.

Conservative inframe insertion: where one or more nucleotide triplets are inserted into the coding sequence, increasing the length of the coding sequence but without changing the reading frame. The same applied for **conservative inframe deletion** where nucleotides are deleted.

Contig: a series of overlapping DNA fragments used to construct a consensus region of DNA.

Downstream: the section of gDNA that is 3′ of the mRNA coding sequence and which is directly adjacent to the 3′ UTR.

Disruptive inframe insertion: where one or more nucleotide triplets are inserted into the coding sequence within an existing codon resulting in increased coding sequence length and altering the reading frame of the downstream sequence. The same applied for **disruptive inframe deletion** where nucleotides are deleted.

Frameshift: the insertion into or deletion from the coding sequence of nucleotides that disrupt the translational reading frame.

Gain-of-function mutation: where a mutation in the gDNA alters the gene product such that its effect gets stronger or even is superseded by a different and abnormal function, including the modification of gene or protein expression. Used interchangeably with "activating" mutation.

Homopolymer-associated indel error: the insertion or deletion of a nucleotide residue in the region of the DNA sequence where ≥ 3 of the same nucleotide residue occur consecutively.

Missense mutation: a non-synonymous nucleotide base change resulting in a codon change which leads to a change in the corresponding amino acid.

Non-coding domains: *KIT* gene 5′ and 3′ UTRs, upstream region, downstream region and splice regions.

Nonsense mutation: a change in the nucleotide base sequence resulting in the gain of a premature termination (stop) codon.

On-target reads: percentage of the total number of nucleotides that aligned within the target region out of the total number of aligned nucleotides.

Sequence coverage: percentage of how much of the targeted region is sequenced.

Sequencing depth: the average number of times a nucleotide has been sequenced.

Splice region variant: intronic alterations that occur within 1–3 nucleotide bases of the exonic coding regions.

Synonymous variant or silent mutation: a nucleotide base change resulting in a codon change, but which does not alter the corresponding amino acid.

Upstream: the section of gDNA that is 5′ of the mRNA coding sequence and which directly adjacent to the 5′ UTR. Includes the promotor.

4.2 Introduction

KIT is a known genetic contributor to the normal and neoplastic biology of mast cells in humans, cats and dogs. In dogs with mast cell tumours (MCTs), *KIT* mutations in the juxtamembrane domain of the Kit protein, specifically exon 11 internal tandem duplications (ITDs), are evident in over 40–50% of high-grade tumours (Chapter 3) (Downing *et al.*, 2002, Tamlin *et al.*, 2017). Mutations also frequently reside in canine *KIT* exons 8 and 9 which encode the Kit protein regulatory domain. Mutations occur less frequently in exons 14 and 17, which encode the enzymatic pocket domain, but are still clinically important (Tamlin *et al.*, 2019a). Many *KIT* mutations have been shown to activate the Kit protein in the absence of Kit-ligand binding (Tamlin *et al.*, 2019a). Whether *KIT* mutations initiate mast cell tumourigenesis, or if the mutations develop as secondary events following primary genetic anomalies is unknown. Further, *KIT* mutations are not involved in every canine MCT case with only 4–13% of Kiupel low-grade tumours possessing a mutation within exons 8, 9 or 11 (**Table 1.1**). The entire *KIT* gene is seldom sequenced in canine MCT molecular investigations, so it is possible that gain-of-function mutations exist outside canine *KIT* mutation hotspots and that these are missed in the majority of studies. Evaluating the *KIT* gene coding sequence in its entirety to identify mutations outside *KIT* gene mutation hotspots using next-generation sequencing (NGS) technology may highlight additional regions of *KIT* involved in MCT pathogenesis.

Advancements in NGS technologies have revolutionised the study of genomics and molecular biology, allowing for quicker and cheaper DNA and RNA sequencing when compared to traditional methods such as Sanger sequencing. AmpliSeq™ Ion Torrent™ Personal Genome Machine NGS technology (referred to herein as Ion AmpliSeq technology) is a highly specific and sensitive amplicon-based enrichment method for targeted NGS. This technology exploits emulsion PCR and pyrosequencing to determine DNA nucleotide base sequences of fragments less than 200 base pairs (bp) (Rothberg *et al.*, 2011). Benefits of Ion AmpliSeq technology over other NGS platforms include minimal DNA input, high data throughput and short running time (Loman *et al.*, 2012, Rathi *et al.*, 2017). Three differently priced sequencing chips are also available, providing flexibility when designing experiments based on the throughput required.

Amplicon-based multiplexed PCR methodologies are ideal for investigating a relatively small number of genes or small target regions and can accommodate formalin-fixed, paraffin-

embedded (FFPE)-degraded DNA (Abed and Dark, 2016, Dedhia *et al.*, 2007). It is for these reasons that Ion AmpliSeq technology was chosen for *KIT* exome sequencing herein to establish the *KIT* mutation profile from canine cutaneous and subcutaneous MCTs.

4.3 Methods: AmpliSeq library preparation and Ion Torrent sequencing

4.3.1 Primer design

The canine *KIT* gene exon coordinates were sourced from University of California Santa Cruz (UCSC) Genome Browser using CanFam3.1 Assembly (UCSC 2011, http://genome.ucsc.edu/, accessed 10/05/2017). In the first instance, primers were designed to flank the *KIT* exonic regions in order to capture all genetic variants existing within the *KIT* coding sequence. Primers flanking intron 11 were also designed to capture the deletion of the entire intron because the deletion of intron 11 has been suggested to be involved in MCT pathogenesis (Reguera *et al.*, 2002). Final amplicons were designed to be less than 150 bp in length to account for fragmented FFPE-extracted DNA. Some exons were covered with one primer pair, whereas other exons required multiple primer pairs with overlapping amplicons to ensure full coverage. Two primer pools were assembled for DNA library construction to avoid overlap between the primers of adjacent amplicons.

4.3.2 Sequencing depth

Sequence coverage of 250X is recommended for detecting low-frequency variants (Rathi *et al.*, 2017). In the present study, the MCT DNA samples were extracted from FFPE blocks of MCT specimens surrounded by normal, non-neoplastic tissue. No attempt was made to isolate the neoplastic cells from the non-neoplastic cells. Tissue sections were estimated to consist of approximately 80% MCT tissue, and 20% surrounding skin and non-tumour cells. It was also estimated that as few as 3% of cancerous mast cells may harbour a *KIT* mutation. For these reasons, a sequencing depth of 500X per sample was desired, limiting the investigation to 100 samples on two sequencing chips (see **Appendix F** for calculations).

4.3.3 Sample selection

Canine cutaneous and subcutaneous MCT DNA samples were randomly selected from the cohort of 280 tumours described in Chapter 2 and were used for Ion AmpliSeq library preparation. Random sample selection was done by listing each MCT DNA sample in an Excel

(2013) spreadsheet. Random numbers between 1 and 280 were then assigned to each sample using the RANDBETWEEN() function. The samples that were assigned numbers 1 to 99, inclusive, were used in subsequent AmpliSeq library preparation and whole exome mutational analysis. The 100th sample in the library preparation was the DNA extracted from the testes of a healthy dog, and this was used as a control by representing the normal sequence of the canine *KIT* gene.

4.3.4 DNA preparation and target amplification

The 99 randomly selected MCT DNA samples and the normal canine testis control DNA sample were used in constructing the libraries according to the Ion AmpliSeq™ DNA Library Kit 2.0 (Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) protocol for two primer pools (**Figure 4.1**). Each library was prepared using a unique barcode (IonCode Barcode Adapters, Ion Torrent).

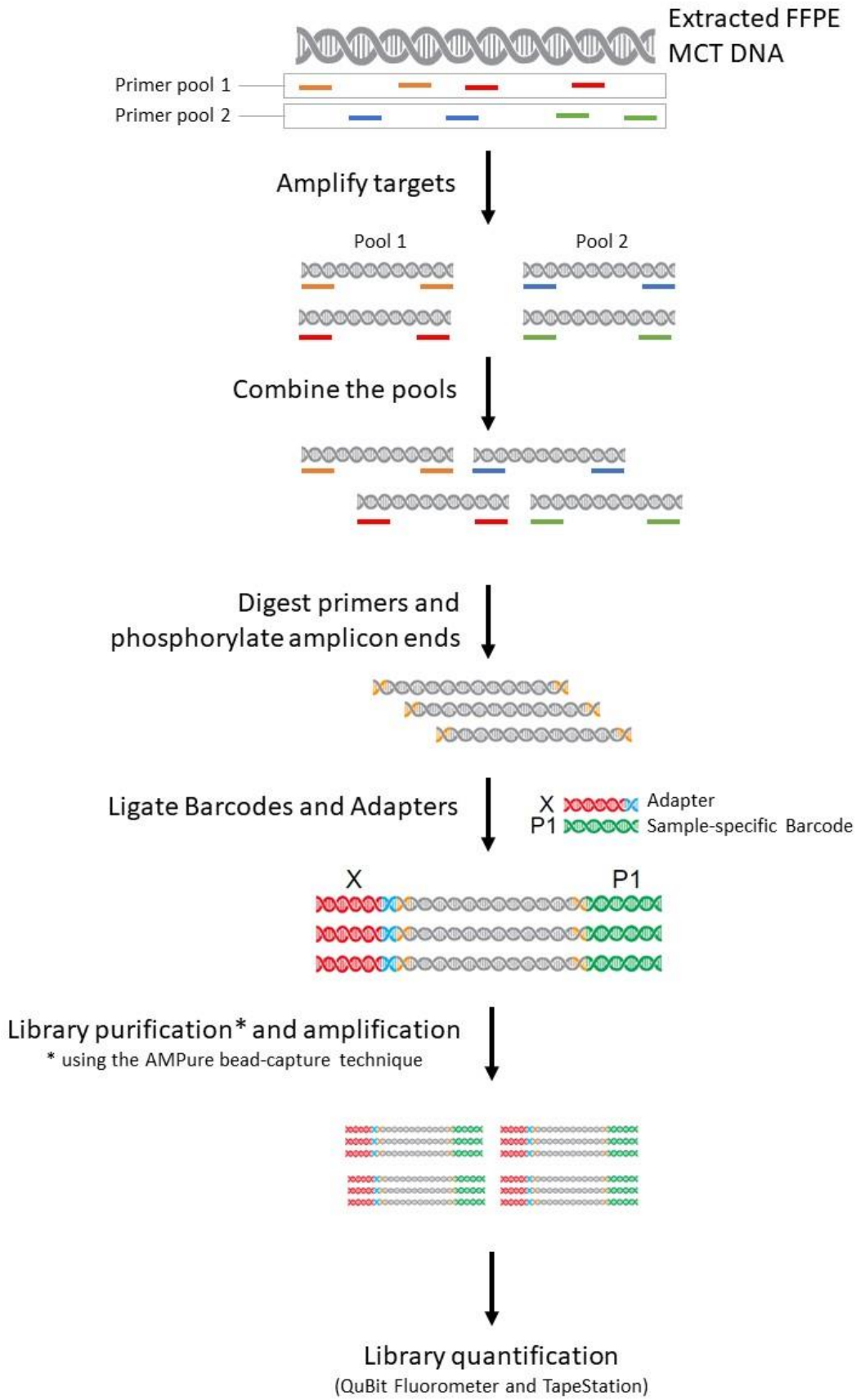

Figure 4.1. Ion AmpliSeq™ library preparation workflow. Adapted from Ion AmpliSeq™ Library Kit 2.0 User Guide. FFPE: formalin-fixed, paraffin-embedded. MCT: mast cell tumour. * See **Figure 4.2** for AMPure bead-capture technique.

A PCR Mastermix was prepared on ice for each primer pool consisting of volumes of 2 µL of 5X Ion Ampliseq™ HiFi Mix and 5 µL of 2X Ion Ampliseq™ Primer Pool (1 or 2) per sample. An aliquot of seven microliters of Mastermix Pool 1 was dispersed into a well of a 96-well twin.tec® real-time PCR Plate (Eppendorf, Hamburg, Germany). Seven microliters of Mastermix Pool 2 were dispersed into the neighbouring column of the 96-well PCR Plate. This was done carefully to ensure no mixing between the primer pools.

The desired amount of DNA in each well was 50–60 ng and the total reaction volume was not to exceed 10 µL. FFPE-extracted MCT DNA sample concentrations were measured on the Qubit 3.0 Fluorometer (Ion Torrent, Life Technologies, Carlsbad, CA, USA) using QuBit dsDNA HS Assay Kit (Ion Torrent) according to manufacturer's instructions. Where necessary, DNA samples were diluted to approximately 20 ng/µL and 3 µL of DNA were added into each well. For samples with a concentration < 16 ng/µL, the number of PCR cycles was increased to ensure adequate amplification of target regions (**Table 4.1**). Once the sample DNA had been added to each pool, the 96-well PCR Plate was sealed with a MicroAmp Optical Adhesive Film (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and the samples were mixed by vortexing at 1,450 rpm for 30 seconds and briefly centrifuged to collect the solution the bottom of the wells before undergoing PCR target amplification in the RealPlex2 MasterCycler (Eppendorf).

Table 4.1. Cycling conditions for target amplification.

Stage	Temp	Time
Hold	99°C	2 min
22-24 cycles*	99°C	15 sec
	60°C	4 min
Hold	10°C	Hold

* The number of cycles required for optimal DNA amplification was adjusted according to DNA sample concentration. Samples measuring ≤ 8 ng/µL, 8–16 ng/µL and ≥ 16 ng/µL were amplified for 24, 23 and 22 PCR cycles, respectively.

4.3.5 Primer digestion and Barcode Adapter ligation

After target region amplification, Pool 1 (column A on the 96-well PCR plate) was combined with Pool 2 (column B on the 96-well PCR plate). Two microliters of FuPa Reagent (**Appendix B**) were added to each sample to partially digest the primers and phosphorylate the extremities of the amplicons. The primers were selectively targeted for digestion because they incorporate uracil instead of thymine and were removed to avoid interfering with

subsequent DNA library construction processes. Phosphorylation of the amplicon ends was important for Barcode adapter ligation. The 96-well PCR plate was sealed with a new optical adhesive film, vortexed at 1,450 rpm for 30 seconds, briefly centrifuged and incubated in the MasterCycler for digestion (**Table 4.2**).

Table 4.2. Cycling conditions for primer digestion.

Temperature	Time
50°C	10 mins
55°C	10 mins
60°C	20 mins
10°C	Hold (up to 1 hr)*

* Libraries were able to be stored overnight at -20°C.

After primer digestion, the pH of each sample was adjusted with 4 µL of Switch Solution (**Appendix B**) to create an environment appropriate for sample-Barcode ligation. Two microliters of sample-specific IonCode Adapters Barcodes were added to each sample and the 96-well PCR plate was sealed with a new adhesive seal, vortexed and centrifuged before adding in 2 µL of DNA ligase to each library. The 96-well PCR plate was sealed with an optical adhesive film, vortexed and centrifuged for amplicon-Barcode ligation in the MasterCycler (**Table 4.3**).

Table 4.3. Cycling conditions for sample-Barcode ligation.

Temperature	Time
22°C	30 mins
68°C	5 mins
72°C	5 mins
10°C	Hold (up to 24 hrs)

4.3.6 Initial library purification

Barcode-ligated AmpliSeq libraries were purified from PCR reagents using the AMPure bead-capture technique (**Figure 4.2**). Agencourt AMPure XP Reagent (magnetic beads; Beckman-Coulter, Brea, CA, USA) was brought to room temperature for at least 30 minutes and 45 µL (1.5X the sample volume) were mixed with each Barcode-ligated library. The plate was sealed then vortexed at a slower speed of 1200 rpm. The mixture was visually checked to ensure proper homogenisation of the magnetic beads with the DNA library mixture. The plate was briefly centrifuged to remove potential residue from the adhesive seal before its removal. The plate was then incubated at room temperature for 5 minutes to allow for the DNA amplicons to bind to the carboxyl molecules on the AMPure beads' surface. The beads were

captured using the 96-well Low Elution Magnet Plate, referred to herein as the magnet (Alpaqua Engineering, LLC; Beverly, MA, USA), leaving PCR contaminants suspended in the solution. The adhesive film was carefully removed, and the supernatant was aspirated and discarded. The 96-well PCR plate was removed from the magnet and 150 µL of freshly prepared 70% ethanol* was added and mixed with the Libraries by gently pipetting up and down five times before placing the plate back on the magnet for 2 minutes to capture the AMPure beads. The supernatant was aspirated and discarded, and the washing step was repeated once more before samples were left to air dry for 5 minutes at room temperature (**Figure 4.2**). Note that the published Ion AmpliSeq™ DNA Library Preparation Quick Guide protocol recommends to "move the plate side-to-side in the two positions of the magnet to wash the beads". The type of magnet used in the current protocol does not have two positions, hence, this step was performed as per the instructions located in the full instruction manual: Ion AmpliSeq™ Library Kit 2.0 User Guide, publication number MAN0006735, revision E.0. See **Appendix G** for a detailed explanation.

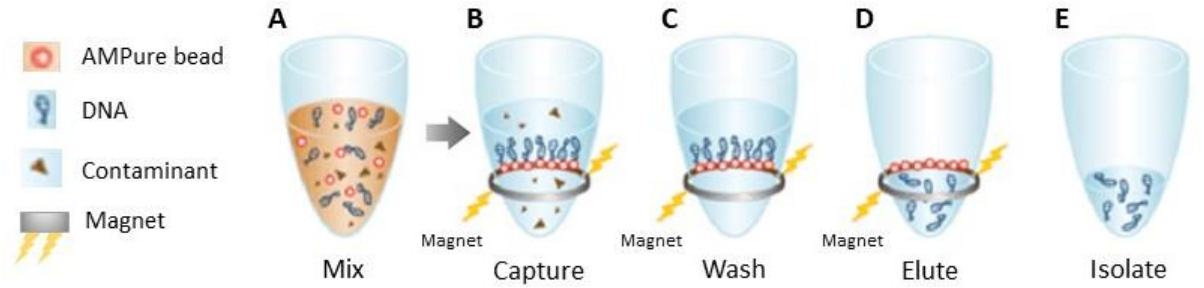

Figure 4.2. Ion AmpliSeq™ library purification workflow using the bead-capture technique. A: AMPure beads (red spheres) are mixed with the crude sample. The AmpliSeq™ libraries (blue DNA helices) bind to the beads and the contaminants (black triangles) remain suspended in the solution. B: the beads are captured using the magnet (grey circle). C: the contaminating solution is removed and the beads are washed with 70% ethanol. D: an aqueous solution is used to elute the Libraries from the beads. E: the Libraries are transferred to a clean tube. Image adapted from http://enseqlopedia.com/2012/04/how-do-spri-beads-work/, accessed 18/12/2019.

4.3.7 Library Amplification

The 96-well PCR plate was removed from the magnet and each library was mixed with 50 µL of Platinum PCR SuperMix HiFi and 2 µL of Library Amplification Primer Mix, creating an aqueous solution to elute the DNA from the beads. The magnet was used to capture the

* The 70% ethanol must be prepared fresh before use to reduce evaporation of the ethanol and absorption of water from the atmosphere. If the ethanol solution is too dilute, the DNA may disassociate from the AMPure beads and dissolve into the supernatant which is aspirated and discarded.

AMPure beads and 50 µL of the supernatant containing the libraries was transferred to a clean well for amplification in the MasterCycler (**Table 4.4**).

Table 4.4. Cycling conditions for library amplification.

Stage	Temperature	Time
Hold	98°C	2 mins
5 cycles	98°C	15 secs
	64°C	1 min
Hold	10°C	Hold*

*samples were able to be stored at -20°C.

4.3.8 Final library purification and quantification

The amplified libraries were purified at room temperature using the magnetic bead-capture technique in a two-round process and according to manufacturer's instructions (Ion AmpliSeq™ Library Kit 2.0 User Guide, publication number MAN0006735, revision E.0). In the first round, 0.5X sample volume (25 µL) of AMPure beads was mixed with each library. At this concentration, high molecular-weight contaminants are bound to the beads, while amplicons and primers remain in the solution. The beads were then captured on the magnet and the supernatant (~75 µL volume containing the amplicon-libraries) was transferred to a clean well. Second round purification involved the addition of 1.2X sample volume (60 µL) of AMPure beads to the libraries and incubation at room temperature for 5 minutes. At this ratio, the amplicons bind to the beads and the primers remain in solution. The AMPure bead-bound libraries were captured by the magnet and the supernatant containing the contaminating primers was aspirated and discarded.

The bead-bound libraries were further purified with the addition of 150 µL of freshly prepared 70% ethanol and incubation at room temperature for 3 minutes. The AMPure beads were then captured by the magnet and the supernatant was removed. This step was repeated a second time before the AMPure beads were left to air dry for 5 minutes.

The 96-well PCR plate was removed from the magnet and 50 µL of Low TE (**Appendix B**) were added to each well, creating an aqueous environment for amplicon-library elution. The plate was sealed, vortexed to disperse the beads and centrifuged to remove the residual solution from the walls of the well. The samples were incubated at room temperature for 2 minutes then the magnet was used to capture the beads. The supernatant containing the final library was transferred to a clean well, being careful not to disturb the AMPure bead pellet.

The AmpliSeq libraries were quantified on the QuBit 3.0 Fluorometer and analysed on a 2200 TapeStation (Integrated Sciences, Agilent Technologies, Santa Clara, CA, USA). TapeStation analyses were performed using the D1000 ScreenTapes (Integrated Sciences, Agilent Technologies) according to the manufacturer's instructions to confirm successful library construction.

4.3.9 Library pooling

AmpliSeq libraries were allocated into one of two pools according to the library sample concentration. Library Pool 1 was prepared using one nanogram of DNA from each of the 50 libraries with the highest concentration. Library Pool 2 was prepared from the 50 libraries with the lowest concentration. Of the samples allocated to Library Pool 2, 20% (n = 10/50) were of such a low concentration that 1 ng of DNA could not be sourced from the sample even if the whole volume was used. For this reason, 0.5 ng of each AmpliSeq library sample were combined for Library Pool 2.

4.3.10 Ion Torrent 316 sequencing chip loading and Ion Torrent Sequencing

Two sequencing chips (Ion 316™ Chip v2 BC, Thermo Fisher Scientific) were used for Ion Torrent PGM sequencing. Using the Ion Chef™ Instrument (Ion Torrent), 6 µg (60 pmol) of Library Pools 1 and 2 were loaded onto respective sequencing Chips 1 and 2. The chips were sequenced at the Lotterywest State Biomedical Facility Genomics at the University of Western Australia using the Ion Torrent™ PGM system (Ion Torrent).

4.3.11 Sequence alignment, data annotations and variant filtering

The sequencing output data were provided in the format of BAM (binary sequence alignment map) files. A unique script was constructed to align the sequencing data to NCBI CanFam3.1 canine reference genome (GCF_000002285.3; NC_006595.3) (NCBI, 2018). All data transformation and variant calling were performed in Ubuntu GNOME Terminal 3.18.3 using VTE version 0.42.5 +GNUTLS and Bash command language. The BAM files were converted to a genomic variant call format (GVCF) and HaplotypeCaller software from the Genome Alignment Tool Kit (GATK, version 4) to identify genetic variants. Thereafter, the data were combined into a single GVCF file using GATK CombineGVCFs function and annotated with GenotypeGVCF. The latter function merges the genotype records for each sample at each gDNA position and gives the likelihood of the genotype at each position. Lastly, the SnpEff tool

(version 4.3T) predicted the consequence of each identified variant.

The final annotated sequencing file was opened in Excel for further evaluation. Variants that aligned to a sequence outside the *KIT* gene were removed from the analysis. Similarly, genetic alterations in the intronic regions of *KIT* were disregarded. To ensure only the highest quality data remained, all variants detected in the *KIT* coding region or in *KIT* gene splicing domains were filtered against pre-determined parameters based on the generic filtering recommendations described by GATK (**Table 4.5**).

Table 4.5. Quality control criteria applied to sequence variants for the removal of erroneous data as recommended by GATK (Van der Auwera, 2013).

Parameter	Cut-off values[1]	Description
Quality Score	< 20	Phred-scaled quality score representing the confidence of the assigned variant by the sequencer. A score of ≥ 20 indicates a ≥ 99% chance of being correct.
Read Depth	< 1,000	Combined depth across the sample.
Excess Heterozygosity	> 54.69	The one-sided Phred-scaled P-value for the exact test of the Hardy-Weinberg Equilibrium. The higher the score, the higher the chance that the variant is a sequencing artefact or that there is inbreeding within the population.
Mapping Quality	< 40	Log-scaled estimation to indicate the confidence that the allele mapped to a particular position in the genome is correct.
Mapping Quality Rank Sum	< -12.5	Estimation of overall mapping quality of the reads supporting a variant call.
Quality by Depth	< 2	Quality Score normalised by the amount of coverage available. Limiting parameter for homopolymer-associated indel errors.[2]
Read Position Rank Sum	< -8 for SNP, < -20 for InDel	Suggests evidence of bias in the position of the alternate alleles within the reads. The ideal result is a value closer to zero, which indicates there is little bias in where the alternate alleles are found relative to the ends of reads.
Strand Odds Ratio	> 3 for SNP, > 10 for InDel	Evaluates sequencing bias between the forward and reverse strands. Higher values indicate one strand is favoured over the other.

[1] The variant is removed if the score is less than (<) or more than (>) the defined cut-off value.
[2] See section 4.1 for the definition of a homopolymer-associated indel error.

4.3.12 *False-positives and homopolymer-associated errors*

A 3% detection frequency threshold was applied to the data as recommended for Ion AmpliSeq NGS (Rathi *et al.*, 2017). Variants that were detected in less than 3% of the sample DNA were deemed to be false-positives and were removed from the dataset.

Homopolymer-associated errors (see definition in section 4.1) were detected by visually reviewing all the indel variants in Integrative Genomics Viewer (IGV; version 2.3.93). Detected erroneous data were removed from the analysis.

4.3.13 *Evaluation against public canine reference SNP and indel databases*

Called variants were searched for in a publicly available multi-breed canine variant discovery research database (UniBE - Institute of Genetics, 2012). This database evaluated 213 genotypes in 12 dog breeds and four wolf populations. The ensembl.org canine variant reference database was also used for comparison (ftp://ftp.ensembl.org/pub/release-68/variation/gvf/canis_familiaris/Canis_familiaris.gvf.gz, accessed 30/08/2018).

4.3.14 *Predicting the mutation effect on KIT mRNA secondary structure*

Secondary mRNA structure and minimum free energy (MFE) of wild-type canine *KIT* and mutant-*KIT* were predicted using the ViennaRNA Package 2.0 version 2.4.12 (Lorenz *et al.*, 2011).

4.3.15 *Investigating the deletion of intron 11*

The deletion of intron 11 in its entirety has been suggested to be implicated in canine MCT pathogenesis (Reguera *et al.*, 2002). Primers flanking intron 11 were designed to capture the deletion. The Ion Torrent sequence output data and the IGV aligned sequences were screened for the deletion.

To explore the possibility of a *KIT*-derived pseudogene, exons 11 and 12 of the *KIT* mRNA gene sequence (GenBank accession number AF044249, nucleotide bases 1737–1899) were aligned to the canine reference genome (CanFam3.1; GCF_000002285.3; NC_006595.3) using NCBI BLAST.

4.4 Results

4.4.1 KIT gene general information

The canine *KIT Proto-Oncogene* is located on CFA13 at position 47,144,084–47,190,029 of the forward strand. The entire canine *KIT* gene coding sequence is 3,090 bp and is comprised of 21 exons which range from 37 to 327 bp in length (**Appendix H**). Two splicing variants exist for canine Kit protein; KIT-201 (ENSCAFT00000049830.2) and KIT-202 (ENSCAFT00000003274.3); **Figure 4.3**. The two transcripts differ in the exon 1 sequence, and by the deletion of GNSK amino acids (12-bp deletion GGTAACAGCAAA) at the 3' end of exon 9 in the KIT-202 variant (**Figure 4.4**).

A

Name	Transcript ID	bp	Protein	Translation ID	Biotype	UniProt
KIT-201	ENSCAFT00000049830.2	5884	975aa	ENSCAFP00000039467.1	Protein coding	O97799
KIT-202	ENSCAFT00000003274.3	3090	963aa	ENSCAFP00000003042.3	Protein coding	F1PUW9

B

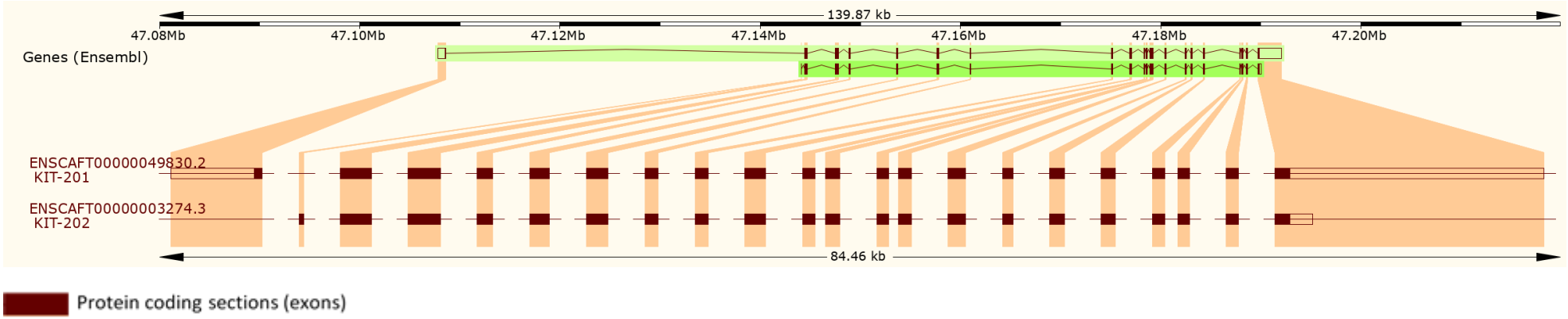

Figure 4.3. Canine Kit protein splice variants; KIT-201 and KIT-202. A: Transcript information for the two variants. Displayed are the splice variants (Name), Transcript ID's, *KIT* mRNA length in nucleotide base pairs (bp), Kit protein length in amino acids (aa), Translation ID, Biotype and UniProt ID. B: Visual representation of the two Kit protein splice variants. Source: screenshots from http://asia.ensembl.org/Canis_familiaris/Gene/Splice?db=core;g=ENSCAFG00000002065;r=13:47144084-47190029;t=ENSCAFT00000003274; accessed 02/08/2019.

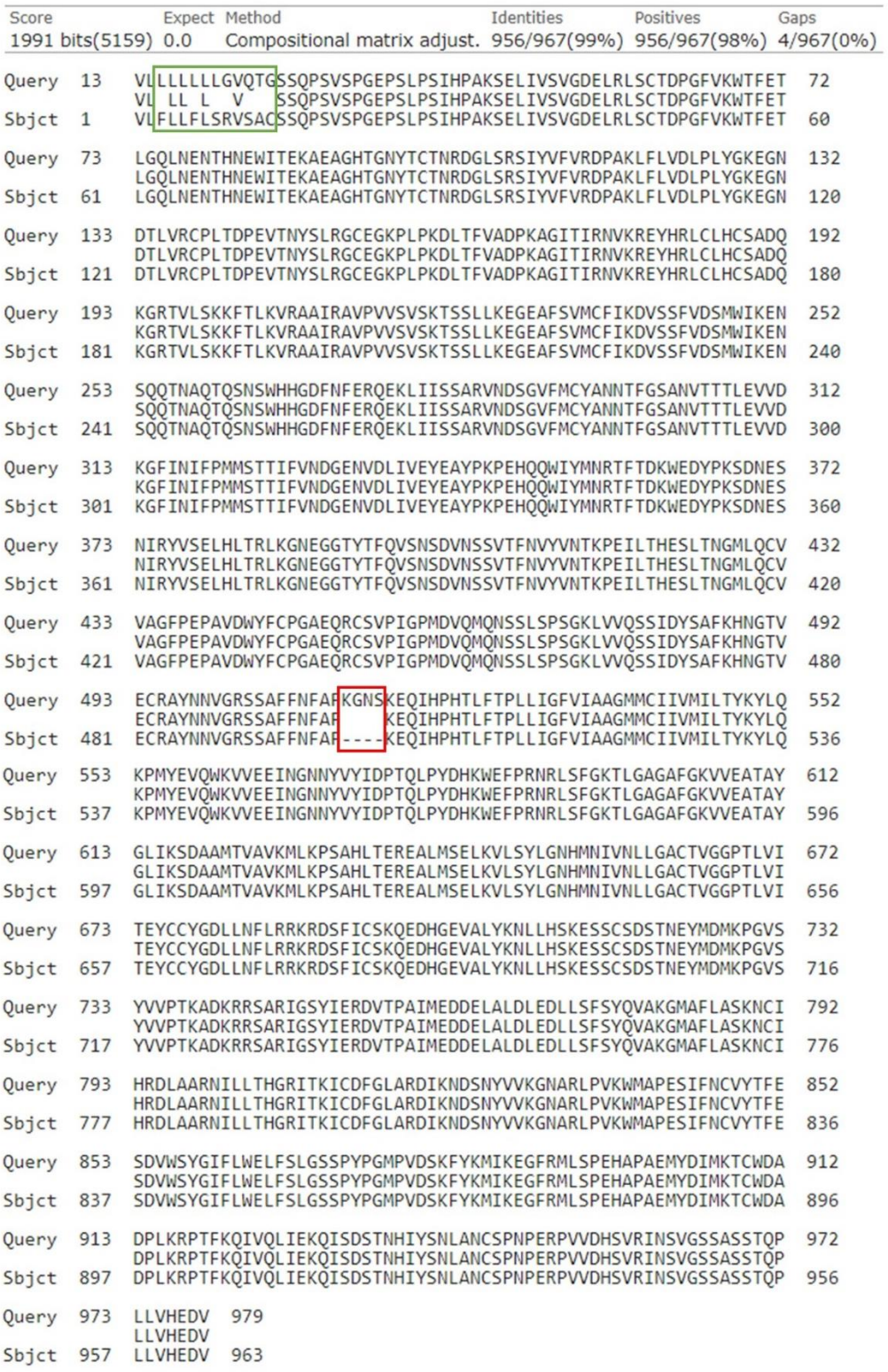

Figure 4.4. Alignment of the two Kit protein splice variants (KIT-201 and KIT-202) using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Query: KIT-201, UniProt ID O97799. Subject: KIT-202, UniProt ID F1PUW9. The green box shows amino acid variations at the beginning of the transcripts corresponding to exon 1 of the DNA sequence. The red box highlights the GNSK-deletion in KIT-202. Nucleotide sequence analysis confirms that this deletion encompasses GNSK amino acids and not the KGNS the sequence depicted here in the BLAST protein alignment.

4.4.2 Primer design

Two Ion AmpliSeq Primer Pools, each with 27 primer pairs, were designed to amplify the entire *KIT* gene coding sequence and intron 11 (**Appendix I**). The 52 resulting amplicons ranged from 125–175 bp in length and covered 96.6% of the target region. Upon primer review and after experimental completion, the designed primers did not capture exon 1 of the KIT-201 splice variant.

4.4.3 Final library quantification and chip loading

AmpliSeq DNA libraries were prepared for 99 MCT samples and the normal canine testis control sample, each with a unique identifying barcode. Final AmpliSeq library concentrations, as measured by the Qubit Fluorometer, were used in allocating AmpliSeq libraries into Library Pool 1 or 2 for loading onto the respective Sequencing Chip 1 or 2 (**Appendix J**).

4.4.4 Ion Torrent PGM sequencer performance

Sequencing Chips 1 and 2 were sequenced by the Ion Torrent PGM system. Chip 1 (**Figure 4.5A**) had a lower loading density than Chip 2 (**Figure 4.5B**), 50% compared to 92%, respectively, but a greater number of successfully sequenced reads, 2.13 million compared to 1.76 million.

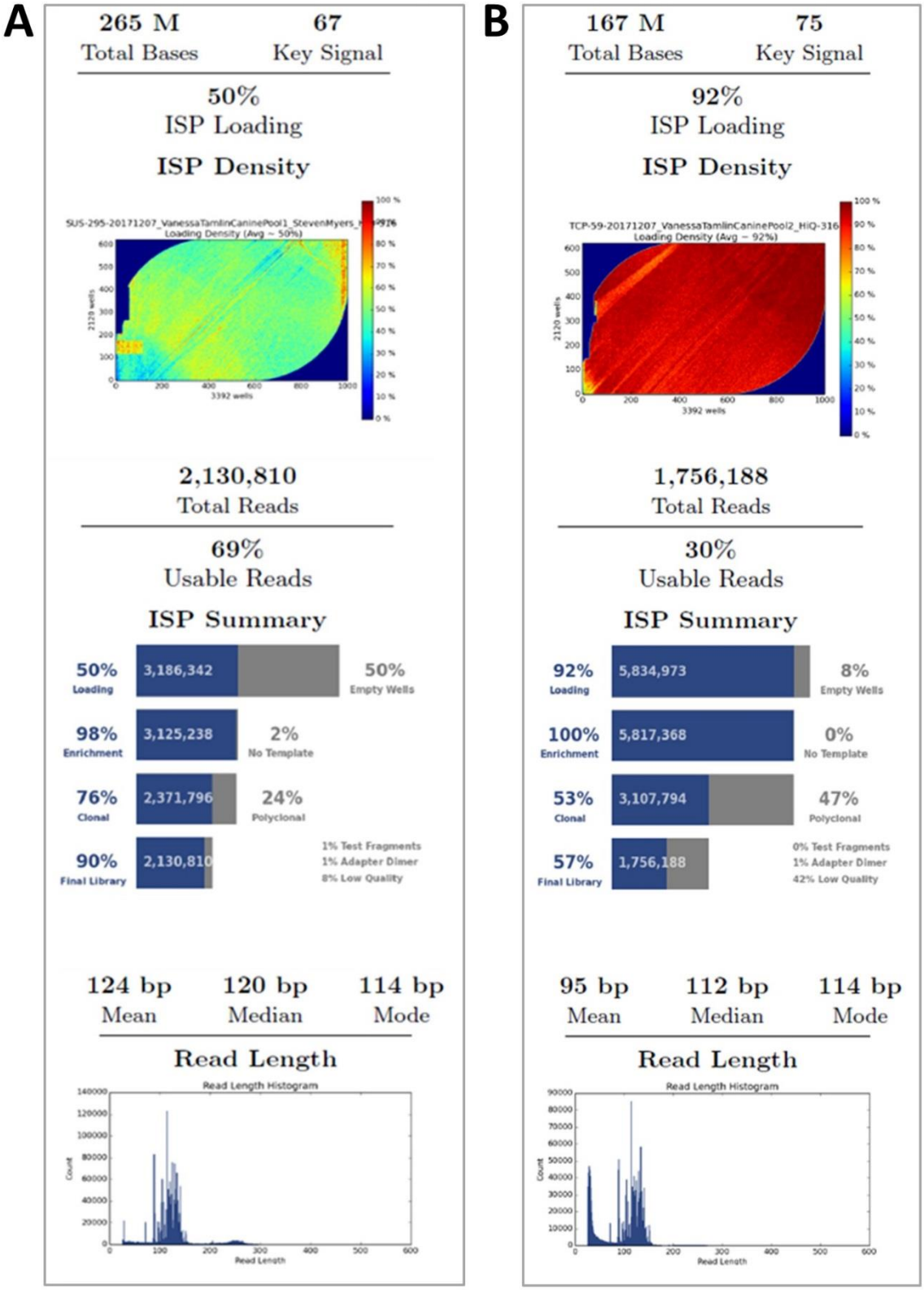

Figure 4.5. Loading density heat map, performance summary and read length of AmpliSeq™ Ion Torrent™ Personal Genome Machine sequencing system of two Ion 316 sequencing chips (Chip v2 BC, Thermo Fisher Scientific) using the Ion Chef™ Instrument. Results are shown for canine cutaneous and subcutaneous mast cell tumour *KIT* gene AmpliSeq™ Library Pools 1 (A) and 2 (B).

Two samples from Chip 1 failed to pass sequencing quality thresholds and were removed from subsequent analyses.

On-target reads from a different sample were below the recommended 95% cut-off and three samples across the two Chips had less than 90% uniformity (**Table 4.6**). Read alignments for these four samples were independently reviewed in IGV to check for strand biases and sequencing error. No flaws in the data were observed and all four samples were determined to be of adequate quality to remain in the dataset for further analyses.

Table 4.6. Summary of sequence coverage parameters for canine mast cell tumour *KIT* gene AmpliSeq™ DNA libraries sequenced on two Ion 316 Sequencing Chips using Ion Torrent™ Personal Genome Machine sequencing system.

	Chip 1 (average; min–max)	**Chip 2 (average; min–max)**	**Recommended**
Number of mapped reads per sample	35,130; 62–102,004	25,487; 5,655–62,660	-
Read length (bp) Mean; Median	124; 120	95; 112	< 150
On-target reads (%)	97.8; 6.45–100	99.3; 98.7–99.7	≥ 95[a]
Sequencing depth (X)	709; 0.062–2,059	504; 107–1246	250[a]
Uniformity (%)	98.2; 82.0–100	98.6; 66.6–100	≥ 90[a]

[a] (Rathi *et al.*, 2017)

4.4.5 Final AmpliSeq dataset

Of the 99 canine MCT *KIT* AmpliSeq DNA libraries, two samples failed sequencing and two samples were not histologically confirmed as MCT (diagnoses could not be concluded from histopathological sections in both cases). These four samples were removed from the dataset. Therefore, sequencing results were available for 95 MCT samples (77 cutaneous MCTs and 18 subcutaneous MCTs) and the normal canine testis control DNA sample.

4.4.6 Sequence annotations and variant filtering

Ion Torrent sequencing detected 178 variants, 144 of which aligned within the *KIT* gene. The remaining 34 alterations aligned to non-target regions within the dog genome and were removed from the dataset. Within the *KIT* gene, 90 variants resided completely within the introns and were removed from the dataset. Fifty-four variants occurred in the *KIT* coding region and the non-coding domain. Filtering parameters were applied to remove low quality

and erroneous data (**Table 4.5**, **Figure 4.6**). Twenty-three variants failed to pass the quality threshold and were removed. The majority of these variants (n = 20) failed because they had a Quality by Depth score of less than 2, which is indicative of a homopolymer-associated indel error; **Figure 4.6F** (Loman *et al.*, 2012). Six of these 20 variants were hereafter concluded to have been wrongly removed. See explanation in section 4.4.7.

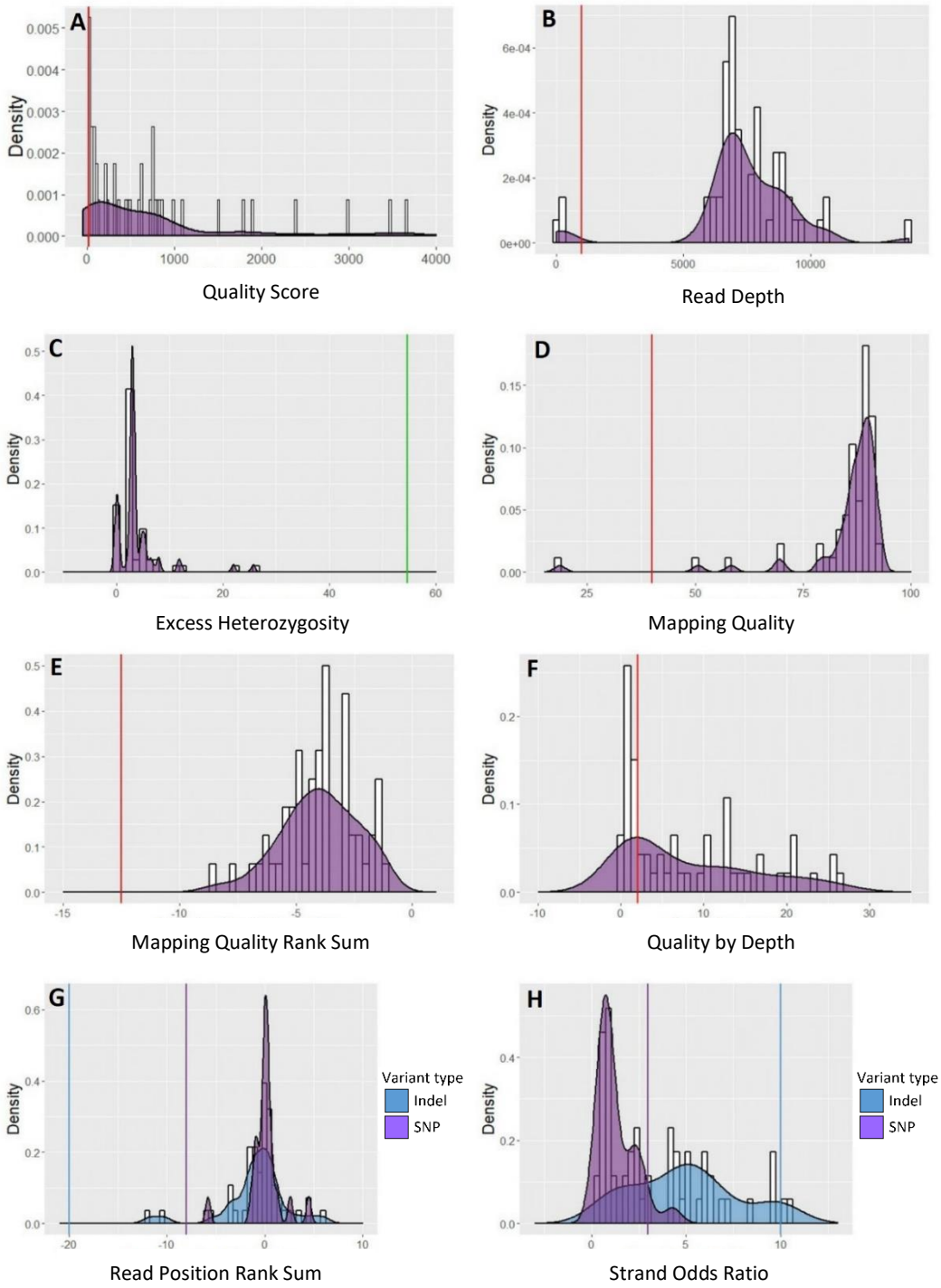

Figure 4.6. Histograms overlayed with density plots to visually represent data filtering parameters applied to canine mast cell tumour *KIT* gene variants for removal of the erroneous data. Variants with values to the left of the red x-axis intercept (A, B, D, E, F) and to the right of the green x-axis intercept (C) were deemed to be of poor quality and were removed from further analyses. The blue and purple x-axis intercepts (G, H) represent the cut-off points for indels and SNPs, respectively. A Read Position Rank Sum score of less than -20 for indels and less than -8 for SNPs are removed (G). Variants with a Strand Odds Ratio value greater than 10 for indels and greater than 3 for SNPs are also removed.

4.4.7 False-positives and homopolymer-associated errors

Variant frequencies in heterozygous samples ranged from 4.08%–80.0% (median = 47.2%). None fell below the 3% threshold.

Thirty-two insertions and deletions within the *KIT* coding region or non-coding domains were called by Ampliseq NGS and Genome Alignment Tool Kit (GATK) HaplotypeCaller software and were visualised in IGV for variant verification (**Figure 4.7**). Insertions and deletions that occurred in a region of ≥ 3 consecutive identical nucleotide residues were determined to be homopolymer-associated indel errors and were subsequently removed from the dataset (Loman *et al.*, 2012, Yeo *et al.*, 2012, Yeo *et al.*, 2014). Of the 32 indels, 14 were correctly removed using the Quality of Depth parameter of < 2 (**Figure 4.6F**). However, after visualisation in IGV, six variants were, in fact, true-positives and had been incorrectly removed by the Quality of Depth filtering parameter. These variants were returned to the dataset. One homopolymer-associated indel detected in IGV was not removed by filtering parameters. This variant was removed from the dataset accordingly.

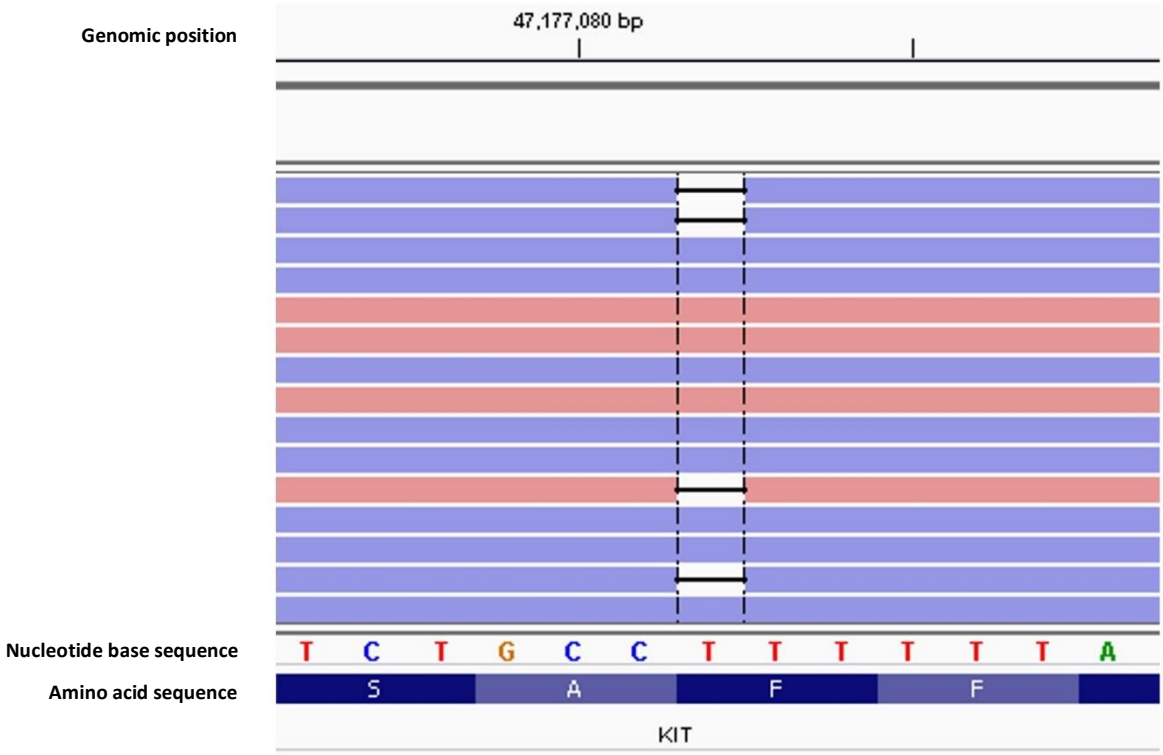

Figure 4.7. Screenshot from Integrative Genomics Viewer (IGV) of the canine *KIT* gene sequence portraying a homopolymer-associated indel error in exon 9 of *KIT* in a canine mast cell tumour extracted DNA sample and called by Genome Alignment Tool Kit (GATK) HaplotypeCaller software after sequencing by Ion Ampliseq™ technology. The horizontal red and blue bars represent the DNA sequence on the forward strand and reverse complement strand, respectively. The beginning of a 6 base pair (bp) poly-thymine sequence is marked by the two vertical, dashed lines. The false-positive thymine residue deletion at this position is shown by the gaps in the coloured bars.

4.4.8 Discovered variants and predicted consequences on Kit protein function

AmpliSeq Ion Torrent technology detected 34 unique variations aligned within the *KIT* gene coding regions or non-coding domain. The non-coding domain included the upstream regions, downstream regions, 3' and 5' UTRs and splicing regions of *KIT.* Additionally, a 3' UTR variant was manually discovered (described in section 4.4.10). Hence, the *KIT* mutation profile for 95 MCT DNA samples consisted of 35 unique variants. Frameshift mutations were the most common mutation type and, in one instance, the frameshift resulted in the gain of a stop codon (**Table 4.7**). The most common SNP was a cytosine to a thymine change (**Table 4.8**).

Table 4.7. Number and type of genetic variants detected in the *KIT* gene from canine cutaneous and subcutaneous mast cell tumours.

Variant type	Predicted impact	Count
3′ UTR variant	Gene expression	3
Conservative inframe deletion	Moderate	1
Disruptive inframe insertion	Moderate	1
Downstream gene variant	Gene expression	2
Frameshift	High	8
Frameshift and splice region variant	High	1
Frameshift and stop gained (nonsense)	High	1
Missense (non-synonymous)	Moderate	6
Splice acceptor intron variant	High	1
Synonymous	Low	8
Upstream gene variant	Gene expression	2
Total		34

UTR: untranslated region

Table 4.8. Single nucleotide polymorphisms detected in the *KIT* gene from canine cutaneous and subcutaneous mast cell tumours.

		Alternate nucleotide				
		A	C	G	T	**Total**
Reference sequence nucleotide	A		-	3	1	4
	C	-		-	7	7
	G	3	-		2	5
	T	-	2	-		2
	Total	3	2	3	10	**18***

*This total is inclusive of both synonymous, non-synonymous and non-coding nucleotide base changes.

Twelve variants were silent, whereby the nucleotide base change did not affect the amino acid sequence. These alterations occurred in *KIT* exons 2, 3, 8, 9, 11 and 16, and in the *KIT* upstream region and the 3′ UTR (**Table 4.9**). These are herein referred to as synonymous variants. All excluding one synonymous variant are documented in publicly available databases. The MCT carrying the novel synonymous variant exhibited a homozygous genotype, hence, this alteration was suspected to be a germline variant and not associated with MCT disease.

Table 4.9. Synonymous and non-coding single nucleotide polymorphisms (SNPs) in the *KIT* proto-oncogene detected in 77 cutaneous and 18 subcutaneous canine mast cell tumour (MCT) DNA samples with a predicted synonymous effect on Kit amino acid sequence. Displayed is the frequency of the alternate allele variant. The variants were detected by Ion AmpliSeq™ technology. NCBI canine reference genome ID NC_006595.3. NCBI canine reference *KIT* gene mRNA ID NM_001003181.1. NCBI canine reference Kit protein ID NP_001003181.1.

Exon	gDNA Position	Variant type	Nucleotide change	Predicted amino acid effects	Alternate allele frequency (%)		Reference database
					Cutaneous MCT	Subcutaneous MCT	
Synonymous germline SNPs							
Upstream	47,144,029	Upstream	c.-55T>C	-	7.14	ND	UniBE - Institute of Genetics (2012)
	47,144,045	Upstream	c.-39C>T	-	5.84	13.9	UniBE - Institute of Genetics (2012)
2	47,144,532	Synonymous	c.159C>T	p.(Gly53Gly)	33.1	27.8	rs851679960[a]
3	47,147,571	Synonymous	c.138C>T	p.(Cys138Cys)	13.6	13.9	rs850580536[a]
	47,147,664	Synonymous	c.507A>G	p.(Lys169Lys)	42.9	41.7	rs852718113[a]
	47,147,778	Synonymous	c.621G>A	p.(Arg207Arg)	ND	2.78	rs852786648[a]
8	47,175,092	Synonymous	c.1275A>G	p.(Thr425Thr)	40.3	41.7	rs22299980[a]
9	47,177,090	Synonymous	c.1524C>T	p.(Asn508Asn)	ND	5.56	-
11	47,178,598	Synonymous	c.1731C>T	p.(Tyr577Tyr)	16.9	13.9	rs853024368[a]
16	47,183,142	Synonymous	c.2355G>A	p.(Lys785Lys)	9.74	8.3	rs851958844[a]
3′ UTR	47,189,868	3′ UTR variant	c.*37G>T	-	21.4	13.9	rs8793422[a]
	47,189,939	3′ UTR variant	c.*108G>A	-	19.5	30.6	rs8793421[a]

[a] (Asia Ensembl).

ND: none detected. UTR: untranslated region.

The asterisk (*) describes nucleotides in the UTR.

Non-synonymous nucleotide base mutations were detected in *KIT* exons 2, 3, 7–9, 11, 13–16, 19 and 21 (**Table 4.10**). These genetic changes were predicted to alter the Kit amino acid sequence and potentially affect Kit protein function. Four of these mutations have been reported in previous canine MCT molecular investigations.

The samples with nucleotide base changes in the 3' splice acceptor region, the 3' UTR and the downstream region of *KIT* were heterozygous at these positions. These mutations are predicted to have a modifying effect on the Kit mRNA (e.g. splicing, stability). Herein, these variants are referred to as non-coding variants.

Table 4.10. Non-synonymous mutations and non-coding variants detected in the *KIT* proto-oncogene in 77 cutaneous and 18 subcutaneous canine mast cell tumour (MCT) DNA samples by Ion AmpliSeq™ technology and the predicted effects on the Kit amino acid sequence. NCBI canine reference genome ID NC_006595.3. NCBI canine reference *KIT* gene mRNA ID NM_001003181.1. NCBI canine reference Kit protein ID NP_001003181.1.

Exon	Position	Variant type	Nucleotide change	Predicted amino acid change	Mutation prevalence (%)		Variant reference
					Cutaneous MCT	Subcutaneous MCT	
Upstream	ND						
5′ UTR	ND						
1	ND						
2	47,144,480	Missense	c.107C>T	p.(Pro36Leu)	2.60	ND	1
3	47,147,500	5′ Splice acceptor variant	c.344-1_344insT	p.(Asp115fs)	18.2	11.1	
	47,147,600	Frameshift and stop gained (nonsense)	c.443_444insG	p.(Tyr148fsTer)	1.30	ND	
4	ND						
5	ND						
6	ND						
7	47,160,989	Missense	c.1187A>G	p.(Gln396Arg)	2.60	ND	
8	47,175,066	Disruptive inframe insertion	c.1251_1262dupAATCCTGACTCA	p.(Thr420_His421insGlnIleLeuThr)	ND	16.7	
9	47,177,002	Missense	c.1436G>T	p.(Ser479Ile)[a]	ND	5.56	2, 3
	47,177,089	Missense	c.1523A>T	p.(Asn508Ile)[a]	3.90	5.56	2, 3, 4, 5
10	ND						
11	47,178,536	Frameshift	c.1670_1677delAGGTTGTT	p.(Lys557fs)	ND	5.56	6
	47,178,547	Frameshift	c.1680_1681insAA	p.(Glu561fs)	ND	5.56	
	47,178,584	Conservative inframe deletion	c.1718_1738delACAGCTTCCTTACGATCACAC	p.(Thr573_His579del)	1.30	ND	
	47,178,591	Missense	c.1724T>C	p.(Leu575Pro)	1.30	ND	7

12	47,179,072	3′ splice acceptor variant	c.1877-42_1844-2 delAAATTTGCTTCTTGCTAAAATGA AATTTTTTTCTGATTTTA		2.60	ND	
13	47,179,115	Frameshift and splice region variant	c.1879delA	p.(Ser627fs)	14.3	5.56	
	47,179,115	Frameshift and splice region variant	c.1879dupA	p.(Ser627fs)	9.09	5.56	
14	47,180,508	Frameshift	c.2082dupA	p.(Asp695fs)	1.30	ND	
15	47,182,526	Frameshift	c.2217dupA	p.(Ser740fs)	1.30	ND	
16	ND						
17	ND						
18	ND						
19	47,188,156	Frameshift	c.2662_2663insC	p.(Met888fs)	2.60	ND	
20	ND						
21	47,189,776	Missense	c.2873C>T	p.(Ser958Phe)	1.30	ND	
	47,189,815	Frameshift	c.2913_2916delACAC	p.(His972fs)	1.30	ND	
3′ UTR	47,189,900	3′ UTR variant[b]	c.*68_89dupGTTCTGTCCTCCTTCG GCTTGC		1.30	5.56	
	47,189,941	3′ UTR variant	c.*111dupC		1.30	ND	
Downstream	47,190,061	Downstream gene variant	c.*230_*231insC		1.30	ND	
	47,190,070	Downstream gene variant	c.*241dupA		1.30	ND	
Variant references	1. rs24756675, source: Asia Ensembl. 2. Letard *et al.* (2008) 3. Mochizuki *et al.* (2017b) 4. Amagai *et al.* (2015) 5. Yamada *et al.* (2011) 6. Zemke *et al.* (2002) 7. Takeuchi *et al.* (2013)						
Footnotes	[a] Kit protein activating. [b] This variant was manually detected whereas all other variants were called by AmpliSeq™ Ion Torrent™ software.						
Definitions	ND: none detected. UTR: untranslated region. fs: frameshift. Ter: indicates terminating codon gain. The asterisk (*) describes nucleotides in the non-translated gDNA sequence (i.e. in the 5′ and 3′ UTRs and in the upstream and downstream regions). An underscore (_) is used to indicate an inclusive range. The minus sign (-) describes nucleotide in the intron preceding the coding DNA sequence.						

*4.4.9 Predicted canine KIT mRNA secondary structural changes from a c.*111dupC 3' UTR mutant*

To investigate the effects of a non-coding gene variant on wild-type *KIT* mRNA secondary structure, the wild-type canine *KIT* sequence and a UTR-mutant *KIT* sequence (c.*111dupC) secondary structures were predicted using ViennaRNA software. Considerable changes in the predicted mRNA secondary structures were noticed between the wild-type canine *KIT* and a UTR-mutant *KIT* sequence. Although, the predicted minimum free energy (MFE) of the mutant sequence was comparable to the wild-type sequence (-1012.9 kcal/mol versus -1015.1 kcal/mol, respectively; **Figure 4.8**).

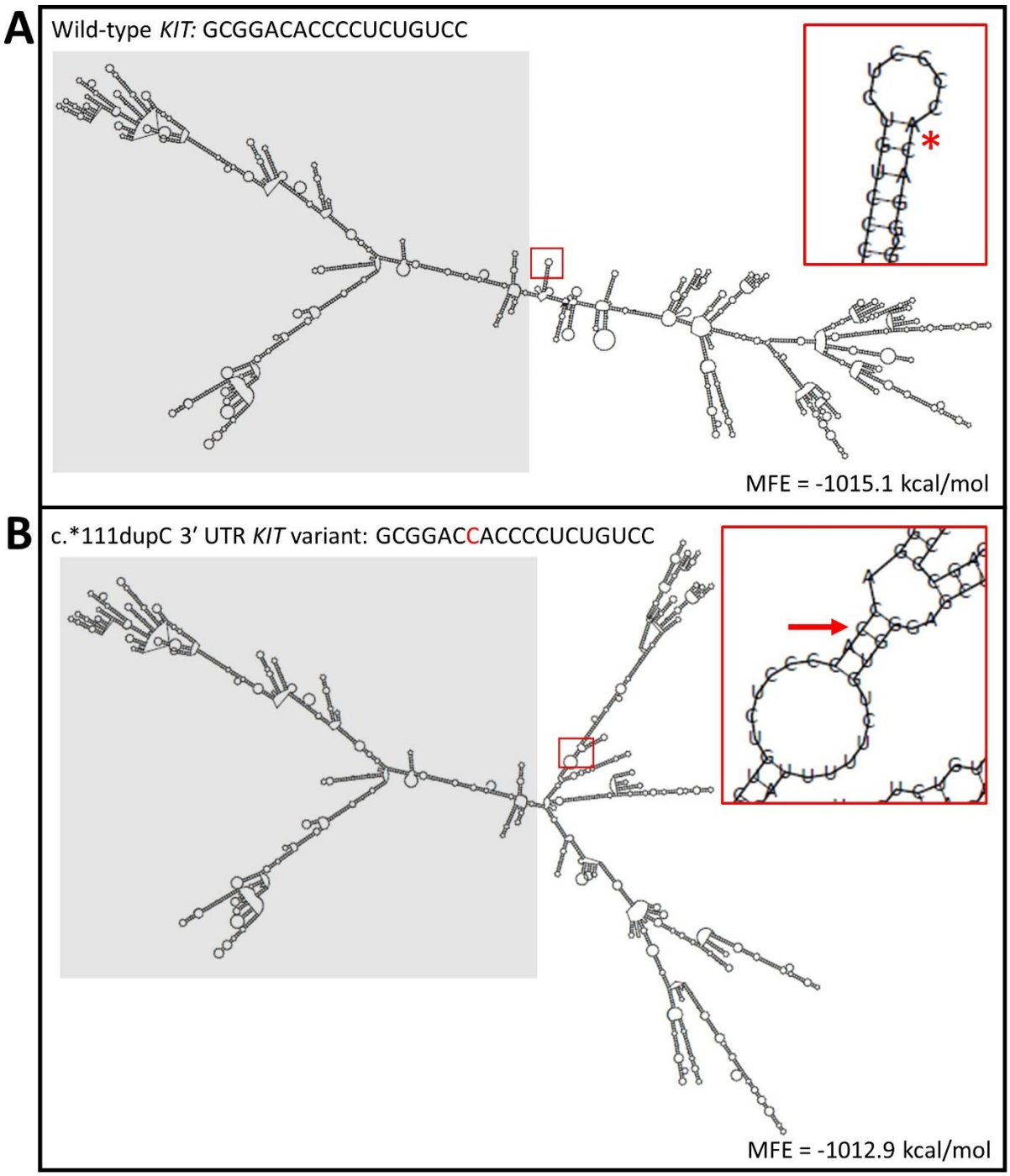

Figure 4.8. Predicted secondary mRNA structure and minimum free energy (MFE) of (A) the wild-type canine *KIT* gene sequence and (B) the c.*111dupC 3' untranslated region (UTR) *KIT*-mutant sequence detected in the DNA from a canine cutaneous mast cell tumour. The structure of the segment highlighted by the grey boxes is identical between the wild-type and mutant *KIT* sequences. The mutant *KIT* sequence differs from the wild-type by a duplication of a cytosine residue 111 bases 3' of the stop codon (red lettering, B). The red asterisk (*) in image A indicates the position where the duplication occurs. The red arrow in image B indicates the duplicated cytosine residue. The secondary structures were predicted using the ViennaRNA Package 2.0 version 2.4.12 (Lorenz *et al.*, 2011).

4.4.10 Exon 11 ITD mutation calling using automated sequencing software

AmpliSeq Ion Torrent sequencing and GATK automated sequencing software analysis correctly called the 21-bp deletion identified in Chapter 3 but did not recognise the exon 11 *KIT* ITDs. To investigate this, the sequence alignment data for the samples known to harbour an ITD, as confirmed by PCR and Sanger sequencing (Chapter 3), were manually inspected in IGV. The ITDs were easily visible and the sequences were confirmed (**Figure 4.9**). Subsequently, all 21 exons of the *KIT* gene from all 95 AmpliSeq library sequence files were reviewed in IGV to ensure no additional large insertion or deletion mutations were missed. An additional 3' UTR 22-bp ITD was detected in two samples (c.*68_89dupGTTCTGTCCTCCTTCGGCTTGC).

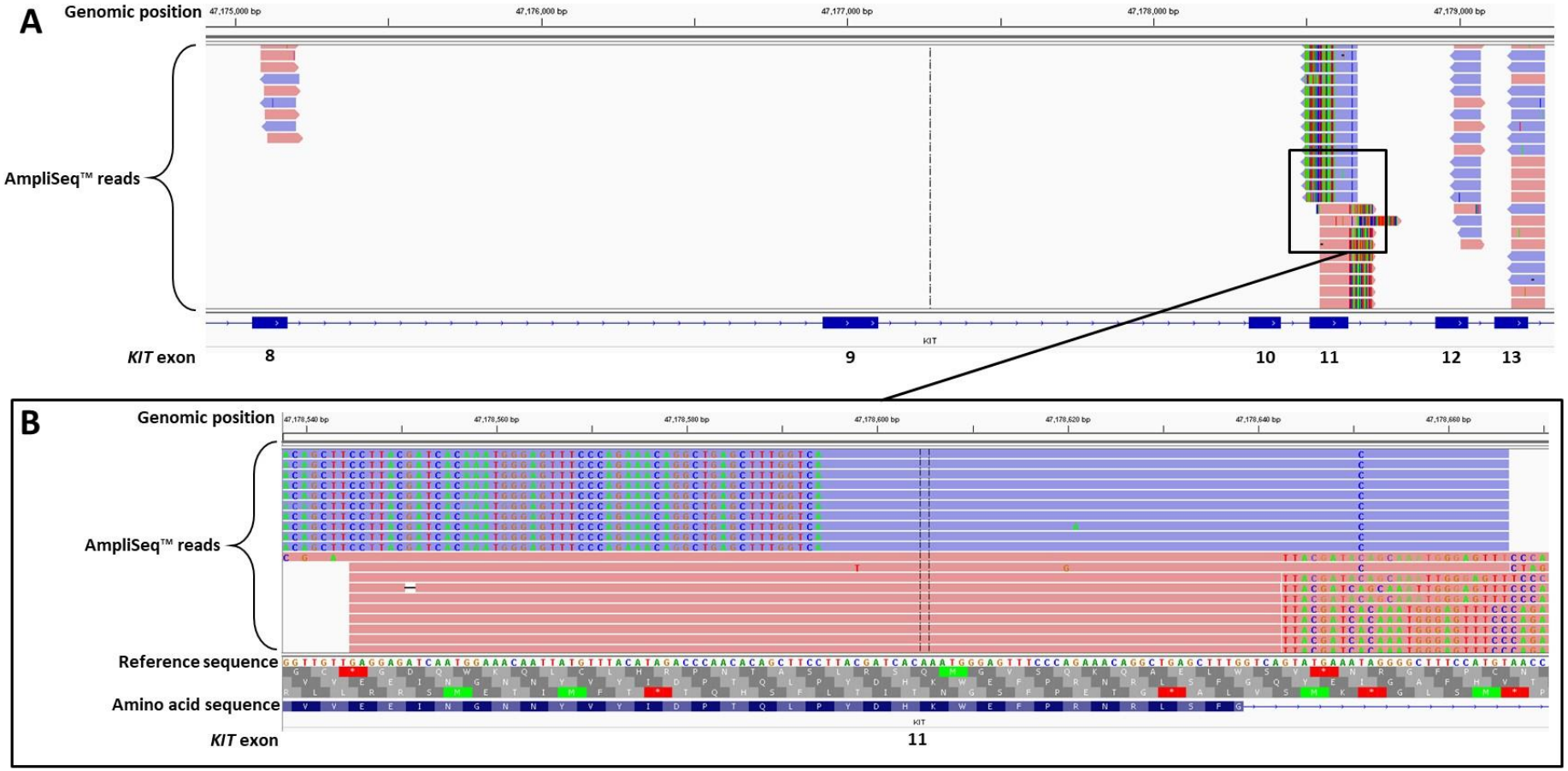

Figure 4.9. Screenshot from Integrative Genomics Viewer (IGV) of a canine cutaneous mast cell tumour AmpliSeq™ DNA sample harbouring a 48-base pair (bp) internal tandem duplication (ITD) in exon 11 of the *KIT* gene. The horizontal red and blue bars represent the sequence on the forward strand and reverse complement strand, respectively. Adenine is denoted by the green bars and the letter 'A'. Thymine is denoted by the red bars and the letter 'T'. Cytosine is denoted by the blue bars and the letter 'C'. Guanine is denoted by the brown bars and the letter 'G'. The "mismatched" nucleotides are noted by the multicoloured segments in the instances where the AmpliSeq™ read sequence does not match the reference sequence. A: highlights the easy visualisation of soft-clipped reads which are characterised by the multicoloured segments of the strands. B: mis-matched nucleotide residues are flagged by coloured letters of the respective nucleotide (A, T, C or G). The segment between the non-aligned regions on the forward and reverse complement strand is the sequence that is duplicated (i.e., is the ITD sequence).

4.4.11 KIT non-synonymous mutation prevalence in canine cutaneous and subcutaneous MCTs

Conventional PCR (Chapter 3) detected exon 11 ITDs in 10 of the 77 cutaneous MCTs in the AmpliSeq cohort (prevalence = 12.9%, 95% CI: 5.48–20.5%). The cutaneous MCT carrying the 21-bp deletion was also included in AmpliSeq analysis. Combining the Ion AmpliSeq mutation results (current chapter) with the ITD mutation results (Chapter 3) determined a non-synonymous *KIT* mutation prevalence of 51.9% in cutaneous MCTs (n = 40/77, 95% CI: 40.8–63.1%). One tumour possessed six *KIT* mutations, three tumours had three mutations, 13 tumours had two mutations and the remaining 23 tumours harboured a single mutation. Of the subcutaneous MCTs, 9/18 (50.0%, 95% CI: 26.9–73.1%) tumours harboured at least one non-synonymous mutation. One tumour harboured three *KIT* mutations, one tumour had two mutations and seven tumours harboured a single mutation.

KIT regulatory-type mutations (exons 1–12, including exon 11 ITDs) were detected in 35.1% (n = 27/77) of cutaneous MCTs and 44.5% (n = 8/18) of subcutaneous MCTs. Enzymatic pocket-type (exons 13–21) mutations were detected in 20.8% (n = 16/77) and 5.56% (n = 1/18) of cutaneous and subcutaneous MCTs, respectively.

When considering only the *KIT*-mutant MCTs, regulatory-type mutations (including exon 11 ITDs) were the most frequent mutation type in both cutaneous and subcutaneous tumours, present in 70% (n = 28/40) and 88.9% (n = 8/9) of the mutant tumours, respectively (**Figure 4.10**).

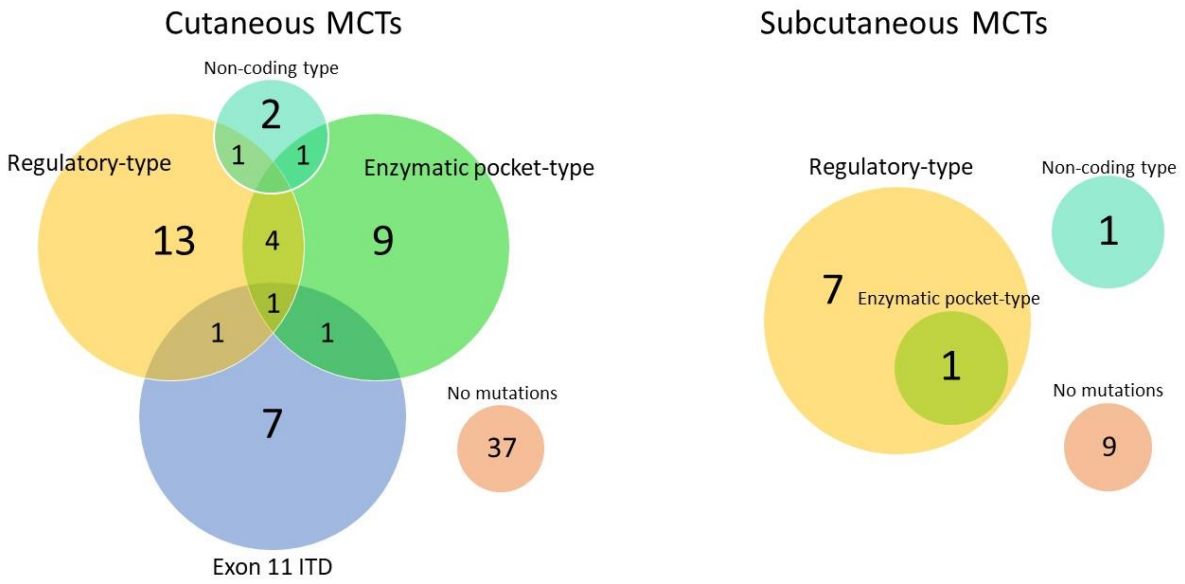

Figure 4.10. Venn diagrams representing the number of canine cutaneous and subcutaneous mast cell tumours (MCTs) with regulatory-type mutations (exons 1–12), enzymatic pocket-type mutations (exons 13–21), non-coding-type mutations (upstream, downstream, splicing and 3' untranslated regions) and/or an exon 11 internal tandem duplication (ITD) within the *KIT* gene.

4.4.12 Mutation prevalence in dogs with multiple MCTs

KIT AmpliSeq libraries were prepared for 77 cutaneous MCTs (61 Kiupel low-grade and 16 Kiupel high-grade) and 18 subcutaneous MCTs from 93 dogs. Two dogs harboured two cutaneous MCTs. Of the dogs with two tumours, only wild-type *KIT* DNA was found in the tumours from one dog. The other dog had one wild-type tumour and one *KIT*-mutant tumour carrying two mutations; an insertion in the 5' slice region of exon 3 (c.308-1_308insT) and a 22-bp ITD in the 3' UTR (c.*68_89dupGTTCTGTCCTCCTTCGGCTTGC).

4.4.13 Intron 11 deletion investigation

The homozygous deletion of intron 11 was not detected in the automated sequencing output data for the AmpliSeq DNA libraries, nor was the deletion observed by manual review of the IGV alignment files.

NCBI BLAST of exon 11 and 12 to the canine reference genome found no alignments outside the *KIT* gene, making the existence of a *KIT*-derived pseudogene unlikely (**Figure 4.11**).

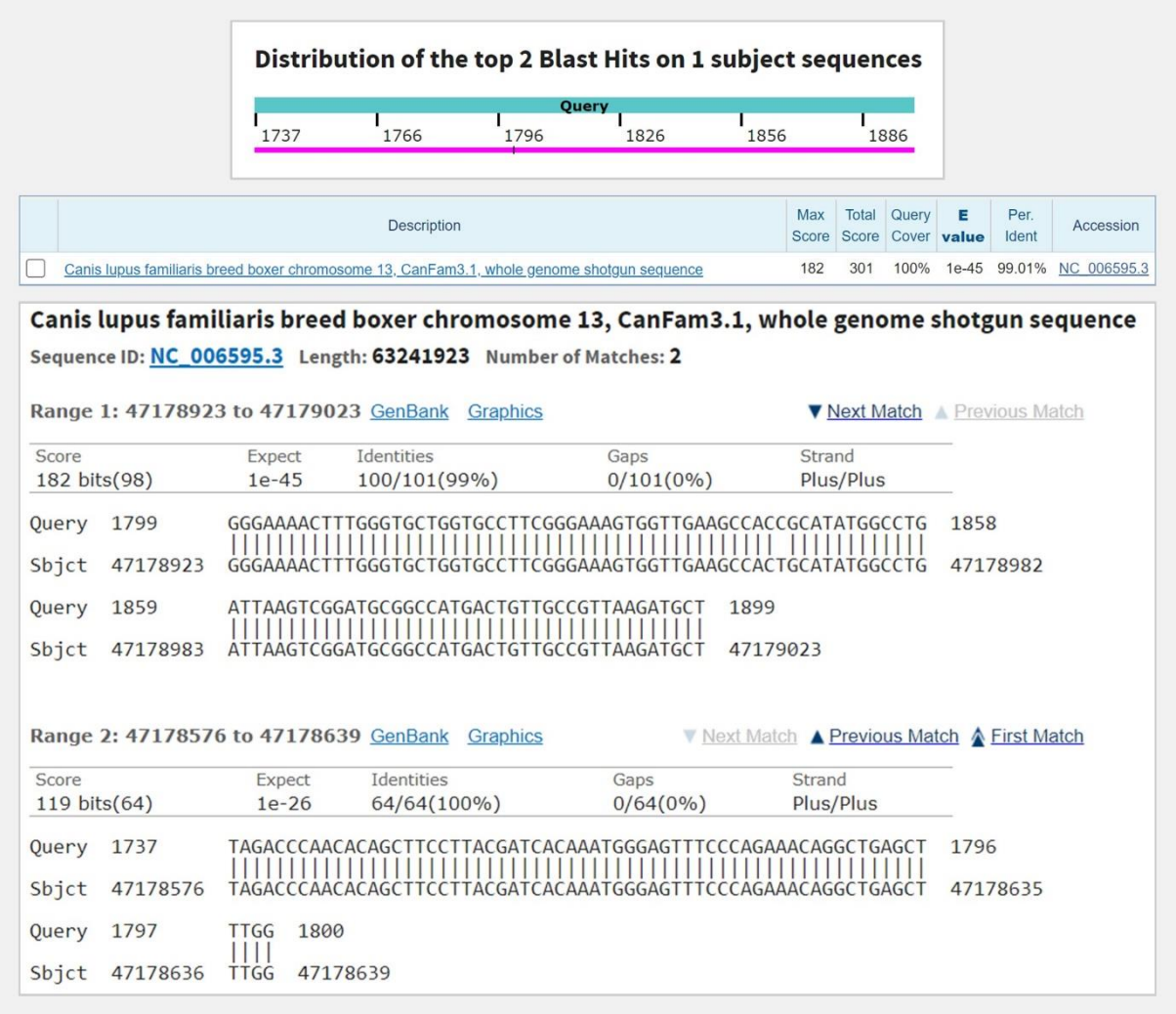

Figure 4.11. Alignment of *KIT* exons 11 and 12 (GenBank accession number AF044249, nucleotide bases 1737–1899) to the canine reference genome (CanFam3.1; GCF_000002285.3; NC_006595.3) using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to test for the presence of a *KIT*-derived pseudogene in the canine genome. The results displayed for Ranges 1 and 2 are the sequences for exons 12 and 11, respectively. No alignments outside the *KIT* gene were discovered.

4.5 Discussion

In this work, I have used AmpliSeq™ Ion Torrent™ PGM NGS technology to establish a *KIT* mutation profile from 95 canine MCTs. Non-synonymous coding and non-coding *KIT* mutations were identified in 51.9% (n = 40/77) of the cutaneous tumours and in 50% (n = 9/18) of the subcutaneous tumours. These mutations included alterations in the regulatory domain (exons 1–11; including exon 11 ITDs), enzymatic pocket domain (exons 12–21) and non-coding domains (non-coding variants; upstream, downstream, 5' UTR and 3' UTR) of the *KIT* gene. The *KIT* DNA sequence of the normal canine testis control sample aligned exactly to the CanFam3.1 NCBI reference sequence. Of the cutaneous MCTs, 13% (10/77) carried a *KIT*

exon 11 ITD, and two of these ITD-mutant tumours carried an additional mutation in the enzymatic pocket domain (**Figure 4.10**). These results suggest that one-fifth of ITD-mutant cutaneous MCTs carry an additional mutation within the enzymatic pocket domain and, therefore, could be resistant to treatment with tyrosine kinase inhibitors (Nakano *et al.*, 2017). This finding requires confirmation with larger numbers of samples from a different population of dogs.

All MCT samples with non-synonymous mutations in the coding sequence (exons) and in the *KIT* gene splicing regions (non-coding domain) were heterozygous for the alternative allele, suggesting a possible somatic origin. Whilst functional protein investigations were not conducted, these mutations are suspected to have a gain-of-function or modifying effects on Kit protein activity due to the similarities they share with previously reported mutations. To date, all investigated missense point-mutations in exons 8, 9, 11, 14 and 17 have demonstrated constitutive Kit protein phosphorylation in the absence of normal ligand binding in neoplastic mast cells (Furitsu *et al.*, 1993, Kitayama *et al.*, 1995, Letard *et al.*, 2008, Nakano *et al.*, 2017). Frameshifts in the protein transcript caused by insertions, deletions and duplications also induce Kit protein autophosphorylation (Letard *et al.*, 2008, London *et al.*, 1999, Ma *et al.*, 1999).

The functional consequences remain unclear for the variants occurring in the *KIT* downstream and 3' UTRs which have not been reported in the canine SNP databases (**Table 4.10**). However, results from investigations on other receptor tyrosine kinase proteins indicate that functional consequences are possible. For example, gain-of-function mutations in the *RET Receptor Tyrosine Kinase Proto-Oncogene* UTR contribute to the hereditary development of medullary thyroid carcinoma in humans. The synonymous Ser836Ser *RET* variant is associated with early disease onset, increased risk for metastasis and is in linkage disequilibrium with the 3' UTR mutations rs3026785 (c.*1969T>C) and rs76759170 (c.*1591G>A) (Ceolin *et al.*, 2016). Research has shown that the 3' UTR variant alleles modulate *RET* expression by increasing mRNA thermostability which is presumed to decrease *RET* mRNA degradation (Ceolin *et al.*, 2016). Supporting this, immunohistochemical analysis of a limited number of samples from medullary thyroid carcinoma patients demonstrated stronger Ret protein staining in samples from patients carrying the Ser836Ser and 3' UTR variants compared to samples from patients a detected wild-type sequence. The effects of the 3' UTR variants on Ret staining in the absence of Ser836Ser was not investigated. Through similar mechanisms of mRNA

modulation, 3′ UTR variants in other protein-coding genes have been linked to risk of cancer development and/or malignancy in cases of breast cancer, colorectal cancer and gastric cancer as either stand-alone mutations or in conjunction with other genetic alterations (Bermano *et al.*, 2007, Jakubowska *et al.*, 2007, Mei *et al.*, 2019, Xiang *et al.*, 2018). The effects of UTR variants in *KIT* on canine MCT pathogenesis are unknown.

The mRNA secondary structure was compared between one canine *KIT* MCT 3′ UTR variant (c.*111dupC) and the wild-type *KIT* sequence (**Figure 4.8**). Despite considerable differences in predicted mutant and wild-type mRNA folding, the minimal free energy of the two configurations was similar. This indirect evidence of comparable thermostability of the two conformations suggests that the 3′ UTR *KIT* mutation is likely to be insufficient to drive mast cell tumourigenesis. This 3′ UTR-mutant was detected in a MCT DNA sample harbouring three other non-synonymous *KIT* mutations and two non-coding variants. It is hypothesised that the 3′ UTR mutation is acting in an additive manner with the other *KIT* mutations to contribute to tumour development.

The other 3′ UTR (a 22-bp ITD) was detected in two MCT DNA samples. One of these DNA samples was extracted from a cutaneous MCT which also harboured a mutation in the 5′ splicing region of exon 3. This MCT was excised from an animal with two concurrent cutaneous MCTs. The second MCT, which was co-excised with the first tumour and which was also investigated in this study, harboured only wild-type DNA. This indicates that 3′ UTR ITD and the 5′ splicing variant in exon 3 are somatic. It is possible that these non-coding variants modify *KIT* mRNA stability and, hence, may contribute to mast cell pathogenesis.

Twelve synonymous and non-coding variants were detected in this study and are predicted to be germline in nature (**Table 4.9**). Eight of the variants occurred in the *KIT* coding sequence, two occurred in the upstream region and two occurred in the 3′ UTR. Genetic alterations that conserve the amino acid sequence are typically considered to be synonymous, or “silent”. However, some synonymous mutations impact post-transcriptional processes and subsequently, affect protein activity (Ceolin *et al.*, 2016, Cortazzo *et al.*, 2002, Supek *et al.*, 2014). In the current investigation, 11 of the 12 synonymous and non-coding variants have been previously identified in non-MCT research investigations and are documented in publicly available databases (Asia Ensembl, UniBE - Institute of Genetics, 2012) (**Table 4.9**). Therefore, these are predicted to be species-specific SNPs and not implicated in canine mast cell pathogenesis. A novel exon 9 c.1524C>T synonymous SNP was discovered in a subcutaneous

MCT which had a homozygous genotype for the alternative (T/T). No attempt was made to purify the neoplastic mast cells from the non-cancerous cells during tissue processing. Hence, it is expected that wild-type DNA and neoplastic MCT DNA were co-extracted. Homozygous allele detection suggests the variant existed in both the normal and cancerous DNA and presumably has a germline origin. This variant, therefore, is presumed to be silent and not involved in canine mast cell tumourigenesis.

The commonest nucleotide base change was a conversion from a cytosine (C) to a thymine (T) (**Table 4.8**). This is known to occur throughout the genome by methylation of cytosine to 5-methylcytosine followed by spontaneous deamination of 5-methylcytosine to thymine (Duncan and Miller, 1980). CpG dinucleotides are methylated at a high rate in mammalian genomes thus making the cytosine to thymine mutation a common event (Lokk *et al.*, 2014). Three of the seven C>T variants occurred at CpG dinucleotides.

The genotypes of the exon 11 ITD sequences for the c.1731C>T SNP (rs853024368) first detected in Chapter 3 were confirmed in this chapter by Ion AmpliSeq technology. However, the actual ITD sequences were not called. This can be explained by the automated sequence alignment algorithm used by Ion Torrent sequencing software which aligns the contig (the sequence assembled from a set of overlapping DNA fragments) to the reference genome according to the first ~20 nucleotides. Once the beginning of a contig is aligned to the reference sequence, the software continues aligning the remainder of the contig, flagging any mismatching bases. The *KIT* ITDs, which ranged from 39-bp to 60-bp, may be too large for correct calling and, therefore, the software predicted them to be errors in the sequence. Fortunately, these sequences were not completely omitted from the dataset and just became "soft-clipped". That is, the aligned sequence of the contig is reported in the dataset and the non-aligned predicted erroneous sequence is electronically masked but remains in the raw sequence file. Soft-clipped sequences can be visualised in IGV and this method was used to confirm the ITD sequences detected by conventional PCR and Sanger sequencing in Chapter 3 (**Figure 4.9**). The entire length of the sequenced regions of the *KIT* gene for the 95 MCT AmpliSeq DNA sequences was screened for large insertions and deletions that may have been missed by automated sequencing software. One additional 22-bp ITD in the 3' UTR of *KIT* was identified in two tumours. In genome-wide mutation analyses or studies investigating several hundreds of samples, it is unrealistic to visually review each sequence data file as it was reviewed here. This is a major limitation of Ion AmpliSeq automated sequencing technology.

Homozygous deletion of *KIT* intron 11 has been detected in 49% of MCT cases and correlated to moderately and poorly differentiated MCTs in one study (Reguera *et al.*, 2002). Intron 11 deletion was also found in 13% of normal, non-cancerous control tissue samples. The contribution of the deletion to mast cell malignancy and its effect on *KIT* splicing is unclear. In the present study, a homozygous deletion of intron 11 was not observed in any instance and this large deletion has not been reported in any other molecular studies evaluating genomic DNA. The presence of a *KIT*-derived pseudogene was suspected as a possible explanation of apparent intron 11 deletion. However, alignment of *KIT* exons 11 and 12 to the canine reference genome found no alignments outside the annotated *KIT* gene, indicating that a *KIT* pseudogene is unlikely to exist (**Figure 4.11**). Sample contamination with RNA may explain homozygous intron 11 deletion. The primers described in the initial publication produce a wild-type, genomic DNA amplicon of 448-bp and a cDNA amplicon of 165-bp (Reguera *et al.*, 2002). Differing concentrations of contaminating RNA between MCT samples could lead to favourable PCR amplification of the smaller PCR product, resulting in apparent homozygous intron 11 deletion in some samples. This is speculative and further research is necessary for clarification.

The performance of Ion AmpliSeq PGM in this investigation was comparable to other studies using Ion AmpliSeq technology for mutation detection across multiple genes in various tumours specimens, and in a study using this technology for bacterial genome sequencing (Loman *et al.*, 2012, Malapelle *et al.*, 2015, Rathi *et al.*, 2017). Ion AmpliSeq primer design achieved 96.6% coverage of the *KIT* gene target region. Any variants in *KIT* existing outside the covered regions will not have been detected. The loading density for Chip 1 was considerably lower than that for Chip 2, 50% compared to 92%, but surpassed the minimum recommendation of 30% (Rathi *et al.*, 2017). Despite a considerably higher loading density from Chip 2, fewer usable reads were available for sequencing due to them being polyclonal and of poor quality. Polyclonality is consequent from overloading the chip with excess DNA library. Chip overloading may have contributed to these results.

A major drawback for Ion AmpliSeq NGS is the high rate of false-positive homopolymer-associated indel errors (**Figure 4.7**). A Quality by Depth filtering threshold of < 2 is recommended to remove false-positive variants (Loman *et al.*, 2012, Van der Auwera, 2013, Yeo *et al.*, 2012, Yeo *et al.*, 2014). Using this threshold, 30% (n = 6/20) of apparently erroneous indel mutations were, in fact, concluded to be true frameshift variants. These

results suggest that using this threshold as a cut-off parameter for indel variants was not totally appropriate in this instance. No patterns suggesting a more suitable threshold for the current data were discerned. To overcome this, resequencing the region of interest either multiple times or using alternative sequencing platforms could verify the nature of these variants. However, additional sequencing negates the cost-effective and time-saving benefits accompanying AmpliSeq technology and therefore, is undesirable.

A 5% variant frequency minimum is generally recommended for Ion AmpliSeq NGS to remove false-positives (Tsongalis *et al.*, 2014, Zhang *et al.*, 2014). In a recent Ion AmpliSeq study, 100% sensitivity and specificity for detecting SNPs and indels in FFPE-extracted tumour DNA was established at a 3% frequency (Rathi *et al.*, 2017). In the current study, sample DNA was extracted from mixed MCT and normal tissue specimens, and hence, the 3% threshold was deemed to be more appropriate than the 5% cut-off for detecting low prevalence. No variants fell below the 3% variant frequency threshold (range: 4.08–80%).

4.6 Conclusion

Non-synonymous mutations and non-coding variants which have a potential gain-of-function effect on Kit protein activity were identified in 51.9% (n = 40/77) of cutaneous MCTs and 50.0% (n = 9/18) of subcutaneous MCTs from Australian dogs. Approximately one-fifth of these tumours carried an enzymatic pocket-type mutation. This is likely to be clinically relevant when considering the use of tyrosine kinase inhibitors in the therapeutic management of unresectable or recurrent high-grade MCTs because mutations of this type are likely to induce tumour resistance to this class of drugs. Wet lab experiments are warranted to determine the true functional consequences of the *KIT* mutations on Kit protein activity and response to treatment with tyrosine kinase inhibitors.

Ion AmpliSeq NGS provided good sequencing depth and coverage of the *KIT* gene, and the output was comparable to that reported in other studies. The high rate of homopolymer-associated indel errors and the false-negative callings for large insertions were major drawbacks to this technology but were rectified by filtering the data using quality thresholds and manually reviewing sequence alignments in IGV.

Chapter 5: Mutations in the intracellular enzymatic pocket domain of *KIT* are associated with increased risk of tumour-related death in Australian dogs with mast cell tumours

5.1 Introduction

Mast cell tumours (MCTs) typically arise in the dermis and account for up to 21% of all skin neoplasms in the dog (Kok *et al.*, 2019, Leidinger *et al.*, 2014, Withrow *et al.*, 2013). MCTs occurring at extracutaneous sites, including the viscera and mucosal areas, are uncommon and are associated with poor clinical outcome (Elliott *et al.*, 2016, Takahashi *et al.*, 2000). MCTs restricted to the subcutaneous fat with no dermal or epidermal involvement are termed subcutaneous MCTs. Subcutaneous MCTs are clinically different than their cutaneous counterparts and are easily misdiagnosed as benign lipomas due to their location under the skin and soft feel on palpation (Withrow *et al.*, 2013). The Patnaik and Kiupel grading schematics do not include subcutaneous MCTs and, as a result, subcutaneous MCTs are occasionally mis-graded as the more malignant Patnaik grade II or III cutaneous tumour (Chapter 3). The intermediately-differentiated histological appearance of subcutaneous MCTs in conjunction with their deep subdermal location can result in them being misinterpreted as infiltrative Patnaik cutaneous tumours (Patnaik *et al.*, 1984). Subcutaneous MCTs are generally clinically indolent and, hence, tumour mis-grading can lead to unnecessarily aggressive treatment (Newman *et al.*, 2007, Thompson *et al.*, 2011a). Metastasis is documented in just 4% of subcutaneous MCTs (n = 13/306) and tumour-related death occurs in 9% of dogs (n = 27/306) (Thompson *et al.*, 2011a). This compares favourably to the prognosis for dogs with grade II cutaneous MCTs in which 25% of dogs (n = 30/119) experience MCT-related mortality (Horta *et al.*, 2018b).

The proposed derivation of subcutaneous MCTs from a mast cell lineage originating in the adipose tissue differs from that of cutaneous MCTs, which are believed to originate from CD34+ stem cells of the bone marrow (Galli and Tsai, 2010, Poglio *et al.*, 2010). These distinct mast cell lineages may, in part, explain the relatively quiescent nature of subcutaneous MCTs.

An anomaly in cutaneous MCTs, which is also now evident in subcutaneous MCTs, is the mutation of the *KIT Proto-Oncogene* and abnormal expression of the Kit transmembrane type III tyrosine kinase receptor protein (Chapters 1 and 4) (Kiupel and Camus, 2019). Immunohistochemical detection of focal or diffuse cytoplasmic Kit protein localisation in MCT tissue is associated with increased mast cell proliferation indices, tumour metastasis and local recurrence of both cutaneous and subcutaneous tumours (Horta *et al.*, 2018b, Kiupel *et al.*, 2004, Thompson *et al.*, 2011b, Webster *et al.*, 2007).

Until recently, mutations of the *KIT* gene in subcutaneous MCTs have not been reported (Vozdova *et al.*, 2019b). My data show that almost half of all subcutaneous MCTs carry a mutation in the *KIT* gene coding sequence or non-coding gene splicing domains, at least in Australian dogs (**Figure 4.10**). The effects of these mutations on Kit protein function and mast cell growth remain unknown, as does the clinical importance of *KIT* gene mutations for dogs with subcutaneous MCT. Comparatively, a substantial amount of research in cutaneous MCTs correlates *KIT* exon 11 internal tandem duplications (ITDs) with Kit protein autophosphorylation and increased cell proliferation (Letard *et al.*, 2008, Webster *et al.*, 2007). Further, tumours harbouring a *KIT* exon 11 ITD are more likely to be of high histological grade, and dogs with these tumours are at a greater risk of MCT-related death when analysed by univariable statistics (Horta *et al.*, 2018b, Takeuchi *et al.*, 2013). The non-exon 11 ITD *KIT* mutations in the *KIT* regulatory domain (exons 1–12), however, are not associated with histological grade (Mochizuki *et al.*, 2017b). This parallels data from cats with splenic MCT whereby *KIT* mutations in exons 8 or 9 were not indicative of increased tumour malignancy or worse patient clinical outcomes (Sabattini *et al.*, 2017).

A minority of dogs with MCT may carry a mutation within the intracellular tyrosine kinase domain of the Kit protein (Hahn *et al.*, 2008, Letard *et al.*, 2008). This domain is encoded by *KIT* exons 13–21 and consists of the ATP-binding region and two tyrosine kinase regions (**Figure 1.1**). It is also referred to as the enzymatic pocket domain. Mutation of this domain is clinically relevant when considering the use of tyrosine kinase inhibitors for dogs with tumours unresponsive to traditional therapeutics. In canine and human studies, neoplastic mast cells harbouring an enzymatic pocket mutation are resistant to most tyrosine kinase inhibitor drugs (Halsey *et al.*, 2014, Nakano *et al.*, 2017, Siebenhaar *et al.*, 2018, Verstovsek *et al.*, 2006). Tyrosine kinase inhibitors bind to the enzymatic pocket domain of tyrosine kinase proteins to block phosphorylation of intracellular proteins (London, 2009). Mutation in the enzymatic pocket domain alters the intracellular Kit protein structure, decreasing the affinity of these drugs for the binding site. Neoplastic mast cells with enzymatic pocket-type mutations are consequently resistant to these drugs. Hence, the location of *KIT* mutations within the gene influences the likelihood of tumour responsiveness or resistance to treatment with tyrosine kinase inhibitors, and therefore, the mutation location is of clinical importance.

Mutation of the *KIT* enzymatic pocket domain causes constitutive Kit protein autophosphorylation in human systemic mastocytosis patients (reviewed in Haenisch *et al.*,

2012). Despite evident gain-of-function effects *in vitro, KIT* enzymatic pocket-type mutations have not been correlated with human prognosis or disease phenotype (Bodemer *et al.*, 2010, Yanagihori *et al.*, 2005). In dogs with MCT, enzymatic pocket-type mutations have been rarely reported (Letard *et al.*, 2008, Webster *et al.*, 2006a). This can be attributed to the lack of research evaluating this region of *KIT*. My research has shown that 20.8% of cutaneous canine MCTs and 5.56% of subcutaneous MCTs harboured at least one mutation within the *KIT* enzymatic pocket domain (Chapter 4), suggesting that mutations in this domain are more common than initially thought.

Herein, the relationships between MCT *KIT* gene mutation status (exon 11 ITD, regulatory-type, enzymatic pocket-type and non-coding variants) with tumour phenotype and canine clinical and prognostic parameters are explored in Australian dogs with cutaneous or subcutaneous MCT.

5.2 Methods

5.2.1 Case selection and data collection

The canine MCT exon 11 ITD mutational data were ascertained as described in Chapters 2 and 3. Mutation profiles of the *KIT* gene coding and non-coding splice sequences (referred to herein collectively as *KIT* gene mutation profiles) were generated for a subset of tumours described in Chapter 4.

For each dog from which a tumour was sourced, the clinical data including signalment (breed, age, sex and neuter status), number of multiple concurrent masses, tumour anatomical location, tumour size, completeness of tumour excision and primary veterinary practice were collected from the histopathological reports held in the databases at respective veterinary pathology laboratories (Adelaide and Melbourne Gribbles Veterinary Pathology laboratories and the University of Adelaide's Veterinary Diagnostic Laboratory). A primary veterinary practice was defined as the clinic which performed the excisional MCT biopsy and submitted the tumour specimen to the pathology laboratory. Patient history data encompassing MCT treatment, second MCT diagnosis, tumour metastasis, history of MCT, overall survival, health status at final follow-up and any patient signalment data missing from the pathology reports were collected through contact with veterinary staff at primary clinical practices. Dogs were grouped as being from a metropolitan area or rural area based on the

geographical location of the primary veterinary practice.

All data were collected in compliance with the ethical statements from Gribbles Veterinary Pathology and the University of Adelaide's Animal Ethics Committee (Project Approval Number: S-2016-131). Initial contact was made with each primary veterinary practice via a telephone conversation where the project was described, and participation was requested. Upon the veterinary practice's agreement to participate, a telephone interview was conducted to obtain patient clinical history. In instances where veterinary staff were time poor or requested written information about the project, an email was sent to practice management describing the project in detail along with the Patient History Questionnaire (**Appendix K**). Each practice was able to choose to respond to the questionnaire. If the practice did not respond to the questionnaire within a week, they were contacted by telephone a second time. The objective of the second phone call was to remind them of the project. Their participation in a telephone interview was also requested. If the interview was not able to be completed, the questionnaire was re-sent in a reminder email. Telephone and email contact was made weekly until data were collected. For some clinics, multiple phone calls each followed by an email reminder were required to obtain canine patient clinical histories. It was ultimately within the discretion of each veterinary practice as to whether they would or would not participate in this research.

Dog breeds were grouped according to the Australian National Kennel Council's (ANKC) recognised breed groups for the survival analyses (e.g. Gun dogs, Terriers, Utility dogs etc.). Dogs were analysed according to breed (e.g. Labrador, Boxer, Pug, etc.) for testing the interactions between animal age and tumour grade with dog breed.

Tumours were grouped as being ≤ 3 cm or > 3 cm based on the largest measured diameter recorded in the pathology reports. The 3 cm cut-off size was chosen based on previous research (Mullins *et al.*, 2006, O'Connell and Thomson, 2013, Pierini *et al.*, 2019).

Metastasis at the time of tumour excisional biopsy was considered in prognostic analyses. Only dogs with cytologically or histologically confirmed metastasis at the time of tumour excision were considered as having metastasis in this study. In cases where the clinical report suggested metastasis, but there was no supporting pathological evidence, the disease was classified as non-metastatic.

Histological MCT diagnosis was determined by board-certified veterinary pathologists

as described in Chapter 2. Briefly, tumours were classified as cutaneous or subcutaneous, and cutaneous tumours were further divided as Kiupel low-grade and Kiupel high-grade MCT (Kiupel *et al.*, 2011, Patnaik *et al.*, 1984). The diagnosis of a subcutaneous MCT was based on the tumour being located completely within the subcutis, with no invasion of the dermis or epidermis.

5.2.2 Survival analysis

For the survival analyses, dogs were grouped as either "young" or "old" based on their age and their breed size (**Table 5.1**). This was to account for the different average life expectancies for different breed sizes (Greer *et al.*, 2007).

Table 5.1. Canine age classification according to breed size.

Breed size (weight in kgs)	**Age considered to be old**	**Breed***
Small (1-10)	≥ 10 years	Boston Terrier, Chihuahua, Fox Terrier, French Bulldog, Jack Russell Terrier, Lhasa Apso, Maltese, Miniature Schnauzer, Pug, Tenterfield Terrier
Medium (11-26)	≥ 8 years	American Bulldog, American/English Staffordshire Bull Terrier, Australian Cattle dog, Australian Shepherd, Beagle, Boxer, British Bulldog, Bull Terrier, Kelpie, Poodle, Whippet
Large (27-44)	≥ 7 years	Bull Arab, Bullmastiff, German Shephard, German Shorthaired Pointer, Golden Retriever, Labrador, Rhodesian Ridgeback, Rottweiler
Giant (≥ 45)	≥ 6 years	Bernese Mountain Dog, Great Dane, Saint Bernard

* This list is not exhaustive. Only the breeds diagnosed with mast cell tumours in the current study were included in this table.

Survival analyses were conducted on the entire canine cohort described in Chapters 2 and 3. The survival analyses were then repeated on the subset of dogs included in the AmpliSeq analysis for which *KIT* mutation profiles were available. For dogs with more than one MCT, the tumour of the highest histological grade for that dog was recorded for the survival analyses. In instances where a dog suffered both cutaneous and subcutaneous MCT, the data regarding the cutaneous lesion was recorded because cutaneous MCTs are generally considered to have a poorer prognosis than subcutaneous MCTs (Newman *et al.*, 2007, Thompson *et al.*, 2011a).

Overall survival was measured in days from the date of tumour surgical excision to the date of last clinical appointment or death. Dogs lost to follow-up, healthy dogs alive at the end

of follow-up and dogs which died or were euthanased due to unknown or non-MCT related causes were right-censored from the survival analyses. Median overall survival was defined as the time (in days) at which 50% of dogs were alive post-surgery.

Due to the lack of data obtainable from the primary practices regarding disease progression, the terms tumour-free survival (TFS), progression-free interval (PFI) and similar were not used in the current evaluation so as to not mislead the reader. Tumour-free survival implies a "disease-free" state. However, the disease state was unconfirmed in many instances, especially for animals with incompletely excised tumours. Technically, these animals still bear neoplastic mast cells. Similarly, progression-free interval implies the tumour has not progressed over a time frame. Accurate data regarding metastases and tumour relapse were unavailable, and hence, true progression-free interval times would be ambiguous.

Instead, time to "second MCT diagnosis" was determined to be a suitable substitute for measuring tumour-free survival in this study. Second MCT diagnosis was measured in days from the date of surgery until the date of suspected diagnosis of a second MCT, providing it was confirmed with histopathology. None were confirmed by cytology. It was not possible to unequivocally determine whether additional MCTs were recurrent disease (local relapse or metastasis) or represented an independent primary tumour. Both were included in the definition of second MCT diagnosis. The median time to second MCT diagnosis was defined as the time (in days) at which 50% of dogs were diagnosed with a second MCT post-excision of the tumour collected for the current investigation. Only dogs diagnosed with a second MCT were included in this statistic.

Patient "history of MCT" was also collected and was defined as any MCT diagnosed before the surgical excision of the MCT collected for the current study. Animals with a history of a MCT were not considered in cases of multiple MCT. Only dogs with ≥ 2 concurrent MCT lesions at the time of surgery of the current MCT were considered to be a case of multiple MCT.

5.2.3 Statistical analysis

All statistical analyses were performed in IBM SPSS statistical software (version 25, Armonk) and determined to be statistically significant at an $\alpha < 0.05$. Results are displayed as mean ± standard deviation and median, minimum–maximum.

An analytic cross-sectional study was performed to assess whether there was an association between tumour exon 11 *KIT* ITD mutation status and various patient prognostic indices including patient signalment (ANKC breed group, age, sex, neuter status), patient geographical location, tumour type (cutaneous Kiupel low-grade, cutaneous Kiupel high-grade, subcutaneous), tumour anatomical location, multiple MCTs at time of surgery, tumour size, completeness of tumour excision, confirmed metastasis at time of surgery, second diagnosis of MCT and previous history of MCT diagnosis. To account for non-normally distributed data and a dichotomous dependent variable (ITD mutation status), a binary logistic regression model was used to test for statistical significance. Generalised binary logistic regression fitted with Wald chi-square analysis was performed to ascertain statistically significant differences in mutation prevalence between prognostic factors.

Pearson's chi-square test was used to examine the relationship between confirmed metastasis at the time of surgery with second MCT diagnosis and history of MCT. The strength of association was indicated by the phi (Φ) coefficient.

The influence of the prognostic variables (listed above) on 6- and 12-month canine MCT-related death was assessed by Cox proportional hazards regression model. All factors were analysed by univariable analyses and only variables having a P-value < 0.25 were included in the multivariable model building, as recommended previously (Bursac *et al.*, 2008). Tumour type (cutaneous Kiupel low-grade, cutaneous Kiupel high-grade or subcutaneous, as determined by histology) was included in all multivariable models, regardless of statistical significance, because distinguishing subcutaneous tumours from cutaneous tumours is prognostically important (Newman *et al.*, 2007, Thompson *et al.*, 2011a). Backward selection methods were used to remove non-significant variables, commencing with the least significant, until all variables in the model reached significance at a level of $P < 0.05$. Survival outcomes were estimated using the Kaplan-Meier estimate. Statistics were repeated for the second diagnosis of a MCT at 6- and 12-month time points. Cases with missing data were omitted from statistical analyses.

Results are displayed as hazard ratios with a 95% confidence interval (CI) for the risk of outcome (outcome being MCT-related death or second MCT diagnosis). A hazard ratio > 1 signifies that the risk factor decreases the time to the outcome. A hazard ratio < 1 signifies that the risk factor increases the time to the outcome. Values for categorical risk factors were interpreted as the ratio of the predicted hazard of one group relative to the reference group.

For example, high-grade MCT is a risk factor for 6-month MCT-related death in dogs; hazard ratio (HR) = 7.69, 95% CI: 2.62–22.5, P < 0.001. This is interpreted as dogs with high-grade MCTs have 7.69 times increased risk for MCT-related death at 6-months when compared to the reference group. In this instance, dogs with low-grade tumours were used as the reference group and the hazard risk was statistically significant (P < 0.001). For continuous predictors, hazard ratios represent the effect of a unit of change in the risk factor for the outcome. For example, the risk for canine 12-month MCT-related death statistically significantly increases by 1.55 times for every additional *KIT* mutation detected in the dog's tumour (HR = 1.55, 95% CI: 1.03–2.33, P = 0.036).

Pearson chi-square analysis (χ^2) was used to evaluate the frequency distributions of histological grade and breed in young and old animals, and then to determine the histological grade frequencies between breeds. Dogs with subcutaneous MCTs were not considered in this analysis due to a limited number of individuals with subcutaneous MCT when segregated by breed. The results for a Pearson chi-square analysis indicate the likelihood that the frequency distribution of events observed in a subgroup of a categorical variable is consistent with the frequency distribution of events in the sample population. For example, when considering breed and age, a P-value < 0.05 implies that the distribution of old and young dogs of a particular breed is statistically significantly different from the age distribution in the entire population cohort. In this instance, the categorical variable "breed" has multiple subgroups (Cross-breed, Labrador, Pug, etc.). The results from the Pearson chi-square test statistical analysis imply that a significant difference in age distributions exists among breeds, however, it does not elucidate which breed(s) is statistically significantly different. To address this, cellwise residual analysis on two-way contingency table method was used to establish which demographic group was statistically different (Beasley and Schumacker, 1995, García-pérez and Núñez-antón, 2016). P-values were adjusted to minimise type-I errors (false-positives) as recommended and previously described (Beasley and Schumacker, 1995).

5.3 Results

5.3.1 Evaluable cases

A total of 280 MCTs from 248 dogs were submitted for this study from 130 primary veterinary practices throughout Australia. Clinical data were collected from 118 (90.8%) of the 130 primary practices upon agreement to participate in the study. Data were not collected

from the remaining 12 clinics due to computer system updates at the clinic resulting in loss of patient records (n = 8), patient-doctor confidentiality restrictions (n = 2), non-consenting pet owners (n = 1) or close of practice (n = 1). Consequently, clinical follow-up data were unavailable for 24 animals. Follow-up data were unavailable for a further four dogs which were not seen at their primary practice after tumour excision. These dogs were excluded from the survival analyses. Consequently, data were available for 220 dogs.

5.3.2 Population demographics

The mean age of the 220 dogs was 8.72 ± 2.79 years (range 3–17 years) and dogs were grouped as young (n = 80, 36.4%) or old (n = 140, 63.6%; **Table 5.1**). There were 49 males (29 intact, 20 castrated) and 171 females (65 intact, 106 spayed). Twenty dogs (9.09%) had multiple concurrent MCTs at the time of surgery; one dog had four cutaneous tumours, three dogs had three cutaneous tumours, 15 dogs had two cutaneous tumours and one dog had one cutaneous tumour and one subcutaneous tumour. The remaining dogs were diagnosed with a single cutaneous (n = 169) or subcutaneous (n = 31) MCT. Geographically, 137 (62.3%) dogs were grouped as being from a metropolitan area and 83 (37.7%) were from a rural area.

The breed demographics for the 220 dogs included 57 Cross-breeds (25.9%), 37 Labrador Retrievers (16.8%), 31 Staffordshire Bull Terriers (14.1%; five known to be American Staffordshire Bull Terriers but the remaining 26 were unspecified as being American or English Staffordshire Bull Terriers), 21 Jack Russell Terriers (9.55%), 20 Boxers (9.09%), 14 Golden Retrievers (6.36%), 12 Pug (5.45%), 4 American Bulldogs (1.81%), 2 Bernese Mountain dogs (0.91%), 2 Fox Terriers (0.91%), 2 Tenterfield Terriers (0.91%) and one of each of Australian Cattle dog, Australian Shetland Sheepdog, Beagle, Boston Terrier, British Bulldog, Bull Arab, Bull Mastiff, Bull Terrier, Chihuahua, French Bulldog, German Short-haired Pointer, Kelpie, Lhasa Apso, Maltese, Miniature Schnauzer, Poodle, Rhodesian Ridgeback, Saint Bernard.

For the survival analyses, dogs were grouped as Cross-breed, Gun dog, Other, Terrier and Utility (**Table 5.2**). This ensured a sufficient number of animals were in each breed group to allow for a statistically significant model for predicting outcome (MCT-related death or second MCT diagnosis).

Table 5.2. Division of dog breeds according to the Australian National Kennel Council's (ANKC) breed groups for Cox proportional hazards regression survival analyses.

Breed group	Number of animals (% of total)	Breed (number of animals)
Cross-breed	57 (25.9)	Includes any cross-breed dog
Gun dog	52 (23.6)	German Short-haired Pointer (1), Golden Retriever (14), Labrador (37)
Other	29 (13.2)	American Bulldog (4), Australian Cattle dog (1), Australian Shetland Sheepdog (1), Beagle (1), Boston Terrier (1), British Bulldog (1), Bull Arab* (1), Chihuahua (1), French Bulldog (1), Kelpie (1), Lhasa Apso (1), Maltese (1), Poodle (1), Pug (12), Rhodesian Ridgeback (1)
Terrier	57 (25.9)	Bull Terrier (1), Fox Terrier (2), Jack Russell Terrier (21), Staffordshire Bull Terrier (5 American and 26 unspecified as American or English), Tenterfield Terrier (2)
Utility	25 (11.4)	Bernese Mountain dog (2), Boxer (20), Bull Mastiff (1), Miniature Schnauzer (1), Saint Bernard (1)
TOTAL	**220 (100)**	

* The Bull Arab is not a recognised ANKC pure breed.

MCT anatomical location was known for 218/220 cases. Tumours most frequently presented on the animals' trunk (n = 84, 38.5%), followed by the limbs (n = 60, 27.5%), multiple locations (n = 20, 9.17%), head/neck (n = 18, 8.26%), genitalia (n = 13, 5.96%), paw (n = 8, 3.67%), tail (n = 5, 2.29%), axilla (n = 5, 2.29%) and inguinal region (n = 5, 2.29%). For statistical purposes, the dogs with tumours with an unknown anatomical location and the dogs with tumours located on the genitalia, axilla and inguinal region were grouped together as "Other". This was due to none of the dogs with tumours located on these regions experiencing MCT-related death at 6- or 12-months post-surgery, hence rendering statistical analyses meaningless. Tumours ranged from 3–100 mm in diameter (mean size of 21.0 ± 16.5 mm). The majority of tumours were ≤ 3 cm in size, n = 179 (81.4%).

Twelve (5.45%) dogs had a history of previous MCT diagnosis and the dates for previous tumour diagnosis were known for eight of these dogs. For the eight dogs, the mean and median time between historically recorded MCT and the MCT recorded in the current study was 1,171 ± 638 days and 1,142 (range 344–2,434) days, respectively. Insufficient data were available to ascertain the recurrent or *de novo* relation between the historically recorded

MCT and the tumour collected for the current study.

All dogs in this study underwent surgery for MCT removal. Complete tumour excision with histologically confirmed tumour-free margins was achieved in 16/31 (51.6%) dogs with subcutaneous MCTs, 89/144 (61.8%) dogs with cutaneous low-grade MCTs and 19/45 (42.2%) dogs with cutaneous high-grade MCTs. Twenty-one (9.55%) dogs had enlargement of the regional lymph node or visceromegaly at time of surgery and were suspected to have MCT metastasis. Cytology confirmed metastasis in 10 of these animals; two dogs had low-grade MCT and eight dogs had high-grade MCT. The cytologic criteria leading to the diagnosis of metastasis were not detailed in the client history records and pathology reports were not available. For the purposes of this analysis, the cytological diagnosis of metastasis is assumed to be consistent with Krick *et al.* (2009). No cases of metastasis were confirmed by histopathology. Post-surgical treatment was administered to 22/220 (10%) dogs. Adjuvant treatments included chemotherapy (n = 8), radiotherapy (n = 5), tyrosine kinase inhibitors (n = 4), combination chemotherapy with tyrosine kinase inhibitors (n = 3) and combination radiotherapy with chemotherapy (n = 2).

5.3.3 Tumour type (histological classification) and KIT exon 11 ITD status

Histological diagnosis of the tumours from the 220 dogs included 144 (65.5%), 45 (20.5%) and 31 (14.1%) tumours respectively classified as cutaneous Kiupel low-grade, cutaneous Kiupel high-grade and subcutaneous MCTs. One dog with a low-grade tumour, 19 dogs with a high-grade tumour and one dog with a subcutaneous MCT carried an exon 11 ITD, totalling an ITD prevalence of 9.55%.

5.3.4 Clinical outcome

The median and mean follow-up time for the 220 dogs was 548 days and 598 (± 474) days, respectively, ranging from 2–3,272 days. The shortest follow-up time was recorded as 2 days for two dogs. One dog was seen for wound re-bandaging and not again thereafter. The other dog experienced systemic hyperhistaminemia and subsequently died.

At final follow-up, 128/220 (58.2%) dogs were alive with no sign of MCT, 15 (6.82%) were alive with confirmed MCT, three (1.36%) were alive but with an unknown health status, 27 (12.3%) died or were euthanased due to MCT, 28 (12.7%) died or were euthanised for reasons unrelated to MCT and 19 (8.64%) died or were euthanased for an unknown cause.

Median survival time was not reached for all dogs. The 6-, 12- and 24-month estimated survival probabilities were 92.3%, 89.5% and 87.7% (Kaplan-Meier). To account for the worst-case scenario, the survival analysis was repeated to include dogs with an unknown cause of death (i.e. dogs with unknown cause of death became uncensored). The 6-, 12- and 24-month estimated overall survival probabilities were 90.5%, 86.4% and 82.3%.

Over the study period, 55 (25.0%) dogs had a second diagnosis of cutaneous MCT. Overall, median and mean time to second MCT diagnosis for these animals was 209 days (range from 6–1,661) and 343 (± 366) days, respectively. One animal with an incompletely excised high-grade MCT of the pinna progressed to an ulcerating mass of the entire ear. This animal was euthanased 6-days following initial biopsy excision due to MCT progression and poor quality of life. This animal was not included in the analysis of second MCT diagnosis. At the end of the follow-up period, the 11.4%, 15.5% and 21.8% of all animals had been diagnosed with a second MCT at 6-, 12- and 24-months, respectively.

When separated by histological grade, the median overall survival time of dogs with a high-grade MCT was 179 days compared to 752 days or 542 days for dogs with low-grade or subcutaneous MCT, respectively (**Table 5.3**). The frequency of MCT-related death or second MCT diagnosis at 6-, 12- and 24- months post-tumour excision, was statistically significantly different between dogs with tumours of different histological grades. At 24-months, 37.8% of dogs with a high-grade MCT had succumbed to the disease compared to less than 10% of dogs with a low-grade or subcutaneous MCT.

Table 5.3. Overall survival (OS) and time to second mast cell tumour (MCT) diagnosis for 220 dogs with cutaneous or subcutaneous MCT and separated according to tumour type (cutaneous Kiupel low-grade, cutaneous Kiupel high-grade and subcutaneous). The statistics were calculated using the Kaplan-Meier Estimator of Survival Probability and determined to be significant at a P-value < 0.05 (*) using the log-rank test.

		Cutaneous Kiupel low-grade MCT	Cutaneous Kiupel high-grade MCT	Subcutaneous MCT	P-value[a]
Mean days (± SD)	OS	717 (± 492)	283 (± 294)	502 (± 368)	-
	Second MCT diagnosis	642 (± 507)	206 (± 222)	412 (± 340)	-
Median days (min–max)	OS	752 (2–3272)	179 (2–1194)	542 (6–1155)	-
	Second MCT diagnosis	621 (2–3272)	134 (2–1155)	417 (6–1078)	-
6-months (95% CI)	OS %	96.5 (93.5–99.5)†	77.8 (65.6–89.9)†	93.5 (84.9–100)	< 0.001*
	Second MCT diagnosis %	9.72 (4.88–14.6)†	22.2 (10.1–34.4)†‡	3.23 (0.00–9.45)‡	0.008*
12-months (95% CI)	OS %	95.8 (92.6–99.1)†	68.9 (55.4–82.4)†‡	90.3 (79.9–100)‡	< 0.001*
	Second MCT diagnosis %	13.9 (8.24–19.5)†	26.7 (13.7–39.6)†‡	6.45 (0.00–15.1)†‡	0.004*
24-months (95% CI)	OS %	95.1 (91.6–98.7)†	62.2 (48.1–76.4)†‡	90.3 (79.9–100)‡	< 0.001*
	Second MCT diagnosis %	19.4 (13.0–25.1)†	31.1 (17.6–44.6)†‡	19.4 (5.45–33.3)†‡	0.001*

[a] The Kaplan-Meier log-rank test statistic implies a statistically significant difference between at least two categorical groups (cutaneous Kiupel low-grade, cutaneous Kiupel high-grade and subcutaneous), however, does not indicate which group(s) is significantly different. To overcome this, the test was repeated using two variables at a time. The groups which are statistically different are indicated († and ‡).

Dogs with a cutaneous Kiupel high-grade MCT had a statistically significantly lower overall survival percentage and increased percentage of second MCT diagnosis at 6-, 12- and 24- months post-surgery than dogs with low-grade MCT. Dogs with subcutaneous MCT had a statistically significantly decreased percentage for second MCT diagnosis at all time points when compared to dogs with cutaneous Kiupel high-grade MCT. The overall survival percentage between dogs with subcutaneous MCT and high-grade MCT was significant at 12- and 24-months, but not at 6-months. The overall survival percentage and the percentage of dogs with a second MCT diagnosis was not statistically different at any time point between dogs with cutaneous Kiupel low-grade tumours or subcutaneous tumours.

5.3.5 Univariable Cox proportional hazards regression analysis to predict MCT-related death and second MCT diagnosis

MCT-related death: *KIT* exon 11 ITD mutation status, tumour type, patient age and metastasis at the time of surgery statistically affected 6- and 12-month survival probabilities for dogs with MCT (**Table 5.4**). Dogs with incompletely excised tumours were significantly more likely to experience MCT-related death at 6-months, but not at 12-months. Tumour anatomical location and the second diagnosis of MCT were statistically significant in predicting

MCT-related death at 12-months but not at 6-months.

Dogs whose MCT harboured a *KIT* exon 11 ITD were 4.64 (P = 0.004) and 6.56 (P < 0.001) times more likely experience MCT-related death at 6- and 12-months, respectively, compared to those whose tumours had only wild-type *KIT*. At the end of the follow-up period, the survival rates for dogs with and without an exon 11 ITD were 76.2% (n = 16/21) and 94.0% (n = 187/199; P = 0.002), respectively, at 6-months, and 61.9% (n = 13/21) and 92.5% (184/199; P < 0.001), respectively, at 12-months. In the 12-month univariable analysis, tumour anatomical location was a significant predictor for MCT-related death. Tumours located on the head/neck and the paw carried respective hazards of 2.78 and 7.18 for the increased likelihood of MCT-related death when compared to dogs with tumours on the trunk.

The second diagnosis of a MCT was a risk factor for MCT-related death at 12-months, however, a history of MCT was not a risk factor. Fifty-five animals were diagnosed with a second MCT after surgical excision of the current tumour. The mean and median time between surgery and second MCT diagnosis for these cases was 343 (± 366) days and 209 (range 9–1,661) days, respectively. Confirmed metastasis at time of surgery was statistically correlated with the diagnosis of a second MCT (P = 0. 009; Φ = 0.176) but not with a history of MCT (P = 0.525; Φ = 0.044).

Patient breed, sex and neuter status, geographical location, the presence of multiple lesions, tumour size and history of MCT were not correlated with MCT-related death at any time interval.

Time to second MCT diagnosis: In the univariable analysis, detection of a *KIT* exon 11 ITD, high tumour histological grade, multiple MCT masses and metastasis at the time of surgery significantly decreased time to the diagnosis of a second MCT at both 6- and 12-months post-diagnosis (**Table 5.4**). Whilst the overall statistic for tumour anatomical location was not a significant factor of second MCT diagnosis at either 6- or 12-months, there was a significant hazard for dogs with tumours at multiple locations to be diagnosed with a second MCT at both time intervals, and dogs with a head/neck tumour were more likely to be diagnosed with a second MCT at 12-months than animals with tumours located on the trunk.

Patient breed, age, sex and neuter status, geographical location, tumour anatomical location, completeness of tumour excision, tumour size and history of MCT were not correlated with second MCT diagnosis at any time interval.

Table 5.4. Univariable Cox proportional hazards regression analysis for determining the hazard ratios (HR) of prognostic factors for 6- and 12-month mast cell tumour (MCT)-related death and second MCT diagnosis for 220 dogs with cutaneous or subcutaneous MCTs. Variables were statistically significant at a P-value < 0.050 (*).

	MCT-related death						Second MCT diagnosis					
	6-month			12-month			6-month			12-month		
Covariate	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
KIT exon 11 ITD	4.64	1.63–13.2	0.004*	6.56	2.77–15.6	< 0.001*	2.81	1.05–7.50	0.039*	3.28	1.42–7.55	0.005*
Tumour type[a]			0.001*			< 0.001*			0.015*			0.008*
Cutaneous Kiupel low-grade[b]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Cutaneous Kiupel high-grade	*7.69*	*2.62–22.5*	*< 0.001**	*10.2*	*3.88–26.6*	*< 0.001**	*2.87*	*1.27–6.48*	*0.011**	*2.81*	*1.36–5.78*	*0.005**
Subcutaneous	*2.12*	*0.411–10.9*	*0.370*	*2.66*	*0.666–10.6*	*0.166*	*0.376*	*0.049–2.86*	*0.345*	*0.537*	*0.126–2.30*	*0.402*
Multiple MCTs	1.38	0.315–6.03	0.670	0.970	0.228–4.12	0.968	3.60	1.44–9.03	0.006*	3.00	1.31–6.90	0.010*
Breed[a]			0.417			0.523			0.736			0.394
Cross-breed[b]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Gun dog	*2.64*	*0.682–10.2*	*0.160*	*1.89*	*0.618–5.79*	*0.264*	*1.70*	*0.480–6.03*	*0.410*	*1.02*	*0.344–3.05*	*0.967*
Other	*0.601*	*0.063–5.78*	*0.659*	*0.356*	*0.042–3.05*	*0.346*	*1.37*	*0.308–6.14*	*0.677*	*1.05*	*0.306–3.57*	*0.945*
Terrier	*1.63*	*0.390–6.84*	*0.501*	*1.18*	*0.361–3.88*	*0.782*	*2.26*	*0.697–7.35*	*0.174*	*2.06*	*0.831–5.10*	*0.119*
Utility	*0.757*	*0.079–7.28*	*0.810*	*1.40*	*0.335–5.87*	*0.644*	*1.72*	*0.385–7.68*	*0.478*	*1.01*	*0.262–3.92*	*0.984*
Old age	9.371	1.24–70.66	0.030*	6.369	1.49–27.2	0.012*	1.06	0.469–2.40	0.888	1.00	0.502–2.00	0.994
Sex and neuter status[a]			0.683			0.593			0.956			0.783
Female entire[b]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Female spayed	*1.53*	*0.478–4.86*	*0.475*	*1.32*	*0.502–3.47*	*0.573*	*1.06*	*0.415–2.68*	*0.910*	*0.980*	*0.445–2.16*	*0.961*
Male entire[c]	-	-	-	*0.403*	*0.049–3.35*	*0.400*	*1.39*	*0.408–4.76*	*0.597*	*1.46*	*0.529–4.00*	*0.468*
Male castrated	*2.54*	*0.569–11.4*	*0.222*	*1.72*	*0.431–6.89*	*0.442*	*0.994*	*0.206–4.79*	*0.994*	*0.693*	*0.152–3.16*	*0.636*
Tumour anatomical location[a]			0.060			0.018*			0.138			0.098
Trunk[b]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Head/neck	*2.78*	*0.664–11.6*	*0.161*	*4.56*	*1.47–14.2*	*0.009**	*2.57*	*0.643–10.3*	*0.182*	*3.13*	*1.02–9.58*	*0.045**
Limb	*0.547*	*0.106–2.82*	*0.471*	*0.912*	*0.257–3.23*	*0.886*	*1.63*	*0.548–4.86*	*0.379*	*1.74*	*0.688–4.42*	*0.241*
Multiple	*1.72*	*0.334–8.87*	*0.516*	*1.41*	*0.285–7.00*	*0.672*	*4.83*	*1.56–15.0*	*0.006**	*4.27*	*1.55–11.8*	*0.005**

Other	*0.660*	*0.077–5.65*	*0.705*	*0.546*	*0.066–4.54*	*0.546*	*0.553*	*0.067–4.59*	*0.583*	*0.803*	*0.171–3.78*	*0.782*
Paw	*7.18*	*1.72–30.1*	*0.007**	*6.29*	*1.572–25.2*	*0.009**	*2.12*	*0.255–17.6*	*0.487*	*1.64*	*0.205–13.1*	*0.641*
Tail	*2.86*	*0.334–24.5*	*0.337*	*2.43*	*0.293–20.2*	*0.411*	*2.36*	*0.284–19.6*	*0.426*	*1.80*	*0.224–14.5*	*0.581*
Tumour size (> 3 cm)	1.51	0.490–4.62	0.475	1.08	0.368–3.19	0.885	1.19	0.447–3.18	0.726	0.867	0.335–2.24	0.769
Incomplete tumour excision	2.79	1.08–7.24	0.035*	1.656	0.702–3.91	0.249	1.38	0.631–3.03	0.418	1.82	0.971–3.60	0.087
Geographical location	0.966	0.357–2.61	0.945	1.163	0.503–2.69	0.724	0.969	0.428–2.19	0.940	1.10	0.549–2.19	0.793
Metastasis at time of surgery	7.13	2.32–21.9	0.001*	11.989	4.88–29.5	< 0.001*	4.37	1.50–12.8	0.007*	4.50	1.73–11.7	0.002*
Second diagnosis of MCT	2.52	0.973–6.54	0.057	4.498	1.95–10.4	< 0.001*	-	-	-	-	-	-
History of MCT	0.972	0.129–7.33	0.978	1.471	0.345–6.28	0.602	0.658	0.089–4.87	0.682	0.467	0.064–3.41	0.453

ITD: internal tandem duplication

[a] For categorical variables with ≥ 3 sub-groups, a P-value is calculated to imply the overall statistical value of the categorical variable. The sub-groups are then analysed independently against a reference group and HRs can be calculated.

[b] Reference group for analysis.

[c] Zero intact males at 6-months had experienced MCT-related death so the statistical comparison was not possible.

5.3.6 Multivariable Cox proportional hazards regression analysis to predict MCT-related death and second MCT diagnosis

MCT-related death: No statistically significant risk factors were identified in the final multivariable Cox proportional hazards regression model for 6-month canine MCT-related death. In the 12-month multivariable model, tumour type, confirmed metastasis at the time of surgery and a second diagnosis of MCT were statistically significant in predicting MCT-related death (**Table 5.5**). Exon 11 ITD mutations were not significant in the final multivariable model (P = 0.667), despite a strong relationship with MCT-related death predicted in univariable analysis (P < 0.001; **Figure 5.1**).

Second MCT diagnosis: The risk for second MCT diagnosis was significantly predicted at 6-months in the multivariable analysis by tumour type (P = 0.018) and the presence of multiple MCTs at diagnosis (P = 0.011). At 12-months, multiple MCTs and metastasis at the time of surgery were significant predictors. Tumour type did not retain significance, but it remained in the final model (**Table 5.5**). The variable reporting a diagnosis of a second MCT is redundant in this model and was not measured.

Table 5.5. Multivariable Cox proportional hazards regression analysis for determining the hazard ratios (HR) of prognostic factors for 6- and 12-month mast cell tumour (MCT)-related death and second MCT diagnosis for 220 dogs with cutaneous or subcutaneous MCTs. Variables were statistically significant at a P-value < 0.050 (*).

	MCT-related death			Second MCT diagnosis		
Covariate	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
6 months						
Tumour type[a]			NSS			0.018*
Cutaneous Kiupel low-grade[b]				*(0.000)*		
Cutaneous Kiupel high-grade				*2.91*	*1.29–6.57*	*0.010**
Subcutaneous				*0.458*	*0.059–3.53*	*0.454*
Multiple MCTs			NSS	3.31	1.31–8.35	0.011*
12 months						
Tumour type[a]			0.005*			0.095
Cutaneous Kiupel low-grade[b]	*(0.000)*			*(0.000)*		
Cutaneous Kiupel high-grade	*5.774*	*2.02–16.5*	*0.001**	*2.24*	*1.00–4.98*	*0.047**
Subcutaneous	*2.847*	*0.711–11.4*	*0.140*	*0.655*	*0.151–2.84*	*0.572*
Metastasis at time of surgery	4.007	1.44–11.1	0.008*	2.95	1.01–8.56	0.047*
Second diagnosis of MCT	2.846	1.19–6.82	0.019*	-	-	-
Multiple MCTs			NSS	3.04	1.30–7.09	0.017*

NSS: not statistically significant.

[a] For categorical variables with ≥ 3 sub-groups, a P-value is calculated to imply the overall statistical value of the categorical variable. The sub-groups are then analysed independently against a reference group and HRs can be calculated.

[b] Reference group for analysis.

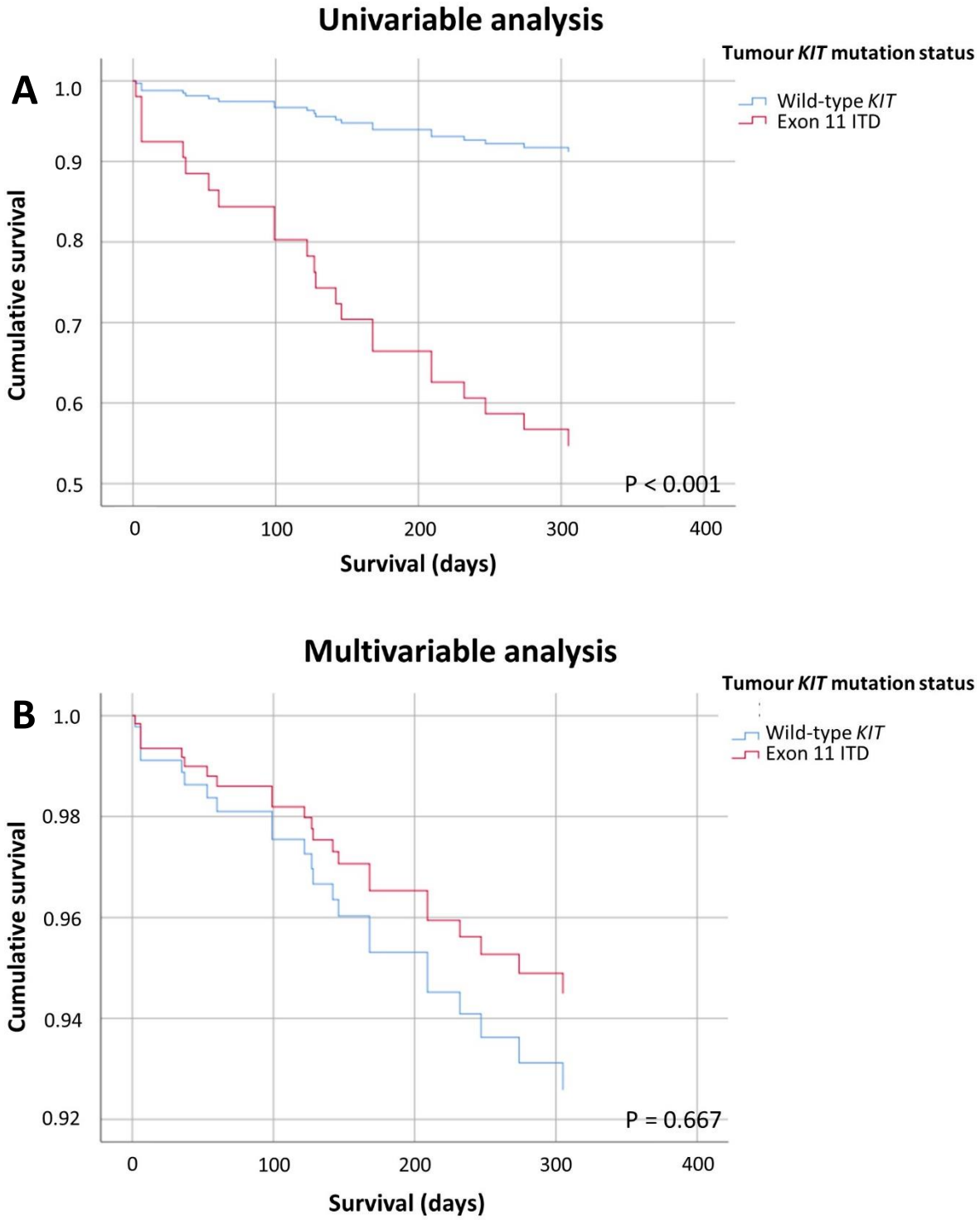

Figure 5.1. Cox proportional hazards regression 12-month survival plot for dogs with cutaneous or subcutaneous mast cell tumours (MCT) with or without a *KIT* exon 11 internal tandem duplication (ITD). Dogs with ITD-mutant tumours had a significantly decreased chance of 12-month survival than dogs with wild-type tumours when analysed with the univariable model (A). After including tumour type, confirmed metastasis at the time of surgery and second diagnosis of MCT in the multivariable model, ITD status was not significantly associated with survival times (B).

5.3.7 Relationships amongst age, breed and histological grade

Age and histological grade: The histological grade frequencies differed significantly between young and old animals (χ^2 [2] = 15.826, P < 0.001). Old animals recorded a higher frequency of high-grade tumours than young animals, 27.9% (n = 39/140) versus 7.50% (n = 6/80), respectively. The distribution of subcutaneous MCTs between young and old dogs was not significantly different (P = 0.368). Subcutaneous tumours were excluded in subsequent analyses.

Dog breed, age and tumour histological grade: When considering the interactions of dog age and tumour grade on breed, the dogs were divided into purebred groups of Boxer, Golden Retriever, Jack Russell Terrier, Labrador, Pug or Staffordshire Bull Terrier and as groups of Cross-breed or Other (**Table 5.6**). This allowed for a statistically significant model for predicting the interactions.

The distribution of age across breeds was significantly different than expected by random chance (χ^2 [7] = 22.7, P = 0.002). The proportion of older dogs was significantly higher than expected in Labrador dogs (90.3%, n = 28/31, P < 0.001) and significantly lower than expected in Pug dogs (16.7%, n= 2/12, P < 0.001). The results remained significant at the reduced alpha level (α = 0.00834), which was adjusted to minimise the risk of making a type-I error (false-positive) (Beasley and Schumacker, 1995).

The distribution of histological grade across the breed was significantly different (χ^2 [7] = 19.9, P = 0.006). The frequency of high-grade MCTs in Labrador retriever dogs was statistically significantly higher than the expected (45.2%, n = 14/31, P = 0.002). In Pug dogs, although 100% (n = 12/12) of animals presented with low-grade MCTs, the difference in the frequency distribution of histological grades was not significant after adjusting alpha (α = 0.00278; P = 0.046).

Table 5.6. Breed demographics for 189 dogs with cutaneous mast cell tumour used for analysing the relationship between canine breed with age and histological grade (Kiupel low-grade and Kiupel high-grade). Dogs with subcutaneous MCTs were not included in the analyses and hence are excluded from this table.

Breed group	Total number of animals (%)	Breed (number of animals)
Cross-breed	49 (25.9)	Includes any cross-breed dog
Boxer	18 (9.09)	Purebred Boxers
Golden Retriever	14 (6.36)	Purebred Golden Retrievers
Jack Russell Terrier	18 (9.55)	Purebred Jack Russell Terriers
Labrador Retriever	31 (16.8)	Purebred Labrador Retrievers
Other	20 (4.09)	American Bulldog (4), Australian Shetland Sheepdog (1), Beagle (1), Bernese Mountain dog (1), Boston Terrier (1), Bull Arab* (1), Bull Mastiff (1), Chihuahua (1), Fox Terrier (2), French Bulldog (1), German Short-haired Pointer (1), Kelpie (1), Lhasa Apso (1), Rhodesian Ridgeback (1), Saint Bernard (1), Tenterfield Terrier (1)
Pug	12 (5.45)	Purebred Pugs
Staffordshire Bull Terrier	27 (14.1)	Includes 5 known purebred American Staffordshire Bull Terrier and 22 dogs unspecified as American or English
TOTAL	**189 (100)**	

* The Bull Arab is not a recognised Australian National Kennel Council (ANCK) pure breed.

5.3.8 ITD correlations with prognostic indices by univariable binary logistic regression analysis

MCT ITD prevalence was significantly correlated with MCT tumour type, completeness of tumour excision and metastasis at the time of surgery in the univariable model (**Table 5.7**). There were no statistically significant associations between ITD prevalence and patient breed, age, sex and neuter status, tumour anatomical location, presence of multiple lesions, tumour size, history of MCT or patient geographical location. The difference in ITD prevalence between dogs with (16.4%, n = 9/55) and without (7.27%, n = 12/165) second MCT diagnosis approached significance, P = 0.053.

ITD prevalence in dogs with cutaneous high-grade MCTs (42.2%, n = 19/45) was

significantly higher than the ITD prevalence in dogs with cutaneous low-grade MCTs (0.694%, n = 1/144) and subcutaneous MCTs (3.23%, n = 1/31) (P < 0.001). ITD prevalence was statistically significantly higher in dogs with incompletely excised tumours (14.6%, n = 14/96) compared to dogs with tumours with histologically tumour-free margins (5.65%, n = 7/124; P = 0.031), and in cases with confirmed metastasis at time of surgery compared to cases without confirmed metastasis at time of surgery, 70% (n = 7/10) versus 6.67% (n = 14/210), respectively (P < 0.001).

5.3.9 ITD correlations with prognostic indices by multivariable binary logistic regression analysis

Tumour type and confirmed metastasis at the time of surgery were statistically significant in predicting the likelihood that MCTs would exhibit a *KIT* exon 11 ITD in the final multivariable model (**Table 5.7**), χ^2 (3) = 64.862, P < 0.001. The model explained 54.6% (Nagelkerke R^2) of the variance in ITD mutation frequency of the data and correctly classified 93.2% of cases. Kiupel high-grade tumours were 80.1 times more likely to exhibit an exon 11 ITD than Kiupel low-grade tumours in the multivariable model (95% CI: 10.0–640, P < 0.001), whereas there was no statistically significant difference in ITD frequency between low-grade and subcutaneous MCTs (P = 0.237). Metastasis at time of surgery was associated with a 12.3 times increased risk of having an ITD (P = 0.013).

The difference in ITD mutation prevalence between completely and incompletely excised tumours was not statistically significant after adjusting for tumour type in the multivariable model. This may be explained by the higher rate of incompletely excised high-grade tumours (57.8%, n = 26/45) compared to the low-grade tumours (38.2%, n = 55/144; P = 0.020), as determined by pairwise comparison. The frequency of incompletely excised tumours was not significantly different between high-grade and subcutaneous MCTs (48.4%, n = 15/31; P = 0.419) or between low-grade and subcutaneous MCTs (P = 0.301). The overall chi-square test for independence approached significance for predicting an association between tumour type and completeness of excision (χ^2 [2] = 5.774, P = 0.056).

Table 5.7. Generalised binary logistic regression analysis with a Wald chi-square model for predicting correlations between *KIT* exon 11 internal tandem duplication (ITD) and prognostic indices for dogs diagnosed with cutaneous or subcutaneous mast cell tumour (MCT). Variables were statistically significant at a P-value < 0.050 (*).

	Univariable analysis				Multivariable analysis		
Covariate	**Exon 11 ITD prevalence (%)**	**SE**	**95% CI**	**P-value**	**HR [Exp(b_i)]**	**95% CI**	**P-value**
Tumour type				< 0.001*			< 0.001*
Cutaneous Kiupel low-grade[a]	*0.69*	*0.007*	*0.098–4.76*		*(0.000)*		
Cutaneous Kiupel high-grade	*42.2*	*7.36*	*28.8–56.9*		*80.1*	*10.0–640*	
Subcutaneous	*3.23*	*3.17*	*0.453–19.6*		*5.47*	*0.327–91.5*	
Multiple MCTs				0.942			
Single	*9.50*	*2.07*	*6.14–14.4*				
Multiple	*10.0*	*6.71*	*2.51–32.4*				
Breed				0.343			
Cross-breed	*7.02*	*3.38*	*2.66–17.3*				
Gun dog	*17.3*	*5.25*	*9.26–30.0*				
Other	*6.90*	*4.71*	*1.73–23.8*				
Terrier	*7.02*	*3.38*	*2.66–17.3*				
Utility	*8.00*	*5.43*	*2.01–26.9*				
Age				0.959			
Young	*9.76*	*4.63*	*3.71–23.3*				
Old	*9.50*	*2.19*	*5.99–14.8*				
Sex and neuter status				0.950			
Female entire	*12.3*	*4.07*	*6.28–22.7*				
Female spayed	*9.43*	*2.84*	*5.15–16.7*				
Male entire	*10.34*	*5.66*	*3.37–27.6*				
Male castrated	*0.00*	*0.00*	*0.00–100*				
Tumour anatomical location				0.573			
Trunk	*7.14*	*2.81*	*3.24–15.0*				
Head/neck	*16.7*	*8.87*	*5.47–40.9*				

Limb	*6.67*	*3.22*	*2.52–16.5*				
Multiple	*10.0*	*6.71*	*2.51–32.4*				
Other	*12.0*	*6.50*	*3.92–31.3*				
Paw	*25.0*	*15.3*	*6.30–62.3*				
Tail	*20.0*	*17.9*	*2.72–69.1*				
Tumour size				0.076			
≤ 3 cm	*7.82*	*2.01*	*4.69–12.8*				
> 3 cm	*17.1*	*5.89*	*8.36–31.7*				
Tumour excision				0.031*			
Complete	*5.65*	*2.07*	*2.71–11.37*				
Incomplete	*14.6*	*3.60*	*8.83–23.1*				
Geographical location				0.663			
Metropolitan	*10.22*	*2.59*	*6.15–16.5*				
Rural	*8.43*	*3.05*	*4.07–16.7*				
Metastasis at time of surgery				< 0.001*			0.013*
No[a]	*6.67*	*1.72*	*3.99–10.9*		*(0.000)*		
Yes	*70.0*	*14.49*	*37.63–90.0*		*12.33*	*1.71–88.76*	
Second diagnosis of MCT				0.053			
No	*7.27*	*2.02*	*4.18–13.4*				
Yes	*16.4*	*4.99*	*8.74–28.6*				
History of MCT				0.883			
No	*9.62*	*2.04*	*6.29–14.4*				
Yes	*8.33*	*7.98*	*1.16–41.32*				

HR: hazard ratio. SE: standard error.

[a] Reference group in multivariate analysis.

5.3.10 Correlations between KIT gene sequence AmpliSeq mutation profiles and risk factors for MCT-related death and second diagnosis of MCT

All previous analyses included the clinical data available for 220 dogs with cutaneous or subcutaneous MCT, unless stated otherwise. In Chapter 4, *KIT* gene mutation profiles were established for a subset of 95 tumours from 93 dogs. In the following sections, risk factors for these dogs are evaluated whilst considering their tumour's *KIT* mutation profile.

Clinical follow-up data were available for 81 animals from the AmpliSeq cohort (Chapter 4). Sixty-seven dogs were diagnosed with cutaneous MCT and 14 dogs were diagnosed with subcutaneous MCT. Tumours ranged from 2–90 mm in diameter (mean size of 21.2 ± 16.7 mm) and 77.8% (n = 63) were ≤ 3 cm. The mean age of 81 dogs in this study was 8.56 years; range: 3–17 years. Dogs were grouped as young (n = 28, 34.6%) and old (n = 53, 65.4%). The median and mean follow-up times for the 81 dogs was 468 days and 556 (± 453) days, respectively, ranging from 7–2,290 days. Over the study period, 29 (35.8%) dogs had a second diagnosis of cutaneous MCT. The median and mean times to second diagnosis of MCT for these animals was 204 days (range from 96–1,348) and 350 (± 351) days, respectively. The estimated probabilities for the diagnosis of a second MCT at 6-, 12- and 24-months were 84.0%, 79.0% and 69.1% for all animals.

KIT mutations were classified according to their functional domain (exon 11 ITD, regulatory-type, enzymatic pocket-type and non-coding). Overall, 51.9% (n = 42/81) of dogs had a tumour with at least one mutation in *KIT.* Of these dogs, 27 had a single *KIT* mutation while ten, four and one dog had 2, 3 and 6 *KIT* mutations, respectively. Of the dogs with a single mutation type, 19 dogs (23.5%) had a non-ITD regulatory-type mutation, seven dogs (8.64%) had an enzymatic pocket-type mutation, six dogs (7.41%) had an exon 11 ITD, and three dogs (3.70%) had a mutation in the *KIT* non-coding domain. Of the dogs with more than one mutation type, four dogs (4.94%) had a non-ITD regulatory-type mutation and an enzymatic pocket-type mutation, one dog (1.23%) had an exon 11 ITD and an enzymatic pocket-type mutation, one dog (1.23%) had an enzymatic pocket-type mutation and a mutation in the *KIT* non-coding domain and one dog (1.23%) had an enzymatic-pocket type mutation, a non-ITD regulatory-type mutation and an exon 11 ITD.

When considering only the dogs with cutaneous MCT, 18 of 67 dogs (26.9%) had at least one non-ITD regulatory-type mutation, 13 dogs (19.4%) had an enzymatic pocket-type

mutation, eight dogs (11.9%) had an exon 11 ITD and three dogs (4.48%) had a mutation in the *KIT* non-coding domain. When considering only the dogs with subcutaneous MCT, six of 14 dogs (42.9%) had at least one non-ITD regulatory-type mutation, one dog (7.14%) had an enzymatic pocket-type mutation and one dog (7.14%) had a mutation in the *KIT* non-coding domain. No dogs with subcutaneous MCT in this investigation harboured an exon 11 ITD.

Univariable analysis: The presence of a *KIT* mutation was not significantly associated with canine MCT-related death or second MCT diagnosis (**Table 5.8**). When the different mutation types were analysed independently, exon 11 ITD-mutant tumours were statistically associated with MCT-related death at 6-months and 12-months post-surgery. At 12 months, mutation of the enzymatic pocket domain increased the risk of MCT-related death by 5.83 times (P = 0.031). An increase in the number of *KIT* mutations by one mutation increased the risk of MCT-related death 12-month post-surgery by 1.55 (95% CI: 1.03–2.33%, P = 0.036). Mutations in the regulatory domain were analysed independently from exon 11 ITDs and were not statistically associated with MCT-related death. Dogs with Kiupel high-grade cutaneous MCTs had a statistically significant increased risk for MCT-related death at 6-months and 12-months post-surgery.

Statistically significant risk factors for a second MCT diagnosis at 6-months and 12-months post-surgery included multiple MCTs (HR = 6.82 and 5.90, respectively) and metastasis at the time of surgery (HR = 7.22 and 5.03, respectively; **Table 5.8**).

Table 5.8. Univariable Cox proportional hazards regression analysis for determining the hazard ratios (HR) of prognostic factors for 6-month and 12-month mast cell tumour (MCT)-related death and a second MCT diagnosis for 81 dogs with cutaneous or subcutaneous MCTs. Variables were statistically significant at a P-value < 0.050 (*).

	MCT-related death						Second MCT diagnosis					
	6-month			12-month			6-month			12-month		
Covariate	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Mutant *KIT*[a]	1.18	0.166–8.39	0.869	2.47	0.452–13.5	0.297	0.692	0.226–2.12	0.518	1.01	0.388–2.61	0.988
KIT exon 11 ITD	10.9	1.52–78.3	0.017*	12.7	2.52–63.6	0.002*	0.867	0.113–6.68	0.891	1.49	0.340–6.55	0.596
Regulatory-type mutation[b]	0.85	0.089–8.19	0.890	1.23	0.225–6.71	0.812	0.202	0.026–1.56	0.125	0.500	0.144–1.74	0.277
Enzymatic pocket-type mutation	5.41	0.760–38.5	0.092	5.83	1.17–29.0	0.031*	2.42	0.745–7.86	0.142	2.38	0.836–6.76	0.104
Non-coding variant	0.046	-	0.777	0.046	-	0.735	0.046	-	0.597	0.046	-	0.555
Number of mutations[c]	1.48	0.860–2.55	0.156	1.55	1.03–2.33	0.036*	1.02	0.617–1.68	0.941	1.12	0.762–1.64	0.570
Tumour type[d]			0.089			0.021*			0.660			0.292
Cutaneous Kiupel low-grade[e]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Cutaneous Kiupel high-grade	*12.7*	*1.32–122*	*0.028**	*19.54*	*2.17–176*	*0.008**	*1.37*	*0.371–5.08*	*0.653*	*2.26*	*0.767–6.63*	*0.140*
Subcutaneous	-	-	-	*4.26*	*0.267–68.2*	*0.305*	*0.482*	*0.061–3.81*	*0.489*	*0.880*	*0.193–4.02*	*0.869*
Multiple MCTs	6.24	0.878–44.3	0.067	3.11	0.570–17.0	0.190	6.82	2.28–20.37	0.001*	5.90	2.23–16.6	< 0.001*
Breed			0.974			0.913			0.307			0.308
Cross-breed[e]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Gun dog[f]	-	-	-	*0.947*	*0.086–10.5*	*0.965*	*1.20*	*0.201–7.19*	*0.841*	*0.615*	*0.124–3.05*	*0.552*
Other[f]	-	-	-	-	-	-	*0.767*	*0.080–7.37*	*0.818*	*0.380*	*0.046–3.16*	*0.371*
Terrier[f]	-	-	-	*2.20*	*0.368–13.2*	*0.387*	*3.39*	*0.876–13.1*	*0.077*	*2.09*	*0.722–6.04*	*0.174*
Utility[f]	-	-	-	-	-	-	-	-	-	-	-	-
Old age	1.63	0.169–15.6	0.674	1.099	0.201–6.00	0.913	0.918	0.300–2.81	0.881	0.843	0.321–2.22	0.729
Sex and neuter status[d]			0.794			0.859			0.773			0.745
Female entire[e]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		

Female spayed	*0.348*	*0.032–3.85*	*0.390*	*0.458*	*0.076–2.74*	*0.392*	*1.62*	*0.419–6.28*	*0.511*	*1.22*	*0.408–3.64*	*0.724*
Male entire[f]	-	-	-	-	-	-	*0.695*	*0.072–6.69*	*0.753*	*0.382*	*0.045–3.28*	*0.380*
Male castrated	*1.31*	*0.119–14.5*	*0.825*	*0.879*	*0.091–8.46*	*0.911*	*1.82*	*0.304–10.9*	*0.511*	*1.085*	*0.210–5.59*	*0.923*
Tumour anatomical location[d]			0.986			0.996			0.862			0.683
Trunk[e]	*(0.000)*			*(0.000)*			*(0.000)*			*(0.000)*		
Head/neck[f]	-	-	-	*0.864*	*0.090*–8.34	*0.899*	*0.590*	*0.069*–5.05	*0.630*	*1.40*	*0.350*–5.62	*0.632*
Limb[f]	-	-	-	-	-	-	*0.527*	*0.126*–2.21	*0.380*	*0.447*	*0.112*–1.79	*0.244*
Multiple	*1.89*	*0.171–20.9*	*0.603*	*1.22*	*-.127–11.8*	*0.861*	*1.51*	*0.293*–7.79	*0.623*	*1.94*	*0.483*–7.78	*0.351*
Other[f]	-	-	-	-	-	-	-	-	-	-	-	-
Paw[f]	-	-	-	-	-	-	*2.30*	*0.265*–12.9	*0.451*	*1.97*	*0.235*–16.5	*0.532*
Tail	*3.30*	*0.298–36.5*	*0.331*	*2.31*	*0.240–22.3*	*0.469*	*1.26*	*0.147*–10.8	*0.834*	*1.06*	*0.128*–8.83	*0.956*
Tumour size (> 3 cm)	1.30	0.134–12.5	0.822	0.841	0.098–7.22	0.875	1.88	0.579–6.13	0.293	1.39	0.451–4.27	0.569
Incomplete tumour excision	2.88	0.299–27.7	0.360	4.58	0.534–39.3	0.165	1.22	0.408–3.62	0.726	1.86	0.686–5.02	0.224
Geographical location	0.966	0.357–2.61	0.945	0.378	0.044–3.23	0.374	1.21	0.396–3.71	0.737	1.39	0.530–3.67	0.501
Metastasis at time of surgery	4.90	0.509–47.2	0.169	2.78	0.324–23.8	0.351	7.22	1.97–26.4	0.003*	5.03	1.44–17.6	0.011*
Second diagnosis of MCT	4.62	0.481–44.5	0.185	7.47	0.872–63.9	0.066	-	-	-	-	-	-
History of MCT[f]	-	-	0.739	-	-	0.672	1.67	0.216–12.8	0.625	1.14	0.151–8.63	0.897

ITD: internal tandem duplication.

[a] Mutant *KIT* was defined as a dog with a tumour with at least one exon 11 ITD, regulatory-type, enzymatic pocket-type or non-coding type mutation.

[b] Excludes exon 11 ITD.

[c] The HR increases proportionally for every additional *KIT* mutation.

[d] For categorical variables with ≥ 3 sub-groups, a P-value is calculated to imply the overall statistical value of the categorical variable. The sub-groups are then analysed independently against a reference group and HRs can be calculated.

[e] Reference group in the analysis and indicated by HRs of 0.000.

[f] Zero animals experienced outcome so the statistical comparison was not possible.

Multivariable analysis: No variables in the multivariable analysis for MCT-related death were significant at 6-months. At 12-months, the covariates remaining significant in the multivariable model included tumour type and enzymatic pocket-type mutation (**Table 5.9**; **Figure 5.2**). The presence of an enzymatic pocket-type mutation increased the risk of MCT-related death at 6-months by 6.17 times.

When considering the risk for second MCT diagnosis, tumour type was not a significant predictor at either 6- or 12-months in the multivariable model. Nonetheless, tumour type was added into both models because Kiupel histological grade is a known important prognostic variable for dogs with cutaneous MCT, and dogs with subcutaneous MCT have a predictably more favourable outcome than do dogs with intermediately- and poorly-differentiated cutaneous MCTs (Horta *et al.*, 2018b, Newman *et al.*, 2007, Thompson *et al.*, 2011a). Hence, tumour type was believed to be an important prognostic indicator. At 6-months, multiple MCTs and confirmed metastasis at the time of surgery were significant hazards for the diagnosis of a second MCT (**Table 5.9**). At 12 months, the presence of multiple MCT masses at the time of surgery was the only statistically significant factor for predicting the risk of a second MCT diagnosis (**Figure 5.2**).

Table 5.9. Multivariable Cox proportional hazards regression analysis for determining the hazard ratios (HR) of prognostic factors for 12-month mast cell tumour (MCT)-related death and second MCT diagnosis for 81 dogs with cutaneous or subcutaneous MCTs. Variables were statistically significant at a P-value < 0.050 (*).

	MCT-related death			**Second MCT diagnosis**		
Covariate	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
6-months						
Tumour type			NSS			0.392
Cutaneous Kiupel low-grade				*(0.000)*		
Cutaneous Kiupel high-grade				*0.525*	*0.113–2.43*	*0.409*
Subcutaneous				*0.229*	*0.024–2.17*	*0.198*
Multiple MCTs			NSS	8.95	2.53–31.6	0.001*
Metastasis at time of surgery			NSS	11.3	1.92–66.6	0.007*
12-months						
Tumour type			0.024*			0.308
Kiupel cutaneous low-grade	*(0.000)*			*(0.000)*		
Kiupel cutaneous high-grade	*19.9*	*2.21–179*	*0.008**	*2.36*	*0.787–7.11*	*0.125*
Subcutaneous	*5.50*	*0.338–90.1*	*0.230*	*1.29*	*0.270–6.13*	*0.752*
Enzymatic pocket-type mutation	6.17	1.21–31.3	0.028*			NSS
Multiple MCTs			NSS	6.07	2.22–16.6	< 0.001*

NSS: not statistically significant

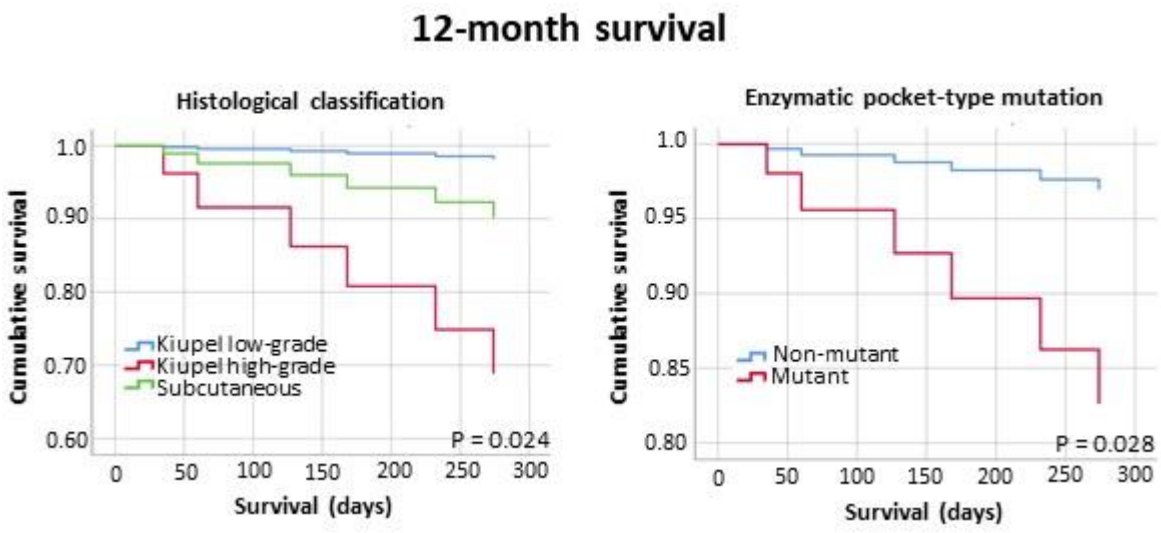

Figure 5.2. Multivariable 12-month Cox proportional hazards regression survival plot for dogs with cutaneous or subcutaneous mast cell tumour (MCT). Illustrated are the survival curves separated according to tumour type (Kiupel low-grade, Kiupel high-grade or subcutaneous) and the mutational status of the *KIT* enzymatic pocket domain (mutant or non-mutant). Variables incorporated into the final survival model included tumour type and enzymatic pocket-type mutation.

5.3.11 Correlations between KIT gene mutation profiles and MCT prognostic indices

Despite mutation of the enzymatic pocket domain being a risk factor for MCT-related death of dogs 12-months post-surgery, this mutation type was not correlated with tumour histological grade or any other prognostic indicator (**Table 5.10**). MCTs with regulatory-type mutations (excluding exon 11 ITDs) or non-coding variants were also not correlated with any prognostic factor.

Table 5.10. P-values for predicting correlations between *KIT* mutation of different functional domains and prognostic indices for 81 dogs diagnosed with cutaneous or subcutaneous mast cell tumour (MCT). No variables were statistically significant with a P-value level < 0.05 using univariable generalised binary logistic regression analysis with a Wald chi-square model.

	P-value			
Covariate	**At least one *KIT* mutation**	**Regulatory domain (excludes exon 11 ITD)**	**Enzymatic pocket domain**	**Non-coding domain**
Tumour type	0.109	0.443	0.560	0.867
Multiple MCTs	0.627	0.761	0.448	0.999
Breed	0.415	0.166	0.952	1.000
Old age	0.103	0.244	0.921	0.963
Sex and neuter status	0.800	0.967	0.799	0.877
Tumour anatomical location	0.982	1.000	0.955	0.999
Tumour size	0.859	0.697	0.167	0.999
Incomplete tumour excision	0.934	0.638	0.413	0.665
Geographical location	0.986	0.193	0.994	0.287
Metastasis at time of surgery	0.225	0.603	0.868	1.000
Second diagnosis of MCT	0.346	0.420	0.546	0.999
History of MCT	1.000	1.000	1.000	1.000

5.4 Discussion

Mirroring previous data, high-grade MCTs were 80.1 times more likely to exhibit an exon 11 ITD than low-grade tumours in this study (Chapter 1, **Table 1.1**). The respective ITD prevalence in dogs with high-grade and low-grade MCT was 42.2% (n = 19/45) and 0.694% (n = 1/144). Dogs in the current study with an exon 11 ITD-mutant tumour were accompanied with a poor prognosis, inferred from the hazard ratios of 4.64 and 2.81 for MCT-related death and second MCT diagnosis at 6-months, respectively, compared to dogs without ITD-mutant tumours when analysed in univariable analyses. In the multivariable generalised binary logistic regression analysis, exon 11 ITD status was statistically significantly associated with histological grade and confirmed metastasis at the time of surgery.

In the multivariable survival models, statistical significance was not retained for exon 11 ITD mutation status for predicting MCT-related death or second MCT diagnosis. This supports the results from a recent investigation in which exon 11 ITD mutation was not independent of histological grade as a statistically significant predictor of overall survival in a multivariable model (Horta *et al.*, 2018b). This contrasts with the results from one research group where *KIT* ITD status

was significant in multivariable analysis for predicting overall canine survival and disease-free interval (Webster *et al.*, 2008). However, that study was limited by a low number of 28 samples, of which only 4 harboured an ITD. A larger multivariable study by the same research group where 9 of 60 tumours (15%) carried a *KIT* ITD concluded that dogs with ITD-mutant MCTs have a statistically significant increased risk for MCT-related mortality and MCT relapse (locally or distally) (Webster *et al.*, 2006b). However, it is not clear whether histological grade was included in the multivariable model. The prognostic value of ITD mutation status independent of histological grade is debatable.

Non-exon 11 ITD mutations in the regulatory domain of *KIT* are also common in canine MCTs. When analysed independently from exon 11 ITDs, other regulatory-type mutations were not associated with histological grade (**Table 5.10**) or MCT-related death and second MCT diagnosis at any time point (**Table 5.8**). This supports earlier reports that regulatory-type activating mutations are not associated with canine survival times or histological grade (Mochizuki *et al.*, 2017b, Takeuchi *et al.*, 2013, Vozdova *et al.*, 2019c). This non-association is particularly curious considering that several studies evaluating the effects of exons 8 and 9 *KIT* mutations on Kit protein function have shown that these mutations cause Kit protein autophosphorylation in every instance (Amagai *et al.*, 2015, Kitayama *et al.*, 1995, Letard *et al.*, 2008, Ma *et al.*, 1999). Further, using cell proliferation assays, one study has shown that two non-ITD regulatory-type mutations (an exon 11 Met555_Glu557delinsVal and an exon 9 Ser479Ile) transfected into Ba/F3 cells (an interleukin-3-dependent hematopoietic cell line) proliferated in the absence of Kit-ligand, whereas wild-type Ba/F3 cells under the same conditions did not (Letard *et al.*, 2008). The absence of a statistically significant association between regulatory-type mutations and canine prognosis perhaps suggests that whilst a non-exon 11 ITD mutation of this domain is likely to be involved in neoplastic mast cell biology, it is perhaps insufficient in causing a malignant disease phenotype. The proposition of mutation type and location within the gene and effects on disease phenotype is documented in other tyrosine kinase proteins. For example, the *RET Receptor Tyrosine Kinase Proto-Oncogene (RET)* is involved in the normal development and maturation of several nerve cell lineages, kidney morphogenesis and spermatogenesis (Arighi *et al.*, 2005). In humans, more than 25 mutations in the *RET* gene have been associated with specific subtypes of multiple endocrine neoplasia type 2 (MEN 2). SNP mutations associated with

MEN type 2A occur within the DNA encoding various cysteine residues (609, 611, 618, 620 and 634), the most common being Cys634Arg (Eng, 1996). Met918Thr is associated with MEN type 2B, and Glu768Asp and Val804Leu SNPs have only been documented in familial medullary thyroid carcinoma MEN subtype. It may be true for dogs with *KIT*-mutant MCTs that the mutation location and mutation type may be significant in disease phenotype. However, in logistic analysis, non-exon 11 ITD regulatory-type mutations were not associated with tumour histological grade, or any other prognostic factor (**Table 5.10**). Analogously, human and feline research has not found any correlations between tumour *KIT* mutation status and mast cell neoplasia disease phenotype or patient clinical outcome (Bodemer *et al.*, 2010, Isotani *et al.*, 2010, Sabattini *et al.*, 2017, Verzijl *et al.*, 2007).

Similarly, non-coding *KIT* variants located in the *KIT* modifying domains were not statistically associated with canine MCT-related death, second diagnosis of MCT or any other prognostic factor evaluated in this study. Furthermore, the functional consequence of these variants on Kit protein splicing and activity has not been investigated.

A major and novel finding of this research was the significance of enzymatic pocket-type mutations for predicting 12-month MCT-related death in both the univariable and multivariable analyses. When considered in conjunction with tumour histological grade, the hazard for 12-month MCT-related death increased significantly by 6.17. Evaluation of the enzymatic pocket domain for mutation supplements histological classification of tumour type and may identify cases that have a high-risk of 12-month MCT-related mortality which histological grade alone may not identify. Remarkably, dogs with enzymatic-pocket domain-mutant MCTs were not at a significantly increased risk for MCT-related death at 6-months or second MCT diagnosis at any time point. Further, enzymatic pocket-type mutation status was not correlated with tumour histological grade nor any other prognostic factor. The use of enzymatic pocket domain mutation status is apparently limited to canine 12-month survival. These results warrant the testing of low-grade MCTs for enzymatic-pocket type mutations to identify tumours with a potentially aggressive clinical course. Further research on a separate cohort of dogs is required to validate these findings

Herein, 20.8% (n = 16/77) of all cutaneous MCTs and 20.0% (n = 2/10) of all exon 11 ITD-

mutant cutaneous MCTs harboured at least one mutation in the enzymatic pocket domain of *KIT* (**Figure 4.10**). Previously, mutation of the enzymatic pocket domain (encoded by *KIT* exons 13–21) has been reported in < 1% of cutaneous canine MCTs, however, the entire tyrosine kinase domain is seldom sequenced (Hahn *et al.*, 2008, Letard *et al.*, 2008, Webster *et al.*, 2006a). Mutations in exons 14, 15, 17 and 18 cause Kit protein autophosphorylation, increased *KIT* gene and Kit protein expression and resistance to tyrosine kinase inhibitor drugs (Halsey *et al.*, 2014, Nakano *et al.*, 2017). If the non-synonymous mutations detected in exons 13–15, 19 and 21 (Chapter 4) also exhibit the gain-of-function properties as other non-synonymous enzymatic pocket-type mutations, it is not unreasonable to suspect that a fifth of tumours with an exon 11 ITD could be, in fact, innately resistant to treatment with tyrosine kinase inhibitor drugs.

The actual effects of the enzymatic pocket-type mutations reported herein (Chapter 4) on Kit protein activity are unknown but are suspected to alter Kit protein structure, and consequently to decrease the affinity of tyrosine kinase drugs to the protein binding site. The 20% enzymatic pocket domain mutation frequency discovered in this investigation may explain tumour unresponsiveness and resistance to tyrosine kinase inhibitors in other research studies, even in the presence of exon 11 ITD mutations in some of the tumours (London *et al.*, 2009, Weishaar *et al.*, 2018). Mast cell resistance to tyrosine kinase targeted drugs through the acquisition of enzymatic pocket-type mutations has been demonstrated *in vitro* in canine C2 cells (an ITD-mutant mastocytoma cell line) after continuous exposure to toceranib (Halsey *et al.*, 2014). Targeting both wild-type *KIT* and *KIT*-mutant neoplastic mast cells using combination conventional chemotherapeutic agents concurrently with tyrosine kinase inhibitor drugs may be most beneficial for most dogs with MCTs (Burton *et al.*, 2015, Olsen *et al.*, 2018, Robat *et al.*, 2012). Results from combination therapeutic trials are limited in the currently available literature. Further research on the efficacy of these drugs against neoplastic mast cells and the tolerability of these agents in dogs with MCTs is warranted.

Mirroring data from previous studies, dogs with a high-grade MCT had a significantly decreased chance of survival at 6-, 12- and 24-months post-surgery compared to dogs with a low-grade or subcutaneous tumour (**Table 5.3**) (Kiupel *et al.*, 2011, Krick *et al.*, 2009, Murphy *et al.*, 2004). The overall survival percentage of dogs with subcutaneous MCTs was similar to that of dogs with cutaneous low-grade MCTs, agreeing with previous conclusions of a good prognosis for

dogs with subcutaneous MCTs (Newman *et al.*, 2007, Thompson *et al.*, 2011a, Thompson *et al.*, 2011b).

The clinical data reported here are from the retrospective review of cases seen in general practice, mainly from clinics in South Australia and Victoria. Notably, the population of dogs seen at the University of Adelaide's Companion Animal Health Centre may have been biased towards the inclusion of more aggressive tumour cases through referral to the veterinary oncologist at the Centre. However, the Centre's cases made up only 5.45% (n = 12/220) of the total cohort and included one high-grade, eight low-grade and three subcutaneous MCTs. The distribution of grades in the Centre's population resembles the grade frequencies of the entire cohort.

As with most neoplastic diseases, increasing age is a risk factor for MCT development with the average age of tumour presentation being approximately 8 years (Giantin *et al.*, 2012, Rothwell *et al.*, 1987, Tamlin *et al.*, 2017, Webster *et al.*, 2006b). Logically, this may be explained by the increased likelihood of somatic mutation occurrence and mutation accumulation in older animals. In this study, dogs were grouped as young or old based on the animal's age and normalised by breed size (**Table 5.1**). This was to account for the shorter average life expectancy of large dog breeds compared to small dog breeds (Greer *et al.*, 2007). In the univariable analysis and reflecting data from other studies, older dogs had a statistically significantly increased risk of MCT-related death at both 6- and 12-months (**Table 5.4**) (Kiupel *et al.*, 2005, Mochizuki *et al.*, 2017a). However, when the histological grade, metastasis at the time of surgery and second MCT diagnosis was accounted for in the multivariable analysis, old age did not retain significance.

The tendency for older dogs to develop high-grade MCTs and younger dogs to develop low-grade MCTs has been documented but not well evaluated (Mochizuki *et al.*, 2017a, Smiech *et al.*, 2018). The relationship between age, breed and tumour malignancy must be carefully considered. Increased risk of MCT-related death in older animals may be attributed to owners electing euthanasia over tumour treatment in senior dogs. However, at a closer examination of the current data, another explanation is apparent. A higher frequency of high-grade MCTs was observed in older animals when compared to younger animals; 27.9% (n = 39/140) versus 7.50% (n = 6/80), respectively (P = 0.001), suggesting that older dogs experience more aggressive disease which is accompanied by an increased rate of mortality. This observation may be a consequence

of selection bias. Pet owners and veterinarians may be less concerned about benign-appearing lumps in older dogs than in younger dogs. Subsequently, an under-diagnosis of benign MCT in older animals may have intrinsically biased the older canine population to an increase in the relative prevalence of high-grade MCT. Still, the discrepancy in histological grade distributions between young and old animals was intriguing and was further investigated. It was hypothesised that dog breed may provide an explanation. Old dogs were observed at a statistically significant higher frequency than expected in Labrador dogs (90.3%, n = 28/31, $P < 0.001$) and a significantly lower frequency than expected in Pug dogs (16.7%, n = 2/12, $P < 0.001$). Furthermore, Labrador dogs had an increased frequency of high-grade MCTs (45.2%, n = 14/31), whereas 100% (n = 12) of Pug dogs presented with low-grade MCTs. This suggests that the significance of age in the univariable survival model may be a result of the predisposition of particular dog breeds for developing MCTs of different malignant phenotypes at different ages. No other correlations with age, breed and histological tumour grade were observed. The results indicate that patient and tumour factors may play a role in tumour histological grade which affects outcome and therefore, should be carefully considered in prognosis.

Breeds reported to be susceptible to MCT development vary considerably. Those commonly regarded as being predisposed to MCTs include breeds of Bulldog origin (American and English Staffordshire Bull Terriers, Boston Terriers, Boxers, French and English Bulldog, Pugs and Shar-Pei dogs), Golden Retrievers and Labrador Retrievers (Jaensch, 2008, Leidinger *et al.*, 2014, McNiel *et al.*, 2006, Mochizuki *et al.*, 2017a, Murphy *et al.*, 2006, Smiech *et al.*, 2018, Warland and Dobson, 2013). Low-risk breeds include German Shepard dogs, Poodles, Dobermanns, Pomeranians and English Cocker Spaniels (Jaensch, 2008, Leidinger *et al.*, 2014, Mochizuki *et al.*, 2017a, Murphy *et al.*, 2006, Warland and Dobson, 2013). High-grade MCTs are more likely to develop in Parson Russell Terriers, Rottweiler, Shar-pei and Shih Tzu dogs than low-grade tumours (Mochizuki *et al.*, 2017a, Reynolds *et al.*, 2019, Smiech *et al.*, 2019, Smiech *et al.*, 2018). In contrast, Boxer and Pug dogs are at high-risk for low-grade MCTs at a younger age (Bostock, 1973, Fonseca-Alves *et al.*, 2015, Jaensch, 2008, McNiel *et al.*, 2006, Mochizuki *et al.*, 2017a, Smiech *et al.*, 2019, Smiech *et al.*, 2018). In general, larger dog breeds ($\geq$ 18 kgs) have a markedly increased chance of developing MCTs compared to small dogs breeds, and small to medium-sized breeds tend to develop MCTs at an older age (Mochizuki *et al.*, 2017a, Shoop *et al.*,

2015, White *et al.*, 2011). The tendency for certain breeds to develop MCT suggests an underlying germ-line genetic predisposition.

In this study, Labrador dogs were at a statistically significant risk for high-grade MCT diagnosis at an older age (≥ 7 years old) which completely opposes the results of two studies by the same research group in Poland where Labradors were found to be at risk for low-grade MCT development at a younger age (4–6 years; P = 0.006) (Smiech *et al.*, 2019). In one recent Australian study, 31.3% (n = 10/32) of MCTs from Labradors were diagnosed as high-grade tumours, although age was not considered in the analysis and the hazard for high-grade MCT development in Labradors was not significant (Reynolds *et al.*, 2019). The discrepancies in Labrador-related risks for MCT development may reflect the geographical differences in MCT genetics in isolated populations throughout the world. A genome-wide association study in Golden Retrievers from Europe and the USA identified distinct differences in predisposing germ-line genetic factors associated with cutaneous MCT development in the two populations (Arendt *et al.*, 2015). Additionally, the *KIT* exon 11 ITD mutation prevalence in canine MCT populations studied in Europe is substantially lower than the prevalence of mutations in canine populations from American studies (**Appendix C**). Additional research is required to further explore the ostensible geographical differences in MCT genetics between different continents.

KIT mutations arise somatically. Therefore, it is not expected that particular dog breeds would be more at risk for developing a *KIT*-mutant MCT. Indeed, it is possible that some breeds may be genetically predisposed to have inferior DNA repair mechanisms which may result in increased risk to somatic mutation. In this study, no statistically significant associations between breed and *KIT* mutation type were discovered (**Table 5.10**). In contrast, one research group who screened 147 primary cutaneous MCTs for *KIT* mutations from a variety of breeds found that Pug dogs were six times less likely to develop *KIT* mutations when compared to other dog breeds in multivariable analysis (P = 0.006) (Mochizuki *et al.*, 2017b). The lack of *KIT* mutations may, in part, explain the paucity of high-grade MCT in Pug dogs.

Recent research indicates that female dogs are at increased risk for developing MCTs, although these tend to be of low histological grade (Smiech *et al.*, 2019, Smiech *et al.*, 2018). Male dogs appear to be at higher risk for high-grade MCT development and are at risk of a poor clinical

outcome (Kiupel *et al.*, 2005, Mochizuki *et al.*, 2017a). Interestingly, there were 3.49 times more female than male dogs in the current study, but no correlations between gender and MCT-related death, second MCT diagnosis or *KIT* mutations were observed.

There is no clear consensus on the effects of gonadectomy on MCT development and prognosis. Many reports suggest neutering increases the risk of MCT development and is associated with short survival times (Kiupel *et al.*, 2005, Mullins *et al.*, 2006, O'Connell and Thomson, 2013, Pierini *et al.*, 2019, White *et al.*, 2011, Zink *et al.*, 2014). In contrast, other reports indicate that intact dogs are at greater risk (Mochizuki *et al.*, 2017a, Shoop *et al.*, 2015), or that there is no association (Leidinger *et al.*, 2014, Reynolds *et al.*, 2019). Agreeing with the latter investigations, the current research found no differences in the risk for MCT-related death, second MCT diagnosis or *KIT* mutation between groups of males and females of different neuter statuses. Regardless, discrepancies in the literature are apparent and the biological contribution of male and female sex hormones in mast cell tumourigenesis is not fully understood. Further research is warranted.

Other factors prompting a more guarded prognosis include the presence of systemic paraneoplastic signs at the time of tumour diagnoses, tumour size, fast tumour growth rate and tumour malignant appearance (e.g. ulceration) (Blackwood *et al.*, 2012). Of these, only data regarding tumour size were available from retrospective analysis. Tumour size (≤ 3 cm versus > 3 cm) was not a significant prognostic indicator for predicting MCT-related death or second MCT diagnosis at any time point (**Table 5.9**). This is in direct contrast to multiple research investigations which report tumours larger than 3 cm as having decreased progression-free survival times and median survival times and higher probability of metastasis to regional lymph nodes (includes pre-metastatic lymph nodes, early metastasis and overt metastasis) (Ferrari *et al.*, 2018, Mullins *et al.*, 2006, O'Connell and Thomson, 2013). Notably, these conclusions were based on univariable analyses. Tumours larger than > 3 cm have not been associated with shorter canine survival in multivariable analyses (O'Connell and Thomson, 2013, Pierini *et al.*, 2019). Further, there are no apparent correlations between tumour size and histological grade (Reynolds *et al.*, 2019). Nonetheless, the association with increased risk of a second MCT event and decreased progression-free survival times as tumour size increases in other investigations exemplifies its use as a negative predictor of canine prognosis.

Incomplete tumour excision is also historically regarded as being a negative factor for canine survival and tumour recurrence (Camus *et al.*, 2016). Some research demonstrates no statistical correlations between completeness of tumour excision with patient survival or tumour recurrence after accounting for tumour histological grade in multivariable analyses (Mullins *et al.*, 2006, Olsen *et al.*, 2018). High-grade MCTs pose the greatest risk for tumour recurrence, with 36% of high-grade MCTs recurring locally, regardless of histologically tumour-free margins (Donnelly *et al.*, 2015, Schwab *et al.*, 2014). Adequate control of Kiupel low-grade tumours can be achieved with margins of < 2 mm (Donnelly *et al.*, 2015), and approximately 23% of incompletely excised Patnaik grade II tumours (n = 7/30) recur locally 12-months post-surgery (Séguin *et al.*, 2006). Supporting latter research, incomplete tumour excision was not statistically significant in predicting the risk of MCT-related mortality or second MCT diagnosis in the multivariable model for dogs in the current study. In the univariable model, dogs with incompletely excised MCTs were at a 2.79 increased risk of MCT-related death at 6-months (P = 0.035), however, those who survived past this time point were not at an increased risk of MCT-related death thereafter (**Table 5.4**). Still, veterinary surgeons should aspire to achieve complete tumour excision, and further local therapy may be otherwise advisable.

Similar to previous reports, majority of MCTs in the current study presented on the trunk (38.5%, n = 84/218) or limbs of the animal (27.5%, n = 60/218) (Leidinger *et al.*, 2014, Mullins *et al.*, 2006, O'Connell and Thomson, 2013, Poirier *et al.*, 2006, Séguin *et al.*, 2006, Simoes *et al.*, 1994, Stefanello *et al.*, 2015). Dogs with tumours located on these anatomical sites have reportedly increased mean survival times and decreased tumour metastatic potential compared to tumours located elsewhere (O'Connell and Thomson, 2013, Stefanello *et al.*, 2015). This outcome may be due to their more noticeable location, permitting early detection and therapeutic management, and a less challenging surgical site, allowing for complete tumour excision. MCTs located on mucosal areas, head, neck, inguinal regions and digits have been reported to carry an unfavourable prognosis (Elliott *et al.*, 2016, Gieger *et al.*, 2003, Hillman *et al.*, 2010, Kiupel *et al.*, 2005, Reynolds *et al.*, 2019, Stefanello *et al.*, 2015). Similarly reported here, dogs with tumours located on the head/neck or paw had an increased risk of 12-month MCT-related death when compared to animals with tumours on the trunk (**Table 5.4**). Dogs with a head/neck tumour were also at an increased risk to be diagnosed with a second MCT at 12-

months post-surgery. The risk for anatomical location in MCT-related death or second MCT diagnosis did not remain statistically significant in the multivariable model.

Only 9% (n = 20/220) of animals in this study presented with multiple concurrent tumours, however, up to 21% of all MCT cases have been documented with multiple lesions (Murphy *et al.*, 2006, Pierini *et al.*, 2019). There is a debate in the literature regarding the prognostic utility of the presence of multiple MCT lesions. In the current study, multiple MCTs did not predict decreased survival, although they were a risk factor for a diagnosis of a second MCT at 6- and 12-months in both univariable and multivariable models (**Table 5.4** and **Table 5.5**). The World Health Organisation (WHO) Clinical Staging scheme for canine MCTs (**Appendix A**) regards dogs with multiple tumours (Clinical Stage III) to have a worse clinical outcome compared to dogs with a single lesion. This is supported by one study in which dogs with multiple concurrent MCTs had a statistically significantly increased risk of MCT-related death compared to dogs with a single lesion as determined by both univariable and multivariable proportional hazards regression analysis (hazard ratio as determined by multivariable regression analysis: HR = 4.6; P = 0.011) (Kiupel *et al.*, 2005). However, histological grade and metastasis were not recorded in that study and case numbers were low (n = 10). It is possible that a proportion of these dogs had high-grade tumours or locoregional metastasis contributing to more malignant disease. Without additional molecular investigation, there is no way to confirm whether the multiple nodules were of *de novo* origin or developed as a result of metastasis from the primary mass. Dogs with high-grade MCTs are at significant risk for metastasis at the time of initial examination when compared to dogs with low-grade tumours (odds ratio = 5.46, P < 0.001) (Stefanello *et al.*, 2015). Further, shorter survival times accompany tumour metastatic spread (odds ratio = 2.5, P = 0.020) (Pierini *et al.*, 2019). Hence, if a MCT lesion grew from the metastasis of a primary malignant mass, the results would be biased towards more aggressive disease (Elliott *et al.*, 2016, Kiupel *et al.*, 2011). For dogs without lymph node metastasis, research advocates the prognosis for dogs with multiple concurrent tumours to be equal to that for dogs with a single mass of the same histologic grade (Horta *et al.*, 2018b, Mullins *et al.*, 2006, Murphy *et al.*, 2006, O'Connell and Thomson, 2013, Pierini *et al.*, 2019). It has been suggested that the outcome for an animal with multiple masses should be based on the prognosis for its tumour of the highest histological grade (Murphy *et al.*, 2006, O'Connell and Thomson, 2013). To address the inconsistencies in the literature surrounding

multiple concurrent MCTs and worse prognosis, one group of investigators has proposed amendments to the current WHO Clinical Stage criteria (Horta *et al.*, 2018b). Horta *et al.* (2018b) present survival data to support amending the current WHO Clinical Stage criteria by separation of non-metastatic single and non-metastatic multiple tumours into Stages I and II, while animals with lymph node metastasis from single or multiple tumours respectively comprise Stages III and IV. This is supported by the work of other research groups which conclude that dogs with multiple concurrent MCTs have statistically similar survival times to dogs with single tumours, and that dogs with ≥ 1 MCT with metastasis to the lymph node have a poorer prognosis than dogs with multiple MCT masses without metastasis (Horta *et al.*, 2018b, Krick *et al.*, 2009, Mullins *et al.*, 2006, Murphy *et al.*, 2006, O'Connell and Thomson, 2013, Pierini *et al.*, 2019, Thamm *et al.*, 1999).

At the time of tumour excision, lymph node metastasis was confirmed in 5.29% (n = 10/189) of dogs with cutaneous MCTs, with a higher frequency of metastasis in dogs with a high-grade (17.8%, n = 8/45) MCT compared to dogs with a low-grade MCT (1.39%, n = 2/144). One of these cases was in a dog with two concurrent Kiupel high-grade, Patnaik grade III MCTs, and the remaining nine instances were in dogs with a single cutaneous lesion (eight dogs with a high-grade tumour and two dogs with a low-grade tumour). Metastasis was not confirmed in any dogs with subcutaneous MCT, reflecting the favourable prognosis for dogs with the subcutaneous variant (Thompson *et al.*, 2011a). Lymph node metastasis has been recorded in 15% (n = 8/54) of dogs with multiple cutaneous lesions (Mullins *et al.*, 2006) and in up to 19% (n = 72/386) of dogs with a single cutaneous mass (Stefanello *et al.*, 2015). However, these findings are from the retrospective analyses of records from a university veterinary teaching hospital and a referring veterinary oncology hospital, whose databases are intrinsically biased toward the inclusion of more malignant cases. The current results are from a retrospective review of cases seen in general practice. It is of note that in the current investigation, many patients' records failed to mention the state of local lymph nodes, pathological testing was not performed in the majority of cases with notably enlarged lymph nodes and parameters used for cytological confirmation of lymph node metastasis are unspecified, limiting the interpretation of these results. The true rate of metastasis is suspected to be higher than reported here, but lower than that deduced from the analyses of referral databases.

Dogs with confirmed metastasis at the time of surgery were 4 times and 2.95 times more

at risk of experiencing 12-month MCT-related death or a second MCT diagnosis, respectively, than dogs without confirmed metastasis as determined multivariable analysis (**Table 5.5**). This supports literature describing the poorer prognosis for dogs with histologically diagnosed metastasis to regional lymph nodes in which the survival of dogs 2-years post-lymphadenectomy was 56% and 90% of dogs with (n = 21) and without (n = 20) lymph node metastasis (Weishaar *et al.*, 2014). Lymphadenectomy in dogs with cutaneous MCT with lymph node metastasis has been associated with a statistically significantly increased chance of 12-month survival (94% versus 79% in dogs with [n = 35] and without [n = 20] lymphadenectomy, respectively) and with a statistically significantly longer median time to MCT progression (median time not reached in 81 dogs with lymphadenectomy versus 170 days in 71 dogs without lymphadenectomy) (Baginski *et al.*, 2014, Marconato *et al.*, 2018). It was not clear whether lymph node metastasis was confirmed by cytology prior to lymphadenectomy in these studies.

This study was limited by lack of data to ascertain the recurrent or *de novo* nature of tumours in dogs diagnosed with a second MCT. However, confirmed metastasis at the time of surgery of the current tumour was statistically correlated with the diagnosis of a second MCT (P = 0.009), but not with a history of MCT (P = 0.525), perhaps alluding to the metastatic development or a local recurrence of the second MCT diagnosed post-excision of the current tumour. Furthermore, a median time interval of less than 12-months between the current MCT and second MCT diagnosis for 55 dogs also supports recurrence of the primary tumour. In comparison, the aforementioned absence of statistical correlation between prior history of a MCT together with > 32-month time interval between historical MCT and current MCT in 8 dogs, may reflect independent *de novo* tumour events. This is purely speculative, and interpretation is restricted due to a small number of dogs with a history of MCT.

Other prognostic variables important in mast cell malignancy and canine MCT prognosis include immunohistochemical evaluation Kit protein staining pattern and assessment of proliferative markers AgNOR, Ki-67, PCNA (Blackwood *et al.*, 2012, Kiupel and Camus, 2019). Analysis of these factors was beyond the scope of the current research, however, their use in prognosis is discussed in Chapter 1.

This research was restricted to the data available from the retrospective review of patient

clinical records from the participating primary veterinary clinics. Consequently, data were limited regarding the cytological parameters used for the detection and confirmation of tumour metastasis at the time of excision and this is further affected by interpreter bias between clinicians and between veterinary practices. Further, the *de novo* or recurrent/metastatic origin of secondary lesions is unable to be determined from the recorded data. Ideally, a prospective study design would have allowed for more accurate data collection. This was not a feasible approach for this project.

5.5 Conclusion

This study confirms the findings of several other reports that canine MCTs are of variable prognosis and histological grade is an important prognostic indicator. A major and novel finding of this research is that mutation of the *KIT* enzymatic pocket domain is statistically significant in predicting 12-month canine MCT-related death, independent of tumour type, and may identify potentially aggressive MCT cases which would have been otherwise overlooked by evaluation of histological grade alone. This contrasts with results suggesting that a *KIT* mutation of any type was not associated with canine prognosis. Further research regarding the significance of enzymatic pocket domain *KIT* mutations on canine MCT prognosis is warranted.

It should be emphasised that no single clinical parameter should be used to accurately define the prognosis for any given patient. Concurrent evaluation of multiple prognostically significant parameters is likely to provide the most educated conclusion concerning prognosis and therapeutic management for each patient. In this study, tumour type, metastasis at the time of surgery, second MCT diagnosis and mutation of the *KIT* enzymatic pocket domain were statistically significant prognostic indicators for canine MCT-related death or second MCT diagnosis in at least one of the multivariable models. The presence of multiple lesions at the time of surgery was predictive of second MCT diagnosis and, therefore, warrant prognostic consideration. Limited prognostic merit surrounds canine age, breed, tumour anatomical location, completeness of tumour excision and tumour *KIT* exon 11 ITD mutation status. Nonetheless, these factors should not be overlooked.

An additional novel finding inferred from this work surrounds the relationship between patient breed, age and tumour histological grade in dogs from different geographical locations.

In this study, the predisposition of Labrador dogs for developing high-grade MCTs at an older age is in direct contrast to the findings of a Polish study. The tendency for certain breeds of distinct populations to develop MCT of different phenotypic malignancies suggests different underlying genetic predisposing elements between the populations.

Chapter 6: *KIT* conservation among species and *KIT* mutations in cheetah and domestic feline mast cell tumours[1]

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[1] Part of this work was conducted with samples from a case study series of cheetah mast cell tumours collected by the Taronga Conservation Society Australia, NSW. The case series is being drafted for publication in the *Journal of Zoo and Wildlife Medicine* (Campbell-Ward *et al.*, 2019).

6.1 Introduction

Mast cell neoplasia is not limited to dogs, but also affects humans, cats and a variety of other mammals, birds, reptiles and amphibians (Tamlin *et al.*, 2019a). The clinical manifestation and biological malignancy of mast cell neoplasia vary considerably not only across species but within species. In human infants, cutaneous mastocytosis presents as urticating lesions isolated to the skin which spontaneously regress in 80% of cases (Ben-Amitai *et al.*, 2005, Kiszewski *et al.*, 2004). In contrast, systemic mastocytosis in adults almost always involves the bone marrow and patients experience frequent and severe symptoms resultant from mast cell mediator release (Coltoff and Mascarenhas, 2019). As seen in the dog, mast cell tumours (MCTs) in cats are clinically erratic, ranging from benign nodules to malignant and metastatic disease (Blackwood, 2015, Blackwood *et al.*, 2012). Despite these differences in biological behaviour, mutation of the *KIT Proto-Oncogene* within the tumour cells is shared between these species (Tamlin *et al.*, 2019a).

It is well established that Kit protein structure and function is disrupted by gain-of-function mutations within the *KIT* gene. *KIT* activating mutations cause constitutive Kit protein dimerization in the absence of ligand binding (Furitsu *et al.*, 1993, Letard *et al.*, 2008). This enables the Kit receptor to phosphorylate various internal substrate proteins, leading to activation of signal transduction cascades which, in turn, regulate cellular proliferation (Lennartsson *et al.*, 2005). In canine cutaneous MCTs, *KIT* mutations are detected in up to 50% of cases (Chapter 4). In cats, 56–68% of MCTs harbour a *KIT* mutation within exons 6, 8, 9 or 11 (Isotani *et al.*, 2010, Sabattini *et al.*, 2013). Exons 12 and 17 of feline *KIT* have been screened for mutations, however, none have been found (Dank *et al.*, 2002). In cats with MCT, correlations between tumour mutation status and prognosis have not been determined. Excluding human and mouse data, *KIT* gene mutation screening in the MCT DNA from other species has not been performed.

A recent case series describes MCT disease in four related captive cheetahs from the Taronga Western Plains Zoo in NSW (Campbell-Ward et al., 2019; unpublished). Case summaries are briefly outlined here. Cheetah Case 1 was a 4-year-old female diagnosed with two cutaneous MCT in 2008. In 2015, this cheetah presented with depression and anorexia and was eventually euthanased. This cheetah was histologically diagnosed with visceral mastocytosis involving the spleen, liver and adrenal glands with accompanying peritonitis and concurrent gastric

Helicobacter colonization, focal glomerulosclerosis and age-related neuronal lipofuscinosis. Cheetah Case 2 was the brother of Case 1, with the same parentage but of a different litter, and was diagnosed with one cutaneous MCT in 2009 at the age of 14 months (**Figure 6.1**). This cheetah was euthanased in 2011 consequent to non-MCT related renal failure. Cheetah Case 3 was a male littermate of Case 2 and was diagnosed with cutaneous MCT in 2010 when he was 2 years old. This cheetah died in 2011 from what was suspected to be a snake bite. Cheetah Case 4 is the offspring of a littermate of Case 1. This female cheetah was 6 years old at the time of cutaneous MCT diagnosis in 2016 and is currently alive with no sign of MCT. In each case, the cutaneous lesion was surgically excised with no reports of local recurrence in any animal, despite incomplete excision of all excluding one lesion from Case 1.

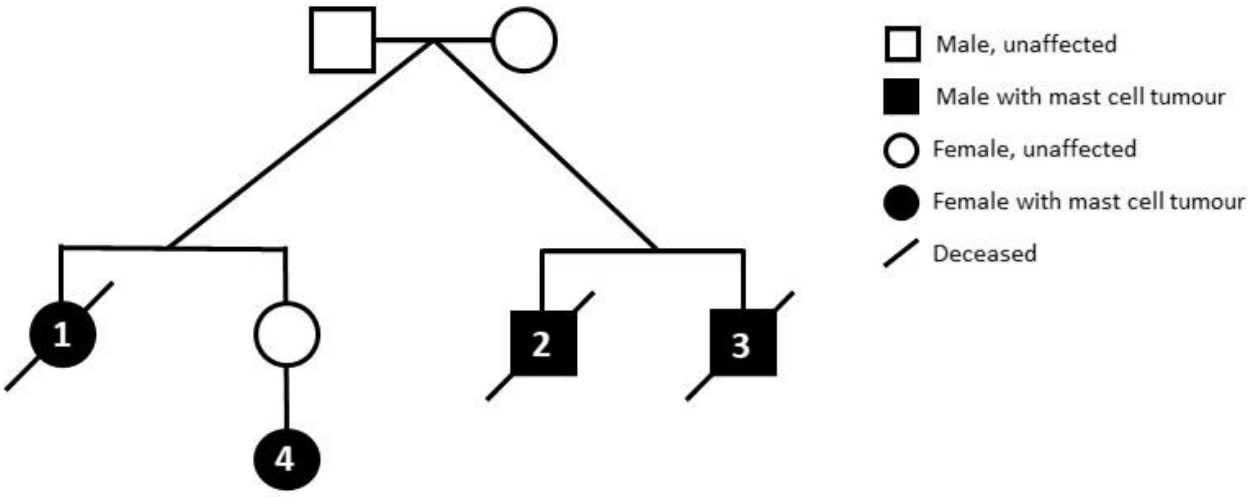

Figure 6.1. Mast cell tumour disease pedigree among four related captive cheetahs at the Taronga Western Plains Zoo, NSW.

The involvement of the *KIT* gene in mast cell tumour biology in big cats is unknown. Based on previous evidence of high exon 11 *KIT* gene and Kit protein sequence conservation between human, dog, cat and mouse orthologs (Dank *et al.*, 2002, London *et al.*, 1999, Ma *et al.*, 1999), it was anticipated that *KIT* sequences would be conserved among a variety of species which have been documented in the literature with a MCT diagnosis (**Table 1.2**) (Tamlin *et al.*, 2019a). High sequence conservation of cheetah with cat and dog *KIT* sequences would support a secondary

hypothesis that non-synonymous *KIT* mutations could exist in MCT DNA of the cheetah and that, if present, these variants would resemble the *KIT* mutations in MCTs from domestic cats and dogs. Given that activating *KIT* mutations confer ligand-independent cellular proliferation in canine, feline and human cell lines, the discovery of *KIT* mutations in cheetah mast cell neoplasms would further elucidate the importance of *KIT* in MCT pathogenesis.

6.2 Methods

6.2.1 KIT gene and Kit protein identity among species

KIT mRNA and Kit amino acid sequence homology between the cheetah and selected mammals including human, mouse, dog and cat was established using National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLASTn or BLASTp, respectively) (Zhang *et al.*, 2000). Respective *KIT* mRNA and Kit amino acid sequence accession numbers used for these analyses were: cheetah XM_027056972.1 and XP_026912773.1 (predicted sequences), human NM_000222.2 and NP_000213.1, mouse NM_001122733.1 and NP_001116205.1, dog NM_001003181.1 and NP_001003181.1, and cat NM_001009837.3 and NP_001009837.3. The annotation prefixes NM and NP are mRNA and amino acid sequence products, respectively, that were derived from the GenBank cDNA and expressed sequencing tag (EST) sequences and supported by RefSeq database. The XM and XP prefixes are the mRNA and amino acid sequence products, respectively, that are predicted from annotated genomes. Only predicted mRNA and amino acid sequences were available for the cheetah.

Complete Kit amino acid sequences from 21 species documented with MCT were aligned using Clustal Omega (version 1.2.4) multiple sequence alignment software (Madeira *et al.*, 2019). These species included human, mouse, dog, cat, cheetah, cougar (XP_025777130.1), Siberian tiger (XP_007075953.1), Pacific walrus (XP_012416406.1), ferret (XP_012918151.1), horse (NP_001157338.2), llama (QAB45132.1), pig (NP_001037990.1), cow (NP_001159956.1), goat (NP_001272653.1), sheep (NP_001295523.1), baboon (XP_021794309.1), rhesus macaque (NP_001253024.1), cynomolgus macaque (XP_005555329.1), chicken (NP_989692.1), burrowing owl (XP_026702780.1) and axolotl (AAS91161.1).

A phylogenetic tree was constructed to predict the evolutionary relationships between the 21 species using the Kit amino acid sequences. Sequences were realigned in Molecular Evolutionary Genetics Analysis software (MEGA X, version 10.0.5) using the ClustalW algorithm and the Neighbour-Joining clustering method (Saitou and Nei, 1987). The confidence of the position of each species branch in the phylogeny was tested using the bootstrap method of 1000 replicates (Felsenstein, 1985). This statistic is calculated from re-runs of the phylogenetic analysis on 1000 independent and random subsets of the data. The reported value is the percentage of bootstrap replicates in which that species was organised into that position. For example, a bootstrap value of 89 indicates the species were clustered in the phylogenetic tree to that branch arrangement in 89% of the runs. The Poisson correction method calculated the evolutionary distances between the species according to Kit amino acid sequence (Zuckerkandl and Pauling, 1965).

6.2.2 Cheetah mast cell tumour cases

Four closely related captive cheetahs at the Taronga Western Plains Zoo were diagnosed with MCT (Campbell-Ward *et al.*, 2019). Briefly, Cheetah Case 1 had two cutaneous nodules excised with a histopathologic diagnosis of mast cell tumour. One tumour was incompletely excised. Cheetah Cases 2, 3 and 4 each had a single cutaneous lesion excisionally biopsied, with neoplastic mast cells extending to the surgical margins in all three cases. Histologically, H&E-stained sections of formalin-fixed tissue of the five cutaneous MCTs from the four cheetahs more closely resembled Patnaik low- and intermediate-grade cutaneous canine MCTs than cutaneous MCTs from domestic cats. The histopathological findings are reported in full elsewhere (Campbell-Ward *et al.*, 2019). Neither local tumour recurrence nor regional metastasis was documented in any case. Six years following excision of the cutaneous MCTs, Cheetah Case 1 was euthanised at exploratory laparotomy due to suspected visceral mastocytosis. Splenic, hepatic and adrenal neoplastic infiltrates were confirmed by histopathology.

Sections from formalin-fixed, paraffin-embedded (FFPE) blocks of the five cutaneous lesions from the four cheetahs and tissue biopsies of the diseased organs from Cheetah Case 1 (liver, spleen, adrenals) were used for DNA extraction. DNA extracted from histopathologically confirmed disease-free small intestine tissue from Cheetah Case 1 was used as presumptive

normal DNA in the molecular investigations.

6.2.3 Feline mast cell tumour cases

Twenty sequential cutaneous feline FFPE MCT blocks were obtained from Gribbles Veterinary Pathology Clayton (VIC, Australia) and Glenside (SA, Australia) laboratories. Tumours were histologically classified by a board-certified veterinary pathologist according to a commonly used schematic described by Kiupel (2016) and a 2-tier histological grading schematic recently proposed by Sabattini and Bettini (2019). The commonly used schematic categorises tumours as mastocytic or atypical (histiocytic) and the mastocytic tumours are further divided into well-differentiated or pleomorphic. Recently, a new subcategory of well-differentiated mastocytic MCTs with prominent multinucleated cells has been described (Melville *et al.*, 2015). Sabattini and Bettini's system classifies tumours as low-grade or high-grade based on a selection of histological criteria (**Table 6.1**).

Tumour mitotic index was calculated based on the total number of mitoses in 10 consecutive high-power fields (× 400) in the most highly cellular areas.

Table 6.1. Histological classification of cutaneous feline mast cell tumours according to unofficial but commonly used recommendations and Sabattini and Bettini's newly proposed 2-tier schematic.

Classification	Criteria
Schematic according to Kiupel (2016)[a]	
Mastocytic	
Well-differentiated	Neoplastic mast cells resemble normal mast cells with no or very little pleomorphism and mitotic figures are rare or absent. Consists of solid sheets and cords of uniform round cells with discrete cytoplasmic margins. Few eosinophils are seen and lymphocytic aggregates may or may not be present. Subtype: well-differentiated with multinucleated cells (≥ 2 nuclei).[b]
Pleomorphic	Large neoplastic cells with eccentric nuclei and prominent nucleoli. Cytomegaly, megalokaryosis, and giant cells with bizarre or multinucleated nuclei may also be present. Eosinophil infiltrates are common.
Atypical	Poorly granulated, histiocytic-like appearance. Comprise large, polygonal to round neoplastic mast cells with abundant amphophilic cytoplasm and large, hypochromatic, slightly indented nuclei. Many infiltrating eosinophils and lymphocytes.
Sabattini and Bettini schematic (2019)[c]	
Low-grade	Cases that do not fit the description of high-grade.
High-grade	> 5 mitotic figures in 10 fields (× 400) and at least 2 of the following: tumour diameter > 1.5 cm irregular nuclear shape nucleolar prominence/chromatin clusters

[a] (Kiupel, 2016)
[b] (Melville *et al.*, 2015)
[c] (Sabattini and Bettini, 2019)

All domestic feline data were de-identified and no clinical information was collected on the cats from which these tumours came. As a control, DNA was extracted from a fresh-frozen domestic feline liver tissue sample already stored in our laboratory which was scavenged from a healthy feral cat and was surplus to the requirements of a different project (University of Adelaide Ethics Approval number S-2013-098).

6.2.4 DNA extraction

DNA was extracted from a single 20 µm section cut from each of the FFPE cheetah and feline tissue blocks using a QIAamp DNA FFPE Tissue Kit (Qiagen; **Appendix B**), according to the manufacturer's instructions. The feline liver control DNA was extracted from fresh-frozen liver tissue using the AllPrep DNA/RNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA quantity (ng/µL) and quality (OD 260:280 absorbance ratio) were analysed using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and samples were stored at 4°C for later use.

6.2.5 KIT amplification

Primers were designed using Primer3Plus online software (version 2.4.2; https://primer3plus.com/) to amplify exons 6, 8, 9 and 11 of feline (NC_018726.3) and cheetah (NW_020834726.1) *KIT* genomic DNA sequences (**Table 6.2**). Primers were designed to complement cat and cheetah homologous sequences in the introns flanking the exons of interest. Thus, all four exons of interest could be analysed in both species using the same primer sets.

Table 6.2. Primer nucleotide sequences for PCR amplification of exons 6, 8, 9 and 11 of feline and cheetah *KIT*.

Exon	5'–3' primer sequence		Product size (base pairs)	$MgCl_2$ concentration
6	6F 6R	GGC TGT GGA TAA TAG TTT CTT CC CAT CCG ATC CTC AGC GTA AG	266	2 mM
8	8F 8R	GGT TTT CCA GCA GTC TGA CG CCC AAA ATC ATC ACC TCA CC	179	1.5 mM
9	9F 9R	TAG TGC CCA TCT GTC CTC C GGC AGA GCC TAA ACA TCC C	387	2 mM
11	11F 11R	CTC CCC TAA TAA GCG CTG TAA TGA CAG GTG CAA CAG AAC AAA GGA AGT	302	2 mM

PCR assays were prepared with approximately 20 ng of target DNA and final reagent concentrations of 250 nM of each primer, 250 µM of dNTP mix (Fisher Biotec Australia), Australia), 33.3 units (0.15 µL of 5 U/µL) of ThermoPrime *Taq* polymerase (Thermo Fisher Scientific), 1.5-2 mM $MgCl_2$ (**Table 6.2**) and 2 µL of 10x Reaction Buffer IV (Thermo Fisher Scientific) made to a total reaction volume of 20 µL with Molecular Biology Reagent H_2O (DNase and RNase free; Sigma Aldrich). The PCR cycling conditions were 3 minutes at 95°C; 35 cycles of 1 minute at 95°C, 30 seconds at 60°C (for exon 6, 8 and 11 primer pairs) or 61°C (for exon 9 primer pair) and 1 minute at 72°C; and a final extension step for 10 minutes at 72°C in the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Resulting PCR amplicons were electrophoresed on a 2% agarose gel.

If no PCR amplicon was detected, the DNA underwent a washing step using a DNA Clean and Concentrator kit (Zymo Research) according to manufacturer's instructions and as described in Tamlin *et al.* (2019b) (Chapter 2). The DNA was eluted in 40 µL of DNA Elution Buffer supplied in the Zymo kit, and DNA quantity and quality were measured before samples underwent a second PCR amplification and gel electrophoresis. If an amplicon was still not obtained, this washing step was repeated a second time and followed by a third PCR and gel electrophoresis.

6.2.6 Sanger sequencing

Gel bands were excised from the agarose gel and purified using QIAquick gel extraction kit (Qiagen) and sent to the Australian Genome Research Facility (AGRF) for Sanger sequencing in the forward and reverse directions using respective primer sets. Sanger sequence results were aligned to the NCBI *KIT* reference gene sequences for the cat (NC_018726.3) or cheetah (NW_020834726.1) using the BLASTn program. All sequence chromatograms were visually reviewed in Chromas (version 2.4.3) for heterozygous variants that may have been missed by BLAST alignment. Nucleotides that altered from the reference sequence but did not lead to an amino acid change are referred to herein as synonymous variants. Nucleotide alterations that resulted in an amino acid change or frameshift are collectively referred to herein as mutations.

6.2.7 Statistical analysis

Statistical analyses were performed in IBM SPSS statistical software (version 25, Armonk) and statistical significance was defined as $P < 0.05$. A generalized linear logistic model with binary logistic regression fitted with a Wald chi-square analysis was used to assess the relationship of

domestic feline MCT *KIT* mutational status with tumour histological grade and mitotic index. One-way analysis of variance (ANOVA) evaluated the relationship between tumour histological classification and mitotic index. Statistical evaluation of the cheetah cases was not performed due to the low numbers of samples.

6.2.8 Predicting effects of KIT mutations on Kit protein structure

To predict the effects of the non-synonymous *KIT* mutations on Kit protein structure, Kyte and Doolittle hydrophobicity plots were constructed using ProtScale on ExPASy Bioinformatics Resource Portal (https://web.expasy.org/protscale/; accessed the 22/07/2019).

Kit protein tertiary structures for the cheetah wild-type Kit and two mutants p.(Glu447Gln) and p.(Asn429Asp) were predicted using ExPASy SIB Bioinformatics Resource Portal's Scratch Protein Predictor 3Dpro Program (http://scratch.proteomics.ics.uci.edu/; accessed 02/09/2019). Output files were visualized in Jmol version 14.29.53 3D molecular modelling software.

6.3 Results

6.3.1 Sequence identity

To determine the degree of conservation of the *KIT* gene and Kit protein, *KIT* mRNA and Kit amino acid sequences were aligned between human, mouse, dog, cat and cheetah sequences (**Table 6.3**). *KIT* nucleotide and Kit protein sequences were moderately to highly conserved between the human, mouse, dog, cat and cheetah. The dog and cat were 91% and 93% homologous for *KIT* mRNA and protein sequences, respectively, but the highest conservation was between the domestic cat and cheetah sequences as expected (99%).

Table 6.3. *KIT* mRNA and Kit amino acid sequence identity between human, mouse, dog, cat and cheetah using NCBI BLASTn and BLASTp, respectively.

	Human	**Mouse**	**Dog**	**Cat**
mRNA (NCBI accession ID)				
Human (NM_000222.2)	-	-	-	-
Mouse (NM_001122733.1)	82.7%	-	-	-
Dog (NM_001003181.1)	87.2%	82.3%	-	-
Cat (NM_001009837.3)	85.3%	82.4%	91.3%	-
Cheetah (XM_027056972.1)	84.5%	82.3%	91.2%	99.0%
Amino acid (NCBI accession ID)				
Human (NP_000213.1)	-	-	-	-
Mouse (NP_001116205.1)	82.5%	-	-	-
Dog (NP_001003181.1)	88.0%	81.8%	-	-
Cat (NP_001009837.3)	88.1%	82.7%	93.0%	-
Cheetah (XP_026912773.1)	88.24%	82.5%	92.9%	99.8%

Multi-species sequence alignment of the Kit amino acid sequences of 21 species was performed to highlight Kit sequence similarities and differences (**Appendix L**). A phylogenetic tree was constructed based on Kit amino acid sequences and illustrates the evolution of mammalian, avian and amphibian Kit sequences from a common ancestral sequence, suggesting Kit to be orthologous among the 21 species (**Figure 6.2**). An especially close evolutionary relationship was observed between the feline species, primate species and ruminants as inferred from the small scaled distance between the branches. These results and the BLAST alignments revealed the high degree of sequence homology shared between species. Given *KIT* mutations are frequently detected in human, canine and domestic feline neoplastic mast cell DNA, it was hypothesised that cheetah neoplastic MCT DNA from the cheetah may also harbour *KIT* mutations.

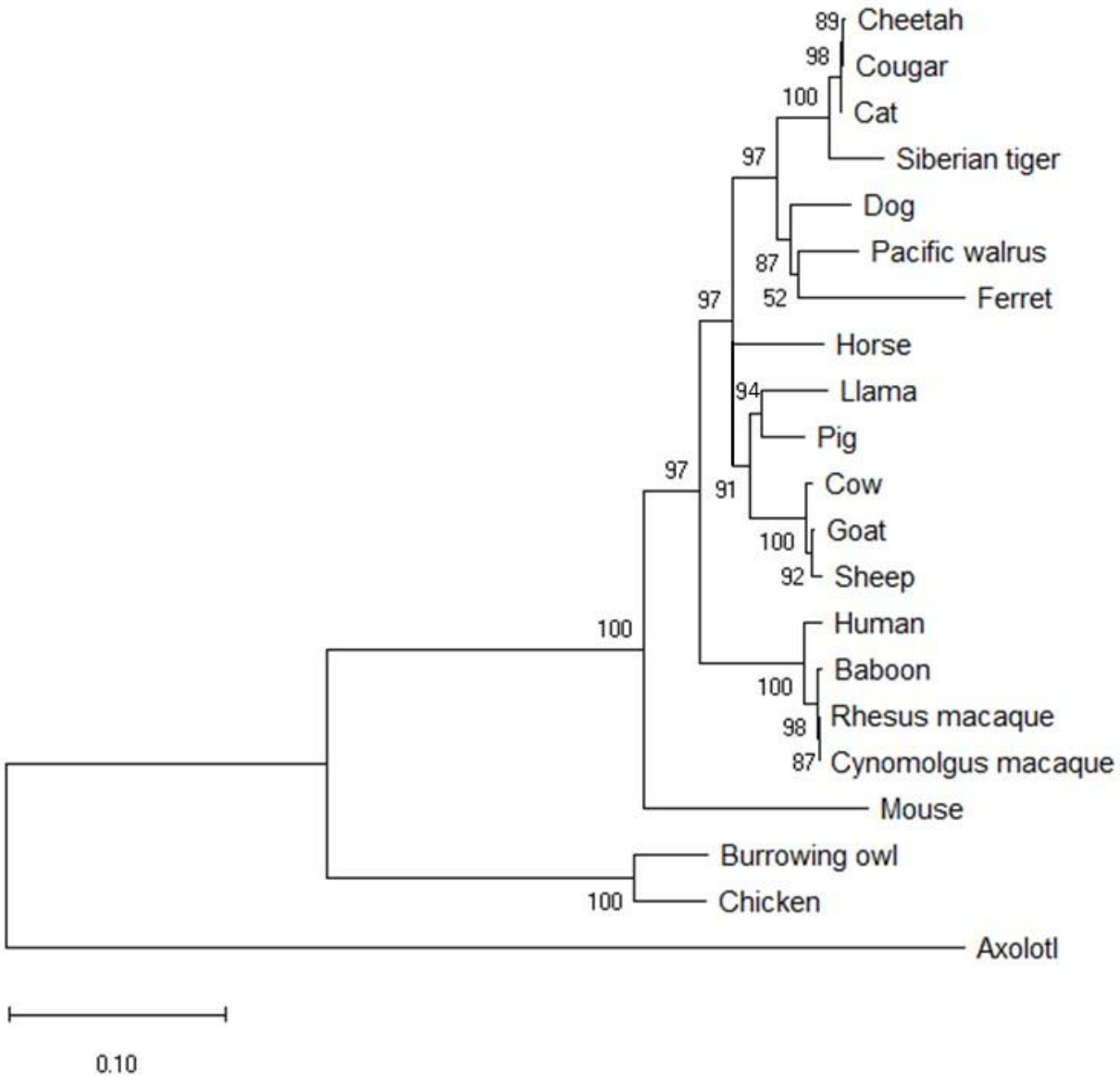

Figure 6.2. A phylogenetic tree illustrating the evolutionary relationship among 21 species based on Kit amino acid sequences. Kit amino acid sequences were aligned in MEGA X (version 10.0.5) using the ClustalW alignment algorithm and the phylogenetic tree was constructed using the Neighbour-Joining method (Saitou and Nei, 1987). The numbers next to the branches represent the percentage of replicate trees in which the associated species clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985). The tree is drawn to scale and implies the evolutionary distance between each species. The evolutionary distances were computed using the Poisson correction method and are written in the units of the number of amino acid substitutions per site (Zuckerkandl and Pauling, 1965).

6.3.2 Domestic feline cases

Feline patient demographics were unknown. Fifteen of the 20 feline cutaneous MCTs (75%) were classified as well-differentiated mastocytic MCTs, three of the 20 (15%) were pleomorphic mastocytic MCTs and two of the 20 (10%) were atypical MCTs. According to the proposed Sabattini and Bettini grading system, 18 of the 20 MCTs (90%) were low-grade and two of the 20 (10%) were high-grade. Of the two high-grade tumours, one was well-differentiated and the other was pleomorphic based on the traditional classification description (Kiupel, 2016). All, but one, of the MCTs (F17) were ≤ 1.5 cm in diameter as measured from the histological sections.

The median mitotic index was 0, ranging from 0–13. Six tumours had counts of 1, 2, 3, 6, 11 or 13 mitoses. The remaining 14 tumours had a 0 mitotic count. There was no statistically significant relationship between tumour mitotic index and histological classification (well-differentiated, pleomorphic or atypical; $P = 0.798$). The difference in the mitotic index between Sabattini and Bettini low-grade and high-grade tumours was statistically significant ($P = 0.001$).

6.3.3 DNA purification

For the cheetah samples, four of the eight FFPE DNA samples (50%) required at least one DNA wash before amplifiable DNA was obtained for PCR with all four sets of primers. Two of these samples required a single wash and two samples required two washes.

For the feline samples, 15 of the 20 FFPE DNA samples (75%) required at least one wash before amplifiable DNA was obtained for PCR with all four sets of primers. Ten samples required a single wash and five samples required two washes. Of the tumours requiring at least one wash, 11 were classified as well-differentiated, two tumours were pleomorphic and two were atypical tumours.

6.3.4 KIT mutations in cheetah and feline mast cell neoplasms

Exon 6 *KIT* mutations were detected in the MCTs from Cheetah Cases 1 and 3 (**Table 6.4**). Cheetah Case 1 was homozygous for a c.1076A>G (predicted Glu447Gln) missense mutation in both its skin lesions and heterozygous for the same mutation in its adrenal tissue. MCT-diseased spleen and liver tissues from the same cheetah harboured c.1284C>T and c.1389G>C synonymous variants which did not alter the predicted Kit amino acid sequence (Asn423Asn and Val458Val,

respectively). The cheetah was homozygous for the Val458Val variant in both tissues. The cheetah was homozygous for the Asn423Asn variant in its spleen but heterozygous for this variant in its liver DNA. In Cheetah Case 3, the cutaneous MCT was heterozygous for an exon 6 c.1300A>G (predicted Asn429Asp) *KIT* missense mutation (**Figure 6.3**). All mutations were confirmed by sequencing in the forward and reverse directions. No mutations were identified in exons 8, 9 or 11 from any of the cheetah mast cell neoplasms.

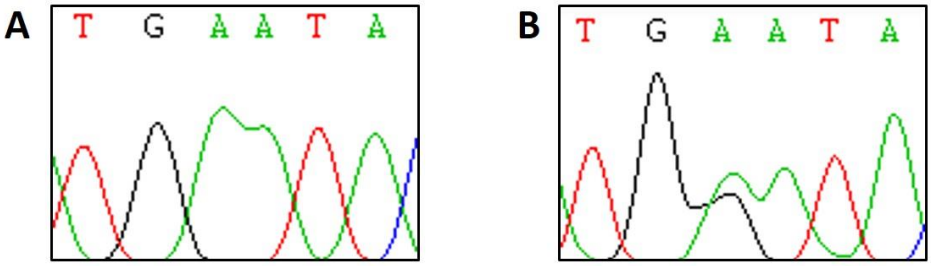

Figure 6.3. Sequence chromatogram of wild-type cheetah *KIT* DNA (A) and an exon 6 c.1300A>G *KIT* mutation in the cutaneous MCT from Cheetah Case 3 (B). The SNP is represented by the overlapping green and black peaks and was confirmed in the reverse direction.

Table 6.4. *KIT* mutations and corresponding predicted Kit amino acid changes in mast cell tumours (MCTs) from four cheetahs. Respective NCBI reference sequence accession numbers for predicted genomic DNA, mRNA and amino acid sequences were NW_020834726.1, XM_027056972.1 and XP_026912773.1, respectively.

Cheetah Case	MCT diagnosis	*KIT* mutation				
		Exon	Type	Genomic position	Nucleotide change	Predicted amino acid change*
Cheetah						
1	Two cutaneous lesions with metastasis to the adrenal gland	6	Missense	43,391,934	c.1355A>G	p.(Glu447Gln)*
	Splenic and hepatic mastocytosis	6	Synonymous	43,391,863	c.1284C>T[a]	p.(Asn423Asn)
		6	Synonymous	43,391,968	c.1389G>C[a]	p.(Val458Val)
2	Cutaneous	ND				
3	Cutaneous	6	Missense	43,391,879	c.1300A>G	p.(Asn429Asp)*
4	Cutaneous	ND				

ND: none detected.

The asterisk (*) denotes conservative amino acid substitutions.

[a] These synonymous variants are predicted to be associated with mast cell neoplasia because they were not detected in the control DNA from the same animal.

In the feline MCTs, non-conservative *KIT* mutations were detected in 60% of samples (n = 12/20, 95% CI: 39.5%–81.5%; **Table 6.5**). One tumour harboured two exon 6 mutations: c.1150T>C (predicted Tyr351His) and c.1187A>G (predicted Tyr363Cys). A different tumour carrying two mutations possessed one mutation in exon 8 and one mutation in the 3' splice acceptor region of intron 8. No exon 11 mutations were identified in any of the feline samples. When considering traditional classification scheme, non-conservative *KIT* mutations were detected in 9/15 (60%) well-differentiated MCTs, 2/3 (66.7%) pleomorphic MCTs, and 1/2 (50%) atypical MCTs. According to Sabattini and Bettini's grading system, 11/18 (61.1%) low-grade and 1/2 (50%) high-grade MCTs carried a non-conservative *KIT* mutation. There was no correlation between tumour *KIT* mutation status with the commonly used histological classification scheme (P = 0.934), Sabattini and Bettini histological grade (P = 0.762) or mitotic index (P = 0.750).

Table 6.5. *KIT* mutations and corresponding predicted Kit amino acid changes in cutaneous mast cell tumours (MCTs) from 20 domestic cats. Respective NCBI reference sequence accession numbers for *KIT* genomic DNA, mRNA and protein were NC_018726.3, NM_001009837.3 and NP_001009837.3, respectively.

	MCT diagnosis			*KIT* mutation				
Cat Case	Traditional schematic	Sabattini and Bettini 2-tier schematic	Mitotic figures	Exon	Type	Genomic position[a]	Nucleotide change	Predicted amino acid change*
Domestic cat								
1–5	Well-differentiated	Low-grade	0	ND				
6	Well-differentiated, cells with prominent nucleoli	Low-grade	11	ND				
7	Atypical	Low-grade	0	ND				
8	Pleomorphic	High-grade	6	ND				
9	Atypical	Low-grade	0	6	Missense	163,986,581	c.1028C>A	p.(Ala310Asp)
10[b]	Well-differentiated	Low-grade	1	6	Missense	163,986,539	c.1070T>C	p.(Ile324Thr)
11[b]	Pleomorphic	Low-grade	0	6	Missense	163,986,539	c.1070T>C	p.(Ile324Thr)
12[c]	Well-differentiated	Low-grade	0	6	Missense	163,986,459	c.1150T>C	p.(Tyr351His)*
13[c]	Well-differentiated, cells with prominent nucleoli	High-grade	13	6	Missense	163,986,459	c.1150T>C	p.(Tyr351His)*
				6	Missense	163,986,422	c.1187A>G	p.(Tyr363Cys)
14	Well-differentiated	Low-grade	3	6	Indel	163,986,474_163,986,475	c.1134_1135delinsCA	p.(Glu345_His346delinsAspAsn)*
15	Well-differentiated	Low-grade	0	8	Insertion, framseshift, premature termination codon	163,968,964_163,968,965	c.1381_1382insA	p.(Cys429ValfsTer19)
16	Well-differentiated	Low-grade	0	8	Deletion, frameshift	163,968,985_163,968,990	c.1356_1361del	p.(Glu420_Ser421del)
17	Well-differentiated	Low-grade	0	8	ITD, frameshift	163,968,992_163,969,003	c.1343_1354dup	p.(Glu415fs)
				Intron 8	Exon 9 5′ splice acceptor variant	163,967,361	c.1447-3T>C	p.?
18	Well-differentiated, cells with	Low-grade	0	9	Missense	163,967,276	c.1529G>T	p.(Ser477Ile)

	prominent nucleoli							
19	Well-differentiated with prominent multinucleated cells	Low-grade	2	9	Missense	163,967,276	c.1529G>T	p.(Ser477Ile)
20	Pleomorphic	Low-grade	0	9	Missense	163,967,286	c.1519G>A	p.(Val474Met)*

ND: none detected. ITD: internal tandem duplication. fs: frameshift. Ter#: termination codon # bases from mutation site. ?: the variant occurs in an acceptor site (within the intron) which may affect mRNA splicing and hence, either alter the amino acid sequence and/or gene expression.

The asterisk (*) denotes conservation conservative amino acid substitutions. Unannotated amino acids changes indicate non-conservative amino acid substitutions.

[a] Reverse strand for feline *KIT* gDNA.

[b] Cases 10 and 11 carry the same mutation (c.1070T>C).

[c] Cases 12 and 13 carry the same mutation (c.1150T>C).

The Kit amino acid changes predicted from the exon 6 *KIT* mutations observed in the cheetah MCT DNA samples were mapped to the corresponding amino acids of the human and feline Kit protein sequences (**Figure 6.4**). The cheetah Kit amino acid changes occurred at the same amino acid or within close proximity to amino acids which have been previously reported to be mutated in human and feline mast cell neoplasia patients.

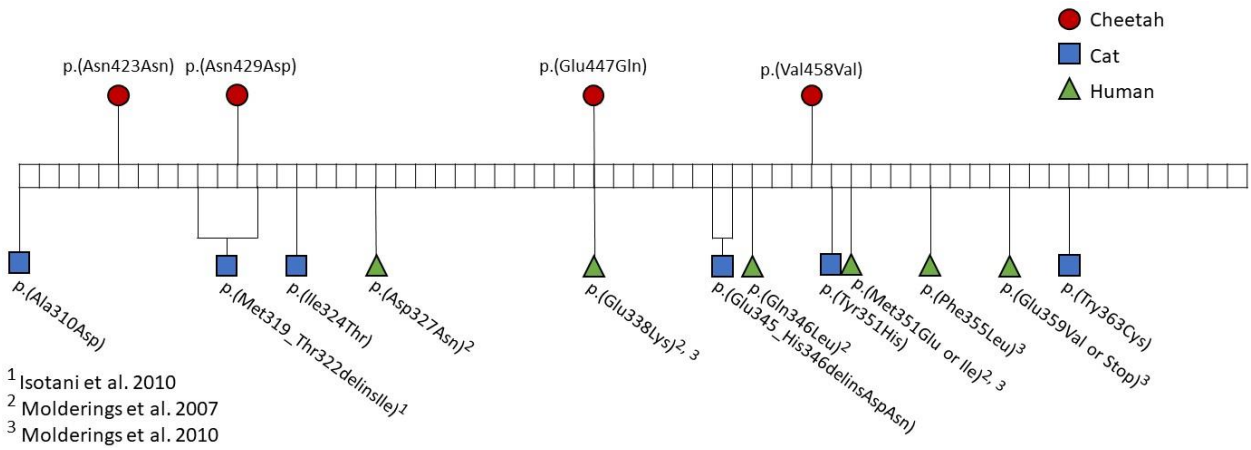

Figure 6.4. Predicted amino acid changes in the extracellular ligand-binding domain of the Kit protein consequent to the mutations occurring within exon 6 of the *KIT* gene observed in neoplastic mast cells from cheetah (red circles), cat (blue square[1]) or human (green triangle[2,3]). Each vertical line represents an amino acid.

Kyte and Doolittle hydrophobicity plots were created to predict the effects of the *KIT* mutations on Kit protein structure (**Figure 6.5**). No obvious differences from the wild-type Kit amino acid hydrophobic/hydrophilic structure (black line) were noticed in the Kit-mutant amino acid sequences (coloured lines) from the cheetah MCTs (**Figure 6.5A**). In the domestic feline cases, all the Kit-mutant amino acid sequences altered the hydrophobicity from the wild-type pattern (**Figure 6.5 B** and **C**). The p.(Glu345_His346delinsAspAsn) mutant appears to have the smallest effect on predicted Kit hydrophobicity (red line, **Figure 6.5B**). The p.(Cys429ValfsTer19) mutant results in a premature stop codon at position 448 (yellow line, **Figure 6.5C**) and therefore, the protein will not be functional. The hydrophobicity structures for mutants p.(Glu415fs) and p.(Glu420_Ser421del) are shifted to the right and left, respectively. The shift in hydrophobicity for these mutants is a consequence of a 12-bp ITD and 6-bp deletion, respectively. However, the reading frames of these mutations are conserved and do not alter the downstream nucleotide codon sequence.

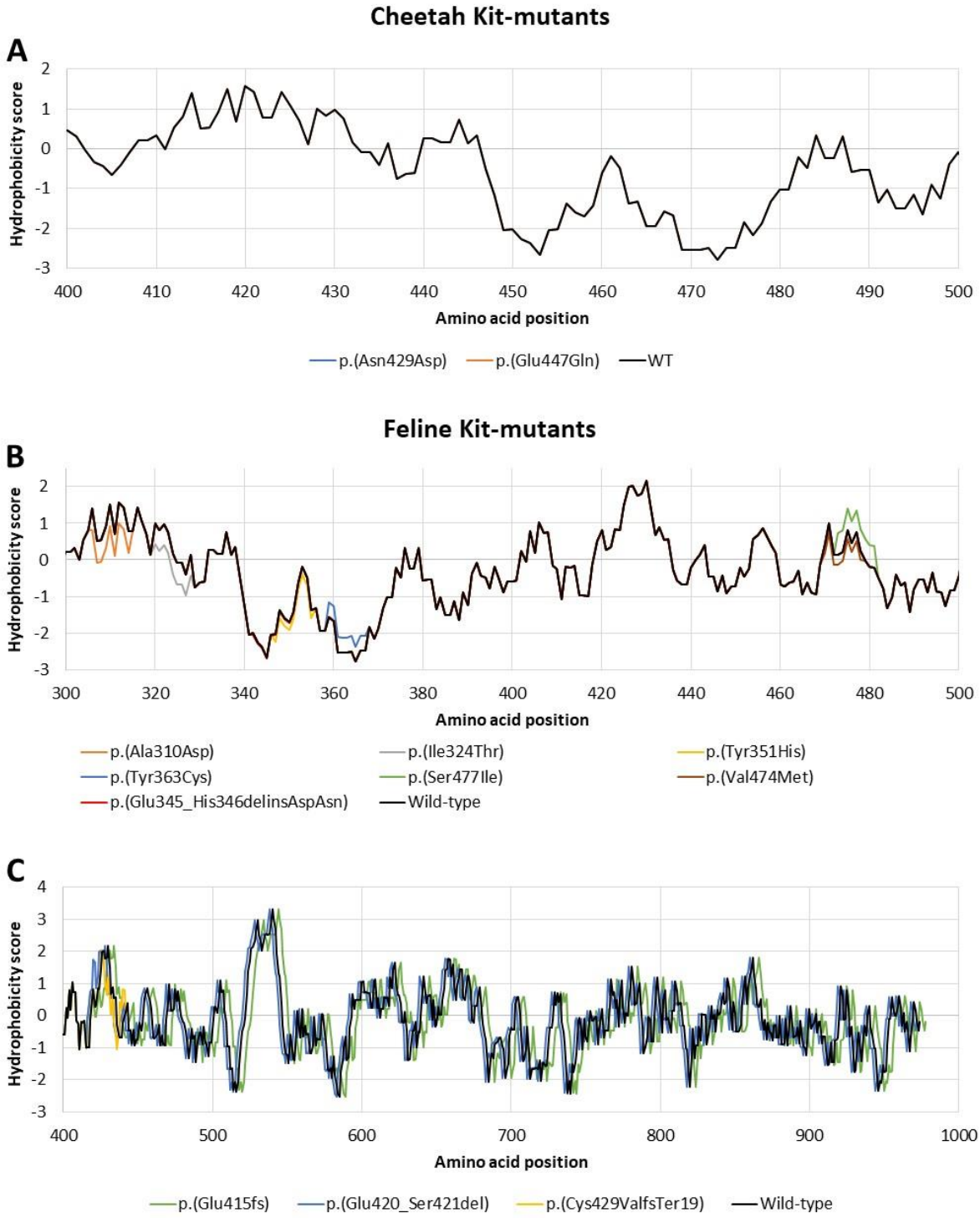

Figure 6.5. Kyte and Doolittle hydrophobicity plots illustrating the predicted changes in mutant-Kit protein hydrophobicity consequent to the non-synonymous *KIT* mutations detected in mast cell tumours from cheetahs (A) and domestic cats (B and C). The wild-type amino acid Kit hydrophobicity structure is represented by the black line in each plot. Mutant-Kit structures are illustrated by the coloured lines. For easy visualisation, the feline Kit-mutations were separated into SNPs and small indels (B) and frameshift mutants (C). Regions with a hydrophobicity score > 0 are hydrophobic and regions with a score < 0 are hydrophilic. The plots were constructed using ProtScale on ExPASy Bioinformatics Resource Portal.

The effects of *KIT* SNPs on Kit protein structure were further investigated in the cheetah. 3D protein modelling software was used to predict the tertiary structure of wild-type Kit and the p.(Glu447Gln) and p.(Asn429Asp) Kit mutants. Small variations in the overall structure of the Kit mutants were observed when compared to wild-type Kit. The protein's structure at the position of each SNP was not altered. The p.(Glu447Gln) variant remained in a beta-pleated sheet structure and the p.(Asn429Asp) mutant remained in an extended structure ("other").

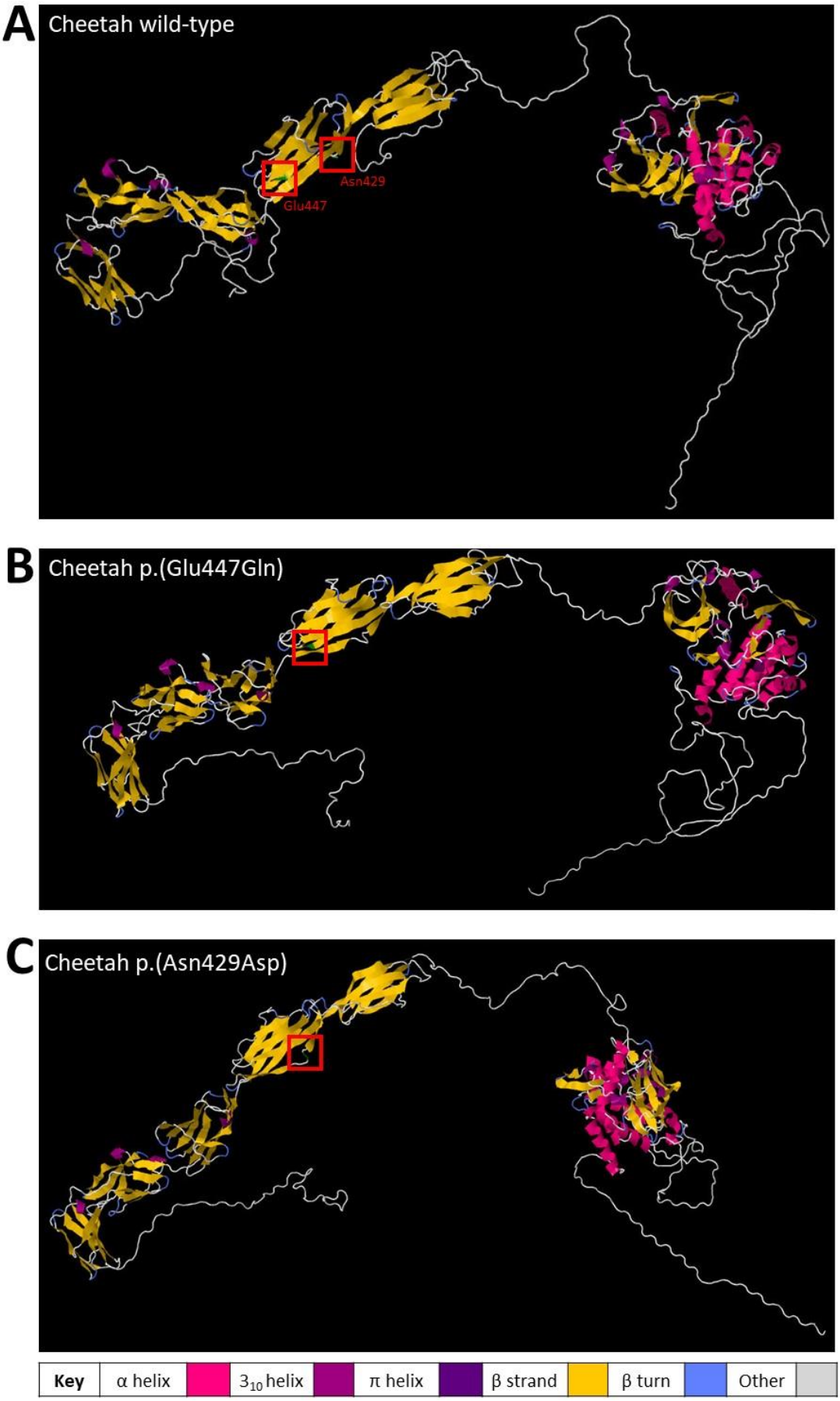

Figure 6.6. Predicted tertiary protein structure for cheetah Kit. A: wild-type Kit. B: p.(Glu447Gln) mutant. C: p.(Asn429Asp) mutant. See the following page for a full description.

Figure 6.6. **Full figure description.** Predicted tertiary protein structure for the cheetah wild-type Kit (A) and two mutants; B: p.(Glu447Gln) and C: p.(Asn429Asp). The left-hand side of the protein depicts the extracellular immunoglobulin-like domains of Kit which is characterised by beta-pleated sheets (yellow ribbons). The right-hand side of the protein depicts the intracellular juxtamembrane and tyrosine kinase domains of Kit which are characterised by alpha-helices (pink coils). The green segment, which is further emphasised by the red box, indicates the mutant amino acid in B and C. The corresponding wild-type amino acid is indicated in the same way in figure A. Structures were predicted using ExPASy SIB Bioinformatics Resource Portal's Scratch Protein Predictor 3Dpro Program (http://scratch.proteomics.ics.uci.edu/; accessed 02/09/2019). Output files were visualized in Jmol version 14.29.53 3D molecular modelling software. Jmol was used to orient each 3D structure to the same position for structural comparison.

Five synonymous variants were observed in the DNA extracted from the feline MCT tissues (**Table 6.6**). The most common variant was a c.939C>T SNP occurring in 95% of tumours. The feline liver control sample was homozygous for this variant allele.

Table 6.6. Prevalence of synonymous single nucleotide polymorphisms (SNPs) detected in exons 6 and 9 of *KIT* from feline cutaneous mast cell tumours (MCTs).

Exon	Genomic position	Nucleotide change	Amino acid position	Number of tumours with this variant (prevalence)	Variant allele frequency*
6	163,986,565	c.939C>T	p.(Asn315Asn)	19/20 (95%)	72.5%
	163,986,460	c.1044C>G	p.(Val350Val)	5/20 (25%)	15.0%
	163,986,505	c.999T>A	p.(Ile335Ile)	1/20 (5%)	2.50%
9	163,967,227	c.1578G>A	p.(Arg493Arg)	1/20 (5%)	2.50%
	163,967,296	c.1509T>C	p.(Ser470Ser)	1/20 (5%)	2.50%

* Variant allele frequency accounts for both homozygous and heterozygous tumour genotypes.

6.4 Discussion

Mast cell neoplasia has been reported in 35 different species of mammals, birds, reptiles and amphibians (**Table 1.2**). Aberrant cytoplasmic Kit protein staining has been demonstrated in MCT tissue from humans, cats, dogs, horses, ferrets, a cow and a macaque (Dobromylskyj *et al.*, 2015, Jordan *et al.*, 2001, Webster *et al.*, 2006b) and has been correlated with decreased survival in cats and dogs (Dobromylskyj *et al.*, 2015, Thamm *et al.*, 2019a). Aberrant cytoplasmic Kit protein localisation in canine MCTs is significantly associated with the presence of a *KIT* exon 11 ITD (Horta *et al.*, 2018b, Webster *et al.*, 2006b). The implication of *KIT* in mast cell neoplasia across a variety of species led to the initial hypothesis that *KIT* gene and Kit amino acid sequences are conserved among species. The results herein indicate

that Kit protein sequences in mammals, birds and amphibians are orthologous and appear to have derived from a common ancestral sequence.

The phylogenetic tree illustrates high Kit amino acid sequence conservation among mammals, and sequence homology with birds and amphibians is also shared (**Figure 6.2**). The shorter the branches between two species in the phylogenetic tree, the greater the sequence similarity and the closer the evolutionary relationship between the two species. Kit amino acid sequences have evolved from a common ancestral sequence and have retained homology despite multiple speciation events. An especially close evolutionary relationship was found between the cheetah and domestic cat, which is not surprising given the high *KIT* mRNA and Kit amino acid sequence homology shared between these species using BLAST analysis (99% and 99.5% sequence identity, respectively; **Table 6.3**). Further, *KIT* mRNA and Kit amino acid sequences are well conserved between more distantly related species including human, mouse, dog, cat and cheetah (**Table 6.3**). Based on high *KIT* conservation, it was hypothesised that the cheetah MCT DNA samples would possess mutations within the *KIT* gene regions which are frequently mutated in the MCTs from domestic cats and dogs. Non-synonymous *KIT* mutations were discovered in these regions for two of the four cheetah MCT cases. The MCTs from the other two cheetahs harboured only wild-type *KIT* in the exons investigated.

Cheetah Case 1 was diagnosed with two cutaneous mast cell tumours six years preceding the diagnosis of visceral mastocytosis affecting liver, spleen and adrenal (Campbell-Ward *et al.*, 2019). The two cutaneous lesions and the adrenal tissue carried the c.1355A>G mutation which leads to a predicted glutamine to glycine amino acid change (**Table 6.4**). Given the temporal and physical co-location of the two cutaneous MCTs, it is likely that the smaller cutaneous nodule was a metastasis from the larger, primary tumour. The relationship between the adrenal gland mast cell neoplasm and the cutaneous tumours is less clear. It is possible that the adrenal gland lesion was a slowly developing or quiescent metastasis from the skin lesions. Alternatively, considering that the cutaneous lesions never recurred, a demonstration of biologically benign behaviour, the adrenal neoplasm may have arisen *de novo* with a coincidental identical *KIT* mutation to the skin lesions. Histologically, this case resembled low-grade cutaneous MCT of the dog (Campbell-Ward *et al.*, 2019), and genetically reflects both feline and canine cases of cutaneous MCT wherein *KIT*-mutant tumours are not indicative of a worse prognosis (excluding the exon 11 ITD mutants) (Mochizuki *et al.*, 2017b, Sabattini *et al.*, 2013).

In the neoplastic hepatic and splenic tissue from Cheetah Case 1, two synonymous *KIT* variants were detected. Both variants were different from the mutation detected in the skin nodules and adrenal tissue. In domestic cats and dogs, different *KIT* mutational status of multiple MCT nodules from the same individual have been documented and are suspected to represent independent disease events (Sabattini *et al.*, 2013, Tamlin *et al.*, 2017). The difference in *KIT* mutational status between the visceral and cutaneous disease in this instance, together with the 6-year time gap between the onset of symptoms, supports independent disease occurrence.

Unexpectedly, only mutant alleles, and not wild-type DNA, were detected in the DNA extracted from the skin tumours and the visceral (splenic and hepatic) tissue from Cheetah Case 1. Since no attempt was made to separate the neoplastic cells from the normal, non-neoplastic tissue surrounding the mast cells during FFPE DNA extraction, it was expected that wild-type and mutant *KIT* DNA would be co-extracted into the same solution and samples would exhibit a heterozygous genotype. One explanation for lack of wild-type DNA detection is the relatively poor sensitivity of Sanger sequencing to detect an allele if it accounts for less than 15% of total alleles in a sample (Tsiatis *et al.*, 2010).

The synonymous variants detected in the liver and spleen of Cheetah Case 1 were not found in the non-neoplastic tissue nor in any other neoplastic tissue DNA from the same animal. It is possible that these variants played a role in the visceral disease of Cheetah Case 1. Genetic alterations that conserve the amino acid sequence are synonymous and typically considered biologically “silent”. However, some synonymous mutations can impact transcript splicing or protein folding and consequently, protein function (Cortazzo *et al.*, 2002, Supek *et al.*, 2014).

The Taronga Conservation Society has been home to 52 cheetahs since 1981. No cases of MCT had been reported in the cheetah until the cutaneous MCT diagnosed in Cheetah Case 1 in 2008 (Campbell-Ward *et al.*, 2019). Since then, three individuals related to the Case 1 cheetah have been diagnosed with MCT (**Figure 6.1**). It is possible that over the last four decades, routine cheetah health examinations at the zoo have improved by becoming more thorough. Therefore, previous cheetahs may have had cutaneous lesions which were undetected. However, the close familial relationship between the four cheetahs reported here suggests an underlying genetic predisposition to MCT. This parallels the familial predispositions of humans to mastocytosis and breed predispositions of Boxer-breed dogs and

Siamese cats to MCT development (Bodemer *et al.*, 2010, Melville *et al.*, 2015, Mochizuki *et al.*, 2017a, Wohrl *et al.*, 2013). The MCTs from these cheetahs did not share the same *KIT* genetic mutations, implying somatic mutation occurrence. Again, this mirrors somatic mutations in humans, cats and dogs where the same *KIT* mutations are not detected in all lesions of an animal with multiple MCTs and where the same *KIT* mutations are not observed in children with mastocytosis of parents with *KIT*-mutant mastocytosis (Tamlin *et al.*, 2017, Wohrl *et al.*, 2013). The genetic cause of inherited predisposition for mast cell disease in this family of cheetahs was not found in exons 6, 8, 9 or 11 of *KIT.* This observation, together with data from humans, domestic cats and dogs, suggests the underlying genetic mechanism predisposing individuals to MCT development is likely located elsewhere in the genome.

In comparison with cheetah, 60% of domestic feline MCTs in this study harboured at least one non-conservative *KIT* alterations in exons 6, 8 or 9. This prevalence is similar to previous international reports (Isotani *et al.*, 2010, Sabattini *et al.*, 2017, Sabattini *et al.*, 2013). No mutations within exon 11 were observed. One tumour harboured two exon 6 mutations, both resulting in an amino acid change (Cat Case 13; **Table 6.5**). A different tumour harboured an exon 8 ITD and a SNP in the 5' splice acceptor region of exon 9 (Cat Case 17; **Table 6.5**). Multiple *KIT* mutations in a single lesion have been reported in mast cell neoplasms from cats, however, this is the first report of the mutations being within the same exon (Isotani *et al.*, 2010, Sabattini *et al.*, 2017).

Non-synonymous *KIT* exon 6 mutations have been reported at a low frequency in human patients with mastocytosis and in a single cat with cutaneous MCT (Isotani *et al.*, 2010, Molderings *et al.*, 2007, Molderings *et al.*, 2010). Exon 6 variants have not been reported previously in MCTs from dogs and were not found in the AmpliSeq study herein (Chapter 4). In the current investigation, half the feline MCT *KIT* mutations (n = 7/14, 50%) and all the cheetah *KIT* variants were clustered within exon 6. Data on exon 6 mutations in previous feline, canine and human MCTs investigations are limited because this region of *KIT* is seldom analysed. The current research exposes exon 6 as a potential mutation hotspot in feline *KIT*. The biological significance of these mutations is unknown.

The cheetah MCT Kit mutants were mapped against human and feline Kit amino acid sequences (**Figure 6.4**). All cheetah mutants occurred either at the same position or within a maximum of three amino acids adjacent to the position corresponding to a mutant Kit amino acid in human and feline patients with mast cell neoplasms. The mutational effects on Kit

protein activity were not investigated in the current study nor have the effects of exon 6 mutations been researched in other feline or human studies. *KIT* exons 6–7 and exons 8–9 encode Ig-like domains 4 and 5, respectively, of the human Kit protein (Lennartsson *et al.*, 2005). The Ig-like domains 4 and 5 are involved in Kit protein dimerization (Lennartsson *et al.*, 2005). Point mutations in exons 8 and 9 cause ligand-independent homodimerization and subsequent Kit autophosphorylation in human, canine and feline neoplastic mast cells (Bodemer *et al.*, 2010, Isotani *et al.*, 2010, Letard *et al.*, 2008). Due to the similar biological function of the Ig-like domains, together with the high Kit protein sequence conservation between these species (**Table 6.3** and **Figure 6.2**), it is reasonable to suspect that the exon 6 *KIT* mutations detected in the feline and cheetah MCTs in this study might cause Kit protein activation.

In domestic feline cases, non-synonymous *KIT* mutations are predicted to alter the hydrophobicity of the Kit protein at the corresponding Kit amino acid residues (**Figure 6.5B** and **C**). This is suspected to induce Kit protein mis-folding and subsequent abnormal Kit protein function. For example, the p.(Cys429ValfsTer19) mutation results in a premature stop codon at position 448, truncating the wild-type amino acid sequence and most likely resulting in a non-functional protein product (yellow line, **Figure 6.5C**). On the other hand, the p.(Glu345_His346delinsAspAsn) mutant appears to have a negligible effect on predicted Kit hydrophobicity (red line, **Figure 6.5B**). This mutation substitutes glutamic acid and histidine for amino acid residues with highly similar structures and chemical properties, aspartic acid and asparagine, respectively. This may explain the minimal change in the predicted Kit-mutant hydrophobicity plot from the wild-type. Notably, this double amino acid substitution occurs in exon 6, a region of the extracellular, regulatory and ligand-binding domain of Kit protein which is highly conserved among mammalian species. The role of these amino acids in Kit-ligand binding is not entirely clear and the effects of this mutation on Kit function cannot be confidently predicted without additional wet-lab experiments.

No obvious differences between the cheetah Kit-mutant and the wild-type Kit amino acid hydrophobic/hydrophilic patterns were observed in the hydrophobicity plots (**Figure 6.5A**). Whilst this suggests that Kit folding pattern is not altered, the exact biological effects of these mutants on Kit protein structure and function cannot be assumed to be null from these data alone. To further investigate the effects of cheetah *KIT* mutations on Kit protein structure, the 3D tertiary structures of wild-type and mutant Kit were predicted (**Figure 6.6**). When

compared to wild-type Kit structure, minor structural changes within the two Kit mutants were observed, although the structure surrounding the altered amino acid was not affected considerably. Whether these structural modifications are sufficient to alter protein function or induce ligand-independent activation cannot be deduced from the current data. It also is possible that these mutations could impact *KIT* post-transcriptional longevity and expression, ultimately altering Kit protein function. Further wet-lab experiments investigating mutant-*KIT* mRNA expression and Kit protein folding patterns and the effect on cell growth are encouraged.

In the domestic cat and big cats, the wild-type amino acid at position 310 in exon 6 of Kit is an alanine residue (**Appendix L**). The wild-type amino acid at this position in non-feline mammalian species corresponds to aspartic acid. In this study, one feline MCT harboured an exon 6 alanine to aspartic acid predicted amino acid change, p.(Ala310Asp). It is possible that this mutation may not be gain-of-function but instead reflect an evolutionary allelic variation. Non-neoplastic tissue from this cat was not available for sequence comparison to determine whether this mutation was present in the normal DNA.

In the wild-type human Kit amino acid sequence, phenylalanine is positioned at residue 355. A SNP mutation causing an amino acid substitution to leucine, p.(Phe355Leu), was discovered in a single human case of mastocytosis (Molderings *et al.*, 2010). In feline (domestic cat and big cats) and avian (chicken and burrowing owl) wild-type Kit sequences, amino acid 355 corresponds to leucine. In the Kit protein from other species, this residue encodes alanine in the pig, serine in the goat, cow, horse, baboon and mouse, and valine in the axolotl (**Appendix L**). Thus, this amino acid is only moderately conserved among the 21 species. Again, the mutational effect on Kit protein autophosphorylation was not explored and primary matched control sample DNA were not evaluated to determine the germline or somatic nature of this amino acid substitution. The p.(Phe355Leu) mutant observed in the human Kit sequence occurs at an amino acid residue that is not well conserved among the 21 species evaluated. Hence, it is suspected that the mutation in the codon encoding this residue may not have functional effects on Kit protein. However, this is purely speculative and rigorous conclusions cannot be drawn from the information presented here.

Synonymous variants at three positions within exon 6 and two positions in exon 9 of feline *KIT* were detected (**Table 6.6**). The most common variant was a c.939C>T substitution occurring at asparagine^{315} and detected with a thymine (T) allele frequency of 72.5%. The

homozygous T/T genotype was detected at this position in the control feline liver sample and nine of the feline MCT DNA samples; hence, this variant is predicted to be a species-specific SNP. The second most common synonymous variant in the cat was a C>G substitution at position c.1044 encoding valine^{350}, with an alternative guanine (G) allele frequency of 15%. The somatic or germline nature of this variant could not be determined, but it is suspected that this is also a species-specific SNP in the cat. One feline MCT was homozygous G/G for this variant and the nucleotide at this position in the closely related cheetah *KIT* sequence corresponds to guanine. Consequently, it is predicted that SNP is not associated with mast cell tumourigenesis of the cat.

The Patnaik and Kiupel histological grading schemes used for classifying canine cutaneous MCTs are not applicable for feline MCTs. Instead, tumours are histologically classified based on cellular and nuclear morphology as either mastocytic well-differentiated or pleomorphic forms, or as atypical (histiocytic) tumours (Johnson *et al.*, 2002, Kiupel, 2016, Sabattini and Bettini, 2010). A mastocytic well-differentiated MCT subtype with prominent multinucleated cells has been recently described and a single case is reported here (Cat Case 19) (Melville *et al.*, 2015). Similar to other feline studies, well-differentiated mastocytic MCTs represented the majority (75%) of cases in this study (Melville *et al.*, 2015, Sabattini and Bettini, 2010). However, there are no official guidelines for feline cutaneous MCT histological classification, and consequently, there are inconsistencies in the definitions of histological sub-types between published reports. These discrepancies in histological classifications and correlations with prognosis have been described previously (Blackwood, 2015). To overcome this issue, Sabattini and Bettini recently proposed a 2-tier histological schematic for feline cutaneous MCTs (Sabattini and Bettini, 2019). This novel grading system was able to predict feline survival, where cats with high-grade tumours had a significantly shorter median overall survival (349 days) compared to cats with low-grade tumours (not reached, $P < 0.001$). However, this system was not successful in predicting the progression-free interval. Follow-up investigation to assess reproducibility in a different, larger cohort of cats is warranted. In the current study, most feline tumours (90%) were histologically low-grade. Patient demographic and survival data were not available for this cohort and correlations between patient prognostic factors and histological grade were not possible.

Tumour *KIT* mutation status was not associated with the commonly used histological classification system (Kiupel, 2016), Sabattini and Bettini 2-tier histological grading system or

tumour mitotic index. This parallels previous research where MCT *KIT* mutation status was not useful in the prognosis of cats with MCT (Sabattini *et al.*, 2017, Sabattini *et al.*, 2013). Similarly, there are no genotypic-phenotypic correlations between *KIT* status and prognosis in human patients with mastocytosis or non-exon 11 *KIT* mutations in MCTs of the dog (Chapter 5) (Bodemer *et al.*, 2010, Mochizuki *et al.*, 2017b). Paralleling canine data, mutation of the *KIT* regulatory domain in feline MCTs does not appear to be sufficient to induce clinical malignancy and is not a useful prognostic predictor for cats with MCT.

Nonetheless, knowledge of tumour mutation status may be clinically advantageous when considering the use of tyrosine kinase inhibitors (TKIs) as a therapeutic option. Tyrosine kinase inhibitors are frequently used in canine and human mastocytosis patients to target mutant-Kit protein (Tamlin *et al.*, 2019a). Tyrosine kinase inhibitors have been also used off-label in treating *KIT*-mutant feline MCTs with some success (Isotani *et al.*, 2006, Isotani *et al.*, 2010). As for the cheetah, the parallels between cat and cheetah MCT clinical behaviour, *KIT* gene and Kit protein sequences and *KIT* mutations indicate that cheetah suffering inoperable mast cell disease may benefit from tyrosine kinase inhibitor treatment. However, the frequently indolent nature of feline and cheetah MCTs, intensive therapeutic requirements of systemic disease management, and lack of research on the use of veterinary drugs in big cats probably deter the use of off-label tyrosine kinase inhibitor drugs in this species.

Limitations of this study are evident by the analysis of only four exons of the *KIT* coding sequence. It is possible that mutations in the *KIT* gene exist outside the exons analysed and that these mutations may influence neoplastic mast cell biology. This study was further restricted by the limited sensitivity of Sanger sequencing and the co-extraction of DNA from both neoplastic and normal tissue. Future investigations may consider laser capture microdissection to isolate neoplastic mast cells from surrounding tissue before DNA extraction to accommodate lower frequency mutant alleles.

6.5 Conclusion

This study determined high *KIT* gene and Kit amino acid sequence homology between a number of species known to suffer mast cell neoplasia, and an especially high sequence identity between the cheetah and the domestic feline *KIT* sequences.

Non-conservative mutations within *KIT* exon 6 were detected in two of the four

cheetah mast cell neoplasms. In one cheetah, the *KIT* mutational status differed between its visceral and cutaneous disease, indicative of somatic mutation occurrence and independent tumour origins. The different *KIT* mutational status between the four related cheetahs suffering mast cell disease further supports the somatic origin of *KIT* mutations. Of the 20 feline cutaneous MCTs, 12 (60%) carried a mutation within exons 6, 8 or 9. No mutations were detected in exon 11. Paralleling published data, the *KIT* mutations did not correlate with tumour histological grade or mitotic index. The contribution of Kit to mast cell oncogenesis and malignancy in MCTs of the cat is unclear. Identifying *KIT* mutations in feline MCTs is not prognostically useful. High *KIT* gene and Kit protein sequence conservation between dog, cat, human and cheetah and the high frequency of genetic mutation in neoplastic mast cell DNA indicate that *KIT* genetic mutations are likely to exist in the neoplastic mast cells of other animals.

Chapter 7: General discussion

Dogs have a peculiar species-specific susceptibility for developing mast cell tumours (MCTs). Particular dog breeds including breeds of Bulldog origin, Golden Retrievers and Labrador Retrievers are commonly regarded as being predisposed to MCT development, suggesting an underlying germ-line genetic contribution to MCT development. It is possible that breeds predisposed to MCT development may exhibit increased concentrations of mast cells at baseline levels. Logically, a higher initial concentration of mast cells would result in increased mast cell progenitor replications, equating to a greater chance of somatic mutation and subsequent abnormal mast cell proliferation. Few results are available regarding the baseline mast cell levels between breeds. One research group comparing mast cell distributions among the organs and gastrointestinal tracts of Beagles (n = 7) and mixed breed dogs (n = 3) report similar mast cell concentrations, albeit numbers were limited (Noviana *et al.*, 2004). Further, species-specific differences in baseline mast cell distributions suggest that increased baseline levels are not a risk factor for MCT development. The domestic cat has substantially greater mast cell levels in normal skin biopsies than the dog, yet it is the dog which is more commonly diagnosed with MCT (**Table 7.1**). Notably, these data must be interpreted with caution due to the methodological heterogeneity between these studies when calculating mast cell numbers.

Table 7.1. Mast cell counts in normal, non-neoplastic skin biopsies from dog, human and cat. Reported mast cell counts were calculated by measuring tryptase activity in fixed specimens.

Species	Mast cells/mm^2 (± SD)	Sample	Reference
Dog	77.9 (68.8)	Skin, ear	(Noviana *et al.*, 2004)
Human	74.9–113 (31.5–46.7)	Skin, various sites	(Janssens *et al.*, 2005)
Cat	357 (96.8)	Skin, ear	(Noviana *et al.*, 2001)

SD: standard deviation

The prognosis for dogs with cutaneous MCT is strongly influenced by histopathological tumour grading. However, histological grading alone does not accurately predict tumour biological behaviour and corresponding survival outcomes for a significant minority of canine MCT cases. Supplementing histological grade by evaluating the *KIT* gene for mutations allows for more precise prognosis and can guide therapeutic management. This work investigated the prevalence of *KIT* gene mutations in cutaneous and subcutaneous MCTs from Australian dogs and focused on the role of *KIT* in prognosis. *KIT* mutations in MCT of the domestic cat and cheetah were also investigated for comparison.

7.1.1 The role of KIT in canine MCT prognosis and treatment

Non-synonymous *KIT* gene mutations were found in the neoplastic mast cell DNA from 50% of canine cutaneous and subcutaneous MCTs. Whilst the presence of any *KIT* mutation type was not correlated with canine prognosis, detection of an enzymatic pocket-type mutation was predictive of an increased likelihood of 12-month MCT-related death. This variable was statistically significant in the multivariable model, highlighting it as a useful prognostic indicator independent of histological grade. Aligning with recent research, detection of an exon 11 internal tandem duplication (ITD) in the tumour DNA was not associated with an increased risk of canine MCT-related death or second MCT diagnosis in the multivariable models (Horta *et al.*, 2018b). This can be attributed to its strong correlation with high histological grade.

In the AmpliSeq study (Chapter 4), mutations in the regulatory domain of *KIT* (including exon 11 ITDs) were the most common type of mutation and were detected in 31 of the 81 dogs with MCTs (38%). Approximately 20% of these 31 dogs (n = 6) harboured an additional mutation within the enzymatic pocket domain. The proportion of dogs with an enzymatic pocket-type mutation was consistent when considering the entire cohort (n = 14/81, 17.3%) and when considering only the dogs with an ITD-mutant MCT (n = 2/10, 20%). The 20% frequency of this mutation type may explain why at least 18% of dogs with ITD-mutant tumours in the London *et al.* studies did not respond to treatment with the tyrosine kinase inhibitors (London *et al.*, 2003, London *et al.*, 2009). Further, the data show that in this sample of the Australian dog population, the prevalence of *KIT* enzymatic pocket-type mutations is significantly higher than the prevalence of exon 11 ITD mutations. The high frequency of enzymatic pocket-type mutations questions the importance of genetic testing of MCT DNA for ITDs in exons 8 and 11. Instead, screening the enzymatic pocket domain for mutations that induce tyrosine kinase inhibitor resistance may provide more valuable knowledge regarding the likelihood of tumour response to tyrosine kinase inhibitor drugs.

Acquired tumour resistance through additional *KIT* enzymatic pocket domain mutations within the tumour DNA has been documented after prolonged exposure to tyrosine kinase inhibitors both *in vitro* and *in vivo* (Kobayashi *et al.*, 2015, Kurita *et al.*, 2019, Nakano *et al.*, 2017). This should be considered in the therapeutic management of tumours with apparently wild-type enzymatic pocket domain DNA. Combination therapy using tyrosine kinase inhibitors in conjunction with traditional chemotherapeutics is a promising treatment

avenue which targets both wild-type and mutant *KIT* tumour cells. It is predicted that targeting neoplastic cells with two drugs that have different modes of action will decrease the chance for the tumour cells to develop resistance to either drug. As an example, toceranib in combination with lomustine or vinblastine to treat canine MCT is currently being explored (Burton *et al.*, 2015, Olsen *et al.*, 2018). The combination appears to be well-tolerated by dogs and demonstrates a favourable response in patients with measurable disease regardless of exon 11 mutation status. This combination therapy may also provide some benefit to dogs with MCTs harbouring an enzymatic pocket-type mutation because cells with these mutations are theoretically still susceptible to chemotherapeutic agents. However, the response of cells with a known enzymatic pocket-type mutation to conventional chemotherapeutics is yet to be investigated.

The use of tyrosine kinase inhibitors for the treatment of canine subcutaneous MCTs has not been explored. In the AmpliSeq study, 8 of 18 dogs with subcutaneous MCT carried a regulatory-type mutation, and only one of these dogs carried an additional mutation within the enzymatic pocket domain. This suggests that the majority of dogs with subcutaneous MCT may derive clinical benefit from tyrosine kinase inhibitor therapy due to the absence of mutation-induced resistance. However, most cases of subcutaneous MCT are cured with surgery alone and postoperative adjuvant therapy is not required (Newman *et al.*, 2007, Thompson *et al.*, 2011a, Thompson *et al.*, 2011b).

Cutaneous MCT is more commonly diagnosed in dogs than the subcutaneous MCT variant. Germline genetic predisposition to cutaneous MCT development is likely in Bulldog-breeds as well as Golden Retrievers and Labrador Retrievers (Mochizuki *et al.*, 2017a, Smiech *et al.*, 2019, Warland and Dobson, 2013). A novel finding from this work involves the relationship between patient breed, age and tumour histological grade in dogs from different geographical locations. The results show Australian Labrador dogs to be at risk for developing high-grade MCTs at an older age (≥ 7 years old), although numbers were somewhat restricted (31 Labrador dogs, Chapter 5). This is in direct contrast to the findings of a Polish research group using two different sets of animals (49 and 57 dogs) drawn from the same population showing that Labrador dogs aged 4–6 years were at an increased risk for low-grade MCT development (Smiech *et al.*, 2019, Smiech *et al.*, 2018). The tendency for certain breeds of distinct populations to develop MCT of different phenotypic malignancies suggests different underlying genetic predisposing elements between the populations. Supporting this, the

overall exon 11 ITD prevalence in canine MCTs from isolated Australian populations more closely resembles the prevalence seen in European populations and is markedly lower than reports from the USA and Japan (**Appendix C**). In one genome-wide association study, the predisposing germ-line genetic factors associated with cutaneous MCT development in European Golden Retriever dogs were different from the factors identified in American Golden Retriever dogs (Arendt *et al.*, 2015). To explore whether the underlying genetic pathology of MCTs from Australian dogs is similar to European canine MCT populations but distinct from American populations, or vice versa, requires further research.

7.1.2 Cheetah and domestic feline MCTs

Based on the results here (Chapter 6) in conjunction with data from other species, it is suspected that the family of cheetahs reported herein is also genetically predisposed to MCT development. This is concerning considering the limited genetic variability of this species. The cheetah is listed as a vulnerable species according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Durant *et al.* 2014). However, the actual population size might be lower than the current estimated population of 6,674 in South Africa and recent papers advocate for cheetah listing to be changed to endangered (Durant *et al.*, 2017, Weise *et al.*, 2017). The cheetah's notoriously low level of genetic diversity can be attributed to the population bottleneck that they experienced approximately 10,000 years ago (Menotti-Raymond and O'Brien, 1993). This has led to the adversely inbred cheetah population, making breeding projects difficult and genetic disease a major concern for ongoing cheetah survival. Knowledge of MCT as a potentially inheritable cancer in the cheetah is important when selecting individuals for breeding programs. Careful monitoring of captive cheetahs, especially those at the Taronga Zoo, could help to gain a better understanding of the genetic and biological behaviour of MCT in this species.

Campbell-Ward *et al.* (2019) concluded that cheetah cutaneous MCTs follow an indolent disease course. This was deduced because the four incompletely excised tumours did not recur, and all tumours histologically resembled low- and intermediate-grade canine cutaneous MCTs. These results should be cautiously considered though because long follow-up times (> 2 years) were not available for two of the four cheetahs. Reports of cheetah MCT are scant. Splenic MCT with metastasis to the larynx was reported in a 13-year-old captive female cheetah (Owston *et al.*, 2008). Incomplete surgical excision of a cutaneous MCT in a female cheetah of an unknown age was described in an unpublished anecdotal blog entry

(Flacke, 2012). In this animal, the tumour recurred at the surgical site one-month post-excision and was removed a second time. Further ultrasounds and radiographs of the cheetah's chest and abdomen were unremarkable (confirmed through email correspondence with the leading veterinarian of this case, Dr. Gabriella Flacke). The recurrent local tumour of this case and the metastatic visceral mastocytosis reported in other cheetahs suggest that mast cell neoplasia of the cheetah can exhibit malignancy. Together with the data presented herein, cheetah MCT behaviour largely resembles MCT of the cat, with mostly benign cutaneous MCT but malignant visceral disease (Chapter 1).

7.1.3 Predisposition of canine KIT to exon 11 ITD mutation

The exon 6, 8 and 9 cheetah and feline MCT *KIT* mutations reported herein (Chapter 6) occurred at positions encoding amino acid residues that are highly conserved across the 18 mammalian species (**Appendix L**). It is expected that these amino acids are important in the correct folding and subsequent function of Kit protein and, therefore, the mutations may play a role in neoplastic mast cell biology. Only wild-type exon 11 *KIT* sequences were detected in all cheetah and feline DNA samples in the current study. A single exon 11 mutation has been reported in just one study of 62 feline cutaneous and visceral MCT specimens (Isotani *et al.*, 2010). In human infants with paediatric mastocytosis, an exon 11 mutation was found in only 1 of 50 patients (Bodemer *et al.*, 2010). In both the cat and human cases, the exon 11 mutation was not an ITD. This contrasts with MCTs of the dog where up to 17% of tumours from non-biased studies exhibit an ITD at the 3' end of exon 11 (**Appendix C**). The unusually high prevalence of exon 11 ITDs in MCTs of the dog is perplexing considering the Kit exon 11 amino acid sequence is 100% conserved between mammalian species (**Figure 7.1**; **Appendix L**).

```
Axolotl              KPKYEDQWKVVDEINGNNYVYIDPTQLPYDHKWEFPRDRLSFG
Burrowing_owl        KPKYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Chicken              KPKYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Mouse                KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Ferret               KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Human                KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Rhesus_macaque       KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Cynomolgus_macaque   KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Baboon               KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Horse                KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Sheep                KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Cow                  KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Goat                 KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Llama                KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Pig                  KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Siberian_tiger       KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Cougar               KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Cat                  KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Cheetah              KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Dog                  KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
Pacific_walrus       KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFG
                     ** ** *****:**************************:*****
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Figure 7.1. Alignment of the Kit exon 11 amino acid sequences for 21 species previously diagnosed with mast cell tumour. Sequences were aligned using Clustal Omega Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/; accessed 07/06/2019). The asterisk (*) indicates that the amino acid residue at that position is conserved among all species. A colon (:) indicates conservation between groups of amino acid with strongly similar properties. A gap indicates no conservation. The red characters represent amino acids in avian and amphibian species which differ from mammalian exon 11 Kit sequence. Reference amino acid sequence accession numbers are listed in Chapter 6, section 6.2.1. See **Appendix L** for the alignment of the entire Kit amino acid sequence.

There are several different possible explanations for the unique genetic pathology of the *KIT* exon 11 ITDs in dog MCTs. The presence of an underlying genetic anomaly within the canine *KIT* genomic DNA sequence, which is absent in feline sequences and well-studied human sequences, might predispose the canine *KIT* gene to duplicate at the 3' end of exon 11. Comparative analysis of the entire *KIT* genomic DNA sequences between species may identify poorly conserved regions within the sequence surrounding exon 11 which may promote sequence instability and predispose mutation. Non-coding regions and poorly conserved sequences may harbour sequence-specific mutation promoting motifs (e.g. simple tandem repeats) and could explain the observed mutability of canine *KIT* exon 11 that is not observed in other species.

Duplicating mutations within the genome are characteristically rare, at least in humans, but can be caused endogenous events such as transposable element replication, double-strand breaks during recombination, replication slippage during DNA replication of

palindromic sequences, and fork stalling leading to template switching (Bouwman and Crosetto, 2018, Carvalho and Lupski, 2016, Pace *et al.*, 2009, Vaughn and Bennetzen, 2014). Alternatively, duplicating mutations may be caused by exogenous factors (e.g. exposure of DNA to ionising radiation, UV, chemotherapy, etc.) (Bouwman and Crosetto, 2018). Elaborate machinery within the cell exists to ensure mismatched nucleotides are amended and replication mistakes are avoided (Hustedt and Durocher, 2016). Permanent errors become mutations, and if the mutation provides the cell with a growth advantage, the cell is selected for and becomes quickly established, potentially resulting in cancer.

To investigate whether an endogenous repetitive element in the canine exonic-intronic regions flanking exon 11 might play a role in *KIT* fragility and ITD formation, the canine sequence was analysed for known repetitive elements (Carvalho and Lupski, 2016, Deininger and Batzer, 1999). Long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and endogenous retroviruses (ENVs) are repetitive retrotransposable elements which employ a "copy and paste" mechanism to distribute and integrate themselves throughout the genome. Retrotransposition of repetitive elements near or within genes can disrupt gene function by altering splicing patterns and gene expression, destabilising the mRNA, introducing breaks within the genomic DNA inducing chromosomal instability, activating oncogenes and silencing tumour suppressor genes (reviewed in Helman *et al.* 2014). Such genetic alterations can result in deleterious phenotypes, such as disease or cancer.

Canine LINEs, SINEs and ENVs were identified in GenBank, NCBI and Ensembl databases (**Appendix M** and **N**). Using NCBI BLAST function, the canine LINE, SINE and ENV sequences were aligned to the canine *KIT* exon 10–12 genomic sequence (NC_006595.3: 47178312–47179028). No repetitive, retroviral or transposable sequences were discovered in this region of the *KIT* gene using BLAST. Lastly, the canine *KIT* exon 10–12 genomic DNA sequence was submitted to CENSOR[1] and Dfam[2] databases and screened for transposons and repetitive DNA elements. None were discovered.

To determine whether obvious structural features of canine *KIT* DNA might predispose a high frequency of exon 11 ITDs, DNA structural comparisons of canine, feline and human *KIT*

[1] CENSOR: a software tool which screens a query sequence against a collection of repeats. Accessed 12/8/2019; https://www.girinst.org/censor/index.php

[2] Dfam release 3.1: a database of transposable and repetitive element libraries produced by the Institute for Systems Biology and the University of Montana. Library for the dog was not available so the sequence was screened against the human database. Accessed 12/8/2019; https://dfam.org/home

sequences were investigated. It was hypothesised that the canine *KIT* gene would exhibit a folding pattern unique from the feline and human *KIT* DNA structures. To investigate this, the exonic-intronic sequences flanking canine *KIT* exon 11 were aligned and compared with the corresponding *KIT* nucleotide sequences of human and cat. The canine sequence shared 82% and 86% identity with the human and feline *KIT* sequences, respectively (**Table 7.2**), and multi-sequence alignment showed that the *KIT* intronic regions are well conserved between the three species (**Figure 7.2**). No tandem repeats or other remarkable differences between the three species were observed in the sequences flanking the start and end positions of the canine ITD.

Table 7.2. *KIT* gene comparative information for dog, human and cat genomic sequences.

Species (reference sequence)	**Chromosome number : number of exons**	**Number of known transcripts**	**Exon 10–12**	
			Genomic location (length, bp)	**Sequence identity with the dog (%)**
Dog (NC_006595.3)	13 : 21	2	47178312 – 47179028 (716)	-
Human (NC_000004.12)	4 : 21	4	54727218 – 54727927 (709)	82
Cat (NC_018726.3)	B1 : 21	1	163965223 – 163965940 (717)	86

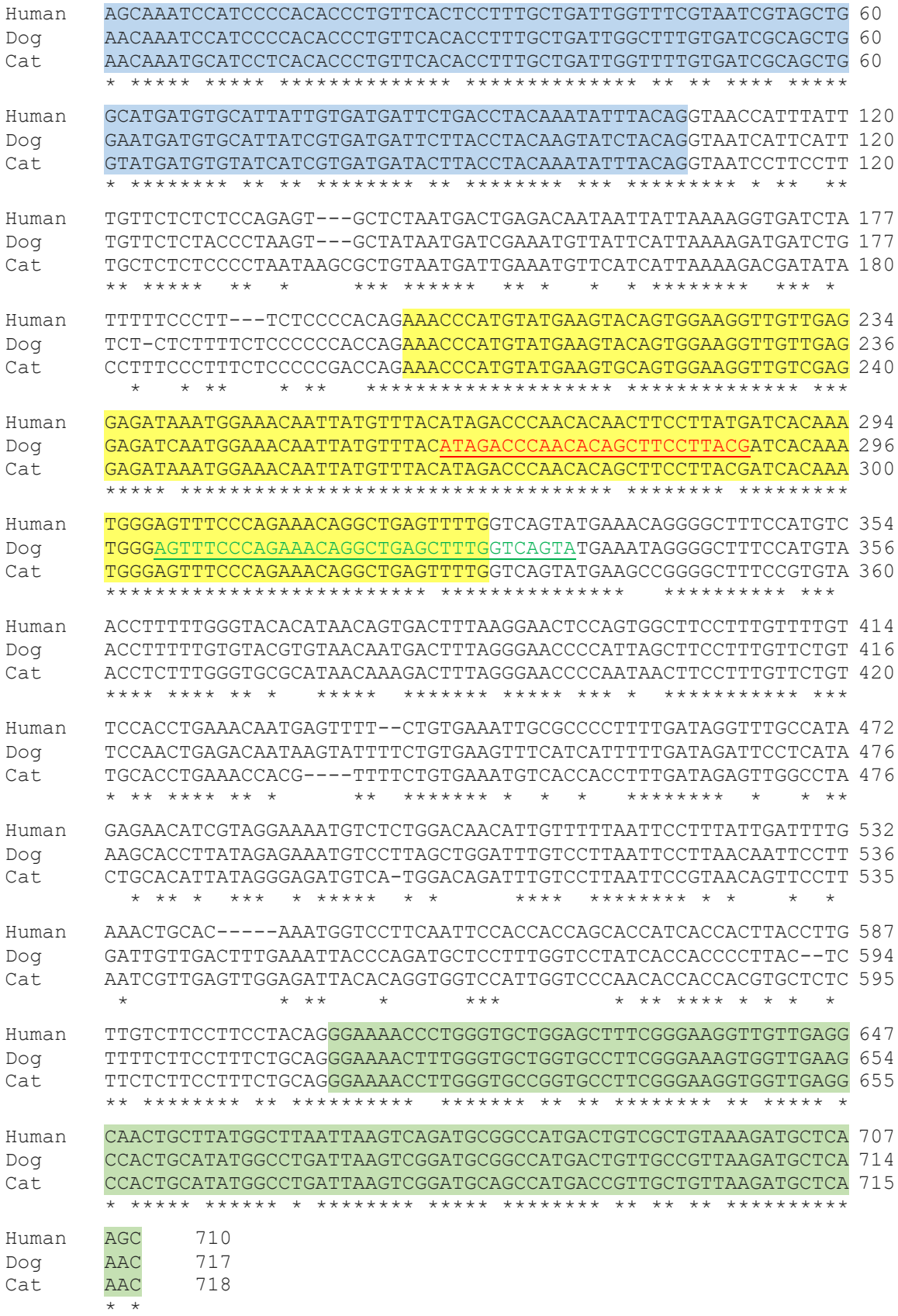


Figure 7.2. Canine, feline and human *KIT* exons 10–12 sequence alignment. See the following page for a full figure description.

Figure 7.2. Full figure description. Alignment of canine, feline and human exons 10–12 from the *KIT* genomic DNA sequences using Clustal Omega Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/; accessed 06/08/2019). The asterisk (*) indicates that the nucleotide residue at that position is conserved among the three species and a gap indicates no conservation. A hyphen (–) denotes no nucleotide. The blue, yellow and green highlighted sequences are exons 10, 11 and 12, respectively. The unhighlighted regions are introns 10 and 11, respectively. The red underlined nucleotides represent the starting positions of internal tandem duplications (ITDs) which were identified in canine mast cell tumour (MCT) DNA (**Appendix D**). The green underlined nucleotides indicate the end positions of the ITDs. Note that ITDs in canine MCTs range from 25 to 63 nucleotides in size and some extend into intron 11. Respective reference sequence accession number and nucleotide locations; dog NC_006595.3 47178312–47179028, human NC_000004.12 54727218–54727927, cat NC_018726.3 163965223–163965940.

Next, the predicted secondary DNA structure of the exon 11 and intron 11 sequence was compared between dog, cat and human. It was suspected that hairpin/looping DNA structures due to palindromic sequences in the lagging DNA strand might be present in the canine structure but absent from human and cat structures. Hairpin/loops can cause "replication slippage" promoting sequence duplication (Kiyoi *et al.*, 1998, Lagunas-Rangel and Chavez-Valencia, 2017, Vaughn and Bennetzen, 2014). Replication slippage tends to occur in genomic regions made up of short tandem repeats and has been suggested to be the mechanism responsible for ITD mutations in the *FLT3 Tyrosine Kinase* gene which is associated with aggressive acute myeloid leukaemia (AML) (Kiyoi *et al.*, 1998, Ni *et al.*, 2019, Port *et al.*, 2014).

No obvious differences in the predicted DNA folding structures between canine *KIT* and human or feline *KIT* were observed that could have explained the mutability of canine *KIT* (**Figure 7.3**). The structure encompassing the exon 11 ITD start and end positions of the predicted canine DNA sequence closely resembled that of the human structure and, in fact, it was the feline structure which was the most distinct of the three species. Those canine ITD sequences that included the intronic region only extended as far as the base of a hairpin/loop in canine *KIT* predicted structure, but no further. However, this does not explain the cause of the sequence duplication.

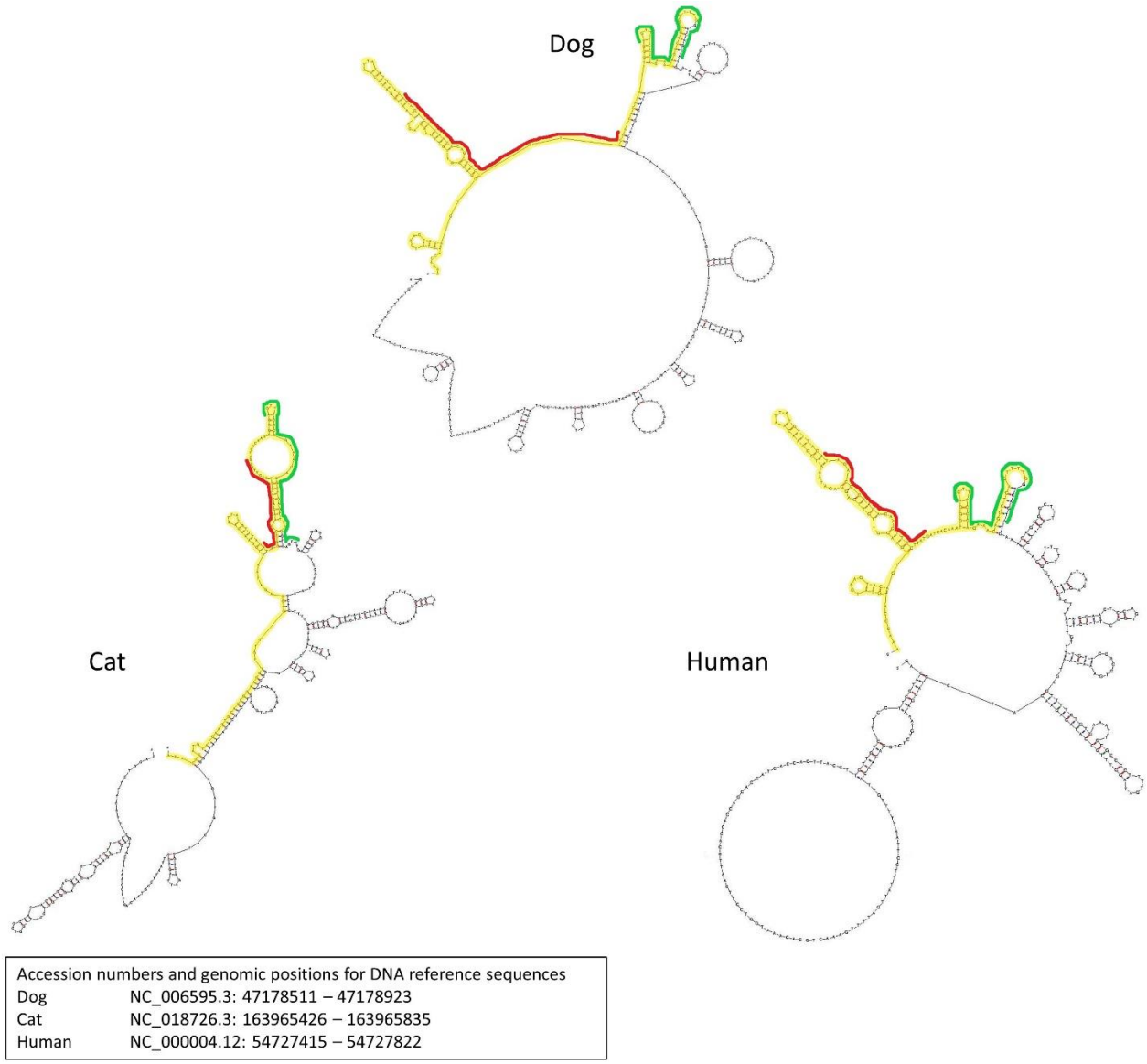


Figure 7.3. Predicted DNA secondary structure of the dog, cat and human wild-type *KIT* exon 11 and intron 11 DNA sequence. The yellow highlight indicates the exon 11 sequence and the remaining sequence is the intronic region. The red outline indicates internal tandem duplication (ITD) start sites in the wild-type canine mast cell tumour (MCT) DNA sequences. The green outline indicates the end positions of ITDs in the dog and the corresponding nucleotides in human and cat *KIT* sequences are also indicated. Note that ITDs in canine MCTs range from 25 to 63 nucleotides in size and some extend into intron 11. Structures were predicted using Mfold Web Server DNA Folding Form as described in **Appendix O** (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form; accessed 09/08/2019) (Zuker, 2003).

The abnormal mutability of the canine *KIT* gene remains a biological anomaly because of the reasonably well conserved genomic DNA sequence. Interestingly, ITD mutations are known to frequently occur in another tyrosine kinase protein-encoding gene in humans, *FLT3,* the *Fms-Like Tyrosine Kinase 3* gene (Levis and Small, 2003, Schnittger *et al.*, 2002).

7.1.4 Similarities and differences in KIT and FLT3 tyrosine kinase genes

Similar to *KIT,* the *FLT3* gene encodes a class III transmembrane receptor-type tyrosine-protein kinase, Flt3 (Shurin *et al.*, 1998). *FLT3* is important in hematopoietic stem cell survival and proliferation. Somatic mutations in *FLT3* are common in human patients with acute myeloid leukaemia (AML) (El Fakih *et al.*, 2018). ITDs in exon 11 of *FLT3* are detected in 20% of human patients and ITDs constitutively activate Flt3 protein (Levis and Small, 2003, Schnittger *et al.*, 2002). AML patients with *FLT3* ITDs have statistically significantly shorter overall and disease-free survival times compared to patients without an ITD when analysed in a univariable model (Moreno *et al.*, 2003, Port *et al.*, 2014, Thiede *et al.*, 2002). In multivariable analyses of known prognostic factors, the presence of a *FLT3* exon 11 ITD has no statistically significant effect on prognosis (Thiede *et al.*, 2002). This parallels results from dogs with exon 11 ITD-mutant MCTs.

Both *KIT* and *FLT3* ITDs frequently occur within the juxtamembrane domain encoded by exon 11. *FLT3* ITDs range from 6 to more than 400 nucleotides in length and the larger duplications extend into the adjacent intron sequence (Schnittger *et al.*, 2002, Thiede *et al.*, 2002). *FLT3* ITDs are always a multiple of three and preserve the reading frame (Thiede *et al.*, 2002). This contrasts with *KIT* ITDs which are not consistently a multiple of three and which occasionally disrupt the reading frame (Chapter 4) (Downing *et al.*, 2002, Letard *et al.*, 2008, London *et al.*, 1999, Zemke *et al.*, 2002). No significant similarity between the human *FLT3* and the canine *KIT* genomic DNA or mRNA sequences was found by NCBI BLAST alignment (**Appendix P**). The peculiar susceptibility of these tyrosine kinase genes to juxtamembrane duplications remains unknown. A 37% sequence identity was determined between Kit amino acid residues 188–938 and Flt3 amino acid residues 232–958. However, when the *KIT* and *FLT3* exon 11 DNA and amino acid sequences were compared on their own, no significant homology was found.

One statistically significant prognostic indicator for AML patients in both univariable and multivariable statistical models is the mutant to wild-type *FLT3* allelic ratio, as determined

from blood-extracted genomic DNA (Thiede *et al.*, 2002). A *FLT3* ITD-mutant to wild-type allelic ratio > 0.78 was negatively associated with the risk of disease relapse (HR = 3.2, P < 0.001) and overall survival (HR = 1.8, P = 0.002) in a study of 258 patients with *FLT3*-mutant AML (Thiede *et al.*, 2002). The prognostic value of the mutant to wild-type allelic ratio remained significant in one other study evaluating the *FLT3* tyrosine kinase I domain (exons 13–15) (Schnittger *et al.*, 2012). A ratio ≥ 1 (i.e. more copies of the mutant allele than the wild-type allele) was prognostically significant for overall survival (P = 0.003) and event-free survival (P = 0.001) in a cohort of 689 AML *FLT3*-mutant patients. Mirroring this, the complete loss of the wild-type *KIT* allele in 15% of in tumour-extracted DNA from human patients with gastrointestinal stromal tumour (GIST) was correlated with malignant tumour behaviour and a worse prognosis than for patients with a heterogeneous disease (Chen *et al.*, 2008).

The prognostic significance of mutant to wild-type allelic ratio in AML and GIST patients suggests that this might be a useful prognostic aid in canine MCTs and is an avenue for future investigation. In the present canine MCT *KIT* study, the mutant to wild-type allelic ratios in the neoplastic MCT DNA could not be deduced from the AmpliSeq NGS analysis. Whilst the allele ratios were reported for each mutation detected, the DNA samples were extracted from FFPE-MCT tissue blocks and no attempt was made to purify the neoplastic mast cells from the non-neoplastic tissue surrounding the tumour. Hence, the wild-type reference alleles would be over-represented in each sample rendering statistical evaluations useless.

The *KIT* mutations resulting in a non-conservative amino acid change which were detected in the canine MCT DNA samples are predicted to have a functional consequence for Kit protein. However, wet-lab experiments are necessary for confirmation of functional consequences. One AML research group investigating the effects of *FLT3* mutations on Flt3 protein function identified nine SNPs resulting in amino acid changes (Frohling *et al.*, 2007). The SNPs were transfected into Ba/F3 cells and cell growth and protein phosphorylation were analysed. Only four of the nine mutations caused ligand-independent cell growth and constitutive Flt3 phosphorylation. These variants were concluded to be gain-of-function. The remaining five mutant Ba/F3 cells lines underwent apoptosis when cultured in the absence of Flt3-ligand, and constitutive Flt3 protein phosphorylation was not observed.

Extrapolating from this work, it is possible that some of the *KIT* mutations detected by Ion AmpliSeq technology may not be associated with malignant protein transformation and might be instead non-malignant passenger mutations. Results from the cheetah

hydrophobicity analysis suggest other non-conservative SNPs may not affect Kit protein structure or function because they do not appear to alter the wild-type hydrophobicity structure (**Figure 6.5**). 3D cheetah Kit protein modelling software predicts minor structural modifications in mutant-Kit protein structure compared to the wild-type (**Figure 6.6**). Whether the other amino acid substitutions discovered herein are sufficient to alter protein function or induce ligand-independent activation cannot be deduced from the current data. Wet-lab experiments are warranted.

7.1.5 Kit protein structure comparison between dogs, cats and humans

To better understand the functional significance of Kit amino acid substitutions on the Kit protein, the predicted secondary Kit amino acid structure was evaluated and compared between wild-type canine, human and feline sequences and the most extreme amino acid mutation found, the canine *KIT* exon 11 ITD. It was hypothesised that the wild-type Kit structures would only exhibit minor differences between the species in the location of alpha-helices, beta-pleated sheets or the positions of turns because of the strong amino acid homology (**Table 6.3**). However, larger differences were expected between the canine wild-type and mutant exon 11 ITD because of the number of amino acids affected.

Minor differences in the Kit amino acid secondary structure were noted between the species. However, no structural differences between the three species occurred within the exon 11 domain (underlined text of wild-type canine Kit, **Figure 7.4**).

The Kit secondary structure was then predicted for a representative canine Kit ITD-mutant with a 17 amino acid duplication consequent from a 51-bp ITD within the *KIT* exon 11 DNA sequence (**Figure 7.4**). The ITD-mutant Kit structure differed significantly from the wild-type structure by the addition of multiple alpha-helices, 3_{10}-helices and beta-pleated sheets, two additional turns/bends and various extending sequence structures. The structure immediately 5' of the 17 amino acid duplication was greatly affected by the ITD, whereas the structure following the duplication remained mostly unchanged. These structural changes must be sufficient to produce Kit protein auto-phosphorylation (Letard *et al.*, 2008).

Wild-type canine Kit:

CCCCHHHHHHHHHHHHHHHHHHTTCCCCCCCTTCCCCCEESSCSSEEEEETTSCEEEEEEC
TTEEEEEEECSSCEEEECSSEEEETTCCGGGCEEEEEEESSSCEEEEEEEEECSSCSBCCCC
CEEEETTSCEEECCCBSCCSCEEEEEEETTSCCCCTTCEEEEETTTEEEEESCCGGGTTCEE
EEEEEETTEEEECCCEEEEEECCCCSCCEEECSCSBCCCCTTCCEEEEEEEEESSTTCEEEE
EEESSCCCCCCEECCEEEEETTEEEEEEEEEESSCCSSSCEEEEEEEECSSCEEEEEEEECC
EECCEEEEEESSCSEEEEETTCCEEEEEEEEEESCCCEEEEEETTEECSSSEEEEESCCSTT
CCEEEEEEEECSCCGGGCEEEEEEEECSSCEEEEEEEEEEEEEEEEEECCSTSEEEEEEE
EESCCEEEEEECCCSSSSCCEEECCSEEEEECCCTTTTCSEEEEEEEECGGGTTTCEEEEEE
EETTEEEEEEEECEEECCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHCSCCHCCCCCCC
CCEEECCSSSTCCCCCCGGGSCCCGGGBCCGGGEEEEEEEECSSEEEEEEEEESSSSTTCE
EEEEEEEECTTCCHHHHHHHHHHHHHHHHCCCTTBCCEEEEECSSSSCEEEEECCTTCBHH
HHHHHTSGGCCCCCCCTTTCCCCCCCCCCCCCCCCCCCCCCCCCCTTCCECCCCCCCCCCC
CCCCCHHCTTCCCCHTSTCCCCCCHHHHHHHHHHHHHHHHHHHTTEECSCCSGGGEEECTT
CCEEECCCTTCEECTTCCTEECCTTCCSCGGGCCHHHHHHCCCCHHHHHHHHHHHHHHTT
SCCSSTTCCHHHHHHHHHTTCCCCCCTTCCHHHHHHHHHHTCSSGGGSCCHHHHHHHHHH
HHHCCCCEHHHHCSCCCCTCCCCCCCCCCCCCCCCCCCCSCCEECCTCC

ITD-mutant canine Kit:

CCCCHHHHHHHHHHHHHHHHHHTTCCCCCCCTTCCCCCEESSCSSEEEEETTSCEEEEEECTTE
EEEEEECSSCEEEECSSEEEETTCCGGGCEEEEEEESSSCEEEEEEEECSSCSBCCCCCEEEET
TSCEEECCCBSCCSCEEEEEEETTSCCCCTTCEEEEETTTEEEEESCCGGGTTCEEEEEEEETTE
EEECCCEEEEEECCCCSCCEEECSCSBCCCCTTCCEEEEEEEEESSTTCEEEEEEESSCCCCCCE
ECCEEEEETTEEEEEEEEESSCCSSSCEEEEEEECSSCEEEEEEEECCEECCEEEEEESSCSE
EEEETTCCEEEEEEEEEESCCCEEEEEETTEECSSSEEEEESCCSTTCCEEEEEEEECSCCGGGC
EEEEEEECSSCEEEEEEEEEEEEEEEEECCSTSEEEEEEEEESCCEEEEEECCCSSSSCCE
EECCSEEEEECCCTTTTCSEEEEEEEECGGGTTTCEEEEEEEETTEEEEEEEECECCCCCCCCC
HHHHHHHHHHHHHHHHHHHHHHHHHHCSCCHCCCCCCCCEEHCHSSSSCEEECCGGCCCCCTTC
BCCGGGEEGGSCCCGGGBCCGGGEEEEEEEECSSEEEEEEEEESSSSTTCEEEEEEEECTTCC
HHHHHHHHHHHHHHHHHCCCTTBCCEEEEECSSSSCEEEEECCTTCBHHHHHHHTSGGCCCCCCC
CTTCCCCCCCCCCCCCCCCCCCCCCCCCCTTCCEECCCCCCCCCCCCCCHHCTTCCCCHTSTC
CCCCCHHHHHHHHHHHHHHHHHHTTEECSCCSGGGEEECTTCCEEECCCTTCEECTTCCTEEC
CTTCCSCGGGCCHHHHHHCCCCHHHHHHHHHHHHHHHTTSCCSSTTCCHHHHHHHHHTTCCCC
CCTTCCHHHHHHHHHTCSSGGGSCCHHHHHHHHHHHHHCCCCEHHHHCSCCCCCCCCCCCCC
CCCCCCCCCCCSCCEECCTCC

Figure 7.4. Predicted secondary protein structure for canine wild-type Kit and the structure for a representative canine Kit-mutant of a 17 amino acid duplication consequent from a 51-base pair exon 11 internal tandem duplication (ITD) the genomic *KIT* DNA sequence (sample A20, **Appendix E**). The structure corresponding to canine exon 11 amino acid sequence is underlined and the position of the representative ITD is in red. Wild-type Kit: the symbols highlighted in grey are the structures which differentiate the dog from human and feline predicted Kit structures. Mutant-Kit: the symbols highlighted in yellow are the structures which differentiated the ITD-mutant from wild-type predicted canine Kit structures. The symbols denote the structure for the corresponding amino acid and are coded as follows; alpha-helix (H), 3_{10}-helix (G), turn (T), beta-bridge (B), extended strand (E), bend (S), the rest non-extended strand (C). Structures were predicted using ExPASy SIB Bioinformatics Resource Portal's Scratch Protein Predictor SSpro8 Program (http://scratch.proteomics.ics.uci.edu/; accessed 02/09/2019).

Next, the wild-type Kit protein 3D structure was predicted for the dog, human and cat (**Figure 7.5**). It was speculated that exon 11 of the canine Kit might be structurally distinct from the human and feline predicted tertiary Kit structures. Not surprisingly, small variations between the three species were observed in the overall tertiary structure of Kit. All structures comprised Kit transmembrane tyrosine kinase protein structure with extracellular immunoglobulin-like domains characterised by beta-pleated sheets and an intracellular kinase domain comprising alpha-helices. The canine Kit amino acid sequence containing the representative exon 11 51-bp ITD was also constructed for comparison with the wild-type canine Kit structure. Only minor structural changes were noted in the overall ITD-mutant canine Kit 3D predicted structure. These structural changes are Kit protein activating (Letard *et al.*, 2008).

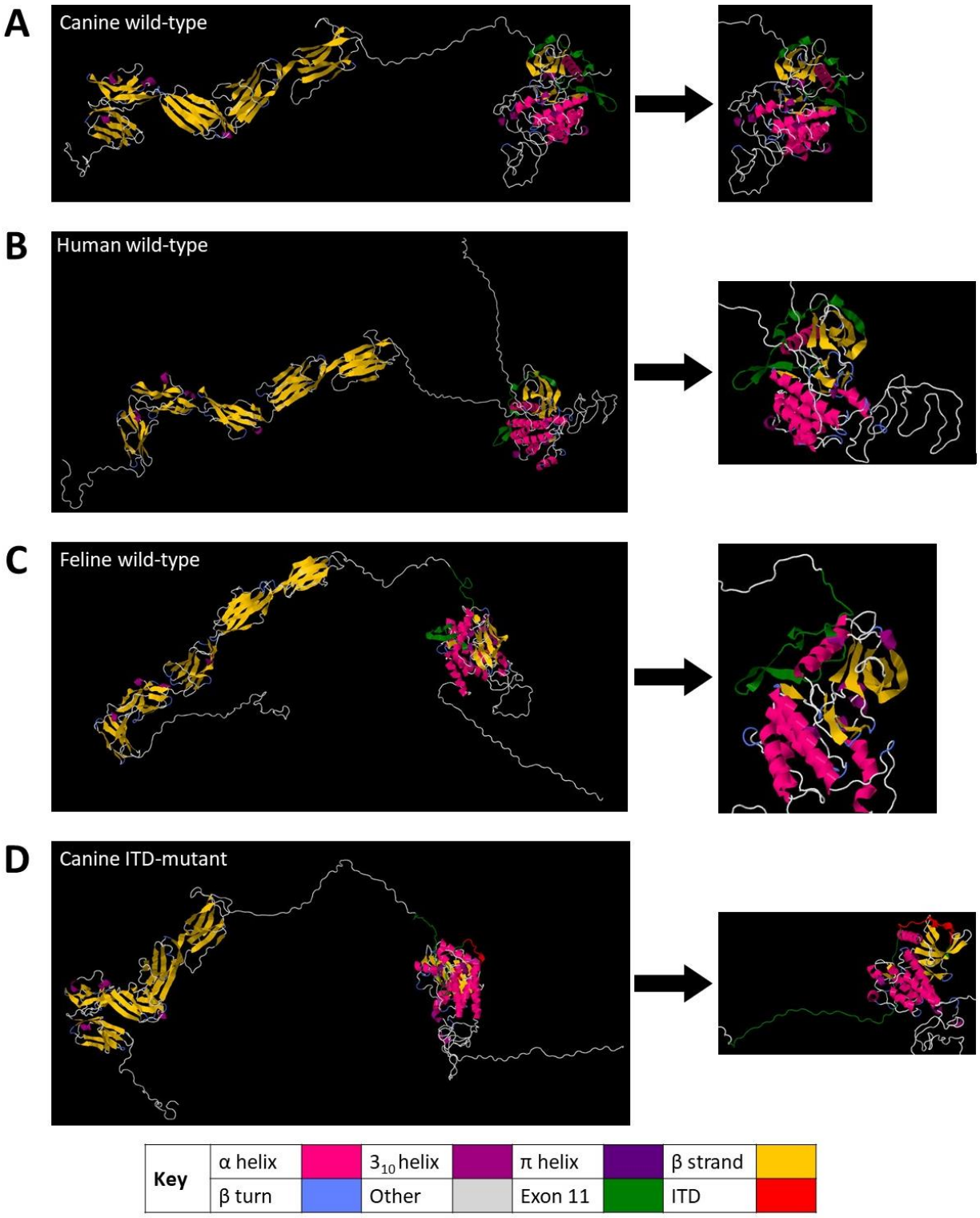


Figure 7.5. Predicted tertiary protein structure for the canine (A), human (B) and feline (C) wild-type Kit amino acid sequences. Also shown is the predicted structure for a representative canine Kit-mutant (D) with a 17 amino acid duplication ITD consequent from a 51-base pair internal tandem duplication (ITD) within the *KIT* exon 11 DNA sequence. A full figure description can be found on the following page.

Figure 7.5. Full figure description. Predicted tertiary protein structure for the canine (A), human (B) and feline (C) wild-type Kit amino acid sequences. Also shown is the predicted structure for a representative canine Kit-mutant (D) with a 17 amino acid duplication consequent from a 51-base pair (bp) exon 11 internal tandem duplication (ITD) in the genomic *KIT* DNA sequence (sample A20, **Appendix E**). The left-hand side of the protein depicts the extracellular immunoglobulin-like domains of Kit which are characterised by beta-pleated sheets (yellow ribbons). The right-hand side of the protein depicts the intracellular juxtamembrane and tyrosine kinase domains of Kit which are characterised by alpha-helices (pink coils). Exon 11 is highlighted in green. The red segment in the canine ITD-mutant structure (D) indicates the duplicated sequence consequent from the 51-bp ITD. Structures were predicted using ExPASy SIB Bioinformatics Resource Portal's Scratch Protein Predictor 3Dpro Program (http://scratch.proteomics.ics.uci.edu/; accessed 02/09/2019). Output files were visualized in Jmol 3D molecular modelling software (version 14.29.53). Jmol was used to orient each 3D structure to the same position for structural comparison. Snapshots were then taken by zooming into the intracellular domain and rotating the image until exon 11 was more easily visualised.

To date, all non-synonymous canine MCT *KIT* SNPs and indel mutations evaluated *in vitro* for their effects on Kit protein function have been found to cause Kit autophosphorylation and some studies have demonstrated ligand-independent proliferation of mutant cells (Furitsu *et al.*, 1993, Kitayama *et al.*, 1995, Letard *et al.*, 2008, London *et al.*, 1999, Ma *et al.*, 1999, Nakano *et al.*, 2017). Despite these gain-of-function effects of mutant Kit, non-exon 11 ITD regulatory-type mutations are not correlated with a worse canine MCT prognosis. A possible explanation for this may be that mutations within specific *KIT* gene domains may confer different Kit protein structures. The different structures, in turn, activate different intracellular signalling cascades. For example, constitutive protein phosphorylation of Erk1/2, Atk, Stat3 and Stat5 is observed in Ba/F3 cells harbouring *FLT3* juxtamembrane mutations (Tyr572Cys or Val592Gly) (Frohling *et al.*, 2007). However, mutation of exon 5 in the extracellular domain (Ser451Phe) and exon 20 in the tyrosine kinase activation loop (Arg834Gln) of *FLT3* result in constitutive activation of Erk1/2. In normal mast cells, Kit receptor protein activation stimulates various signalling cascades including Erk1/2, Akt, Mapk, Ras, Jak, Stat3/5 and Pi3k signalling pathways mediating mast cell proliferation, migration, differentiation and apoptosis (Cletzer *et al.*, 2019, Ronnstrand, 2004). In a canine mutant VI-MC neoplastic mast cell line harbouring a *KIT* Asn508Ile exon 9 activating mutation and a recombinant rVI-MC1 cell line, carrying the exon 9 mutation and an activating exon 17 Asp815His, Kit and Erk proteins were constitutively phosphorylated, whereas Akt and Stat3 proteins were not phosphorylated (Kobayashi *et al.*, 2015). In the positive control cell lysate prepared from a canine osteosarcoma cell line with wild-type *KIT*, all evaluated proteins were phosphorylated. The phosphorylation of different target signalling proteins between wild-

type and mutant *KIT* highlights the functional consequence of particular *KIT* mutations. If a particular mutation was to activate cellular proliferation pathways but not cascades leading to cellular apoptosis, this could contribute to the malignant mast cell transformation. Conversely, mutations insufficient in activating cell growth signalling pathways may exist purely as bystander mutations. It is impractical to accurately predict the effects of various *KIT* mutations on intracellular signalling cascades without laboratory experiments.

Implications of findings and future directions

The dog is particularly susceptible to MCT disease and *KIT* mutations are frequent within their neoplastic mast cell DNA. *KIT* mutations were detected in approximately 50% of canine MCTs in this study. The DNA from the remaining tumours appeared to harbour only wild-type *KIT* sequence, indicating that *KIT* is unlikely to be the underlying genetic driver for their MCT initiation. *KIT*-independent genetic mechanisms implicated in canine MCT pathogenesis have been suggested (Chapter 1), however, the exact molecular events and genetic predisposition contributing to the establishment and growth of canine MCTs remain unknown. Relatively recent research evaluating chromosomal copy number variations and structural rearrangements in canine MCTs have identified clonal copy number gains and losses of various cancer-related genes (Mochizuki *et al.*, 2017b, Vozdova *et al.*, 2019a). These genes and corresponding signalling pathways should be investigated for their effects on neoplastic cell growth. This will enhance the understanding of mast cell biology and may also identify novel targets for molecular therapy.

An important finding of this research is the implication of enzymatic pocket-type mutations in prognosis. Mutation of the *KIT* enzymatic pocket domain is statistically significant in predicting 12-month canine MCT-related death in multivariable analysis. Oddly, when comparing dogs with *KIT*-mutant MCT (at least one mutation in the regulatory domain, enzymatic pocket domain or non-coding domain) to dogs with only wild-type *KIT* MCT, the presence of a *KIT* mutation was not associated with canine prognosis.

Wet-lab experiments are necessary to verify the effects of the novel *KIT* mutations identified by this research on Kit protein activation and neoplastic cell proliferation. It is especially important to investigate the mutations occurring within the enzymatic pocket domain and to characterise their effects on the cell's susceptibility or resistance to tyrosine kinase inhibitor drugs. The development and implementation in veterinary diagnostic laboratories of genetic tests for mutations that demonstrate tyrosine kinase inhibitor resistance could be very useful in veterinary cancer medicine. Dogs being considered for treatment with tyrosine kinase inhibitors should be screened before initiating treatment with these drugs. For any dog with a tumour testing positive for a mutation of this type, non-tyrosine kinase inhibitor treatment options should be considered. Identification of enzymatic pocket-type mutations causing drug resistance is arguably more important than identifying

exon 11 ITD mutations because ITD status carries only a limited predictive value for tumour response to tyrosine kinase inhibitor treatment. Further, the presence of an exon 11 ITD mutation is strongly correlated with high tumour histological grade (Chapters 1 and 5). Histological grade can be assessed more quickly and inexpensively then *KIT* genetic testing, perhaps making exon 11 mutation screening unnecessary.

The functional consequences for the variants occurring in the *KIT* splicing domains, downstream regions and 3' UTRs also remain unclear. Research into splicing patterns, mRNA expression, protein structure and cell growth consequent of these mutations should be considered.

An additional novel finding inferred from this work involves the relationship between patient breed, age and tumour histological grade in dogs from different geographical locations. In this study, the predisposition of Australian Labrador dogs for developing high-grade MCTs at an older age is in direct contrast to the findings of a Polish study where Labrador dogs were at an increased risk for low-grade MCT development at a younger age. Underlying genetic predisposing elements between the populations are proposed but further research is required to confirm geographical differences.

Conclusions

The results presented herein describe numerous novel *KIT* mutations detected in canine cutaneous or subcutaneous MCTs in dogs presented to first opinion veterinary practices in south-eastern Australia. *KIT* mutations in subcutaneous MCTs have been identified, including an exon 11 ITD in one case. Non-synonymous coding mutations and genetic alterations within the *KIT* gene non-coding domains were detected in 51.9% of 77 cutaneous MCTs and 44.4% of 18 subcutaneous MCTs from Australian dogs. An enzymatic pocket-type mutation was detected in 20.8% (n = 16/77) of the cutaneous MCTs, perhaps explaining why a substantial minority of tumours do not respond to treatment with tyrosine kinase inhibitors.

Dogs with tumours harbouring mutation in the *KIT* enzymatic pocket domain were at an increased risk for 12-month canine MCT-related death in multivariable survival models. Conversely, and aligning with recent data, exon 11 ITD status did not add any prognostic value to that already achieved by histological grade.

The exact role of *KIT* in mast cell oncogenesis in dogs remains unclear. The data suggest that many mutations within the *KIT* gene are not sufficient for malignant transformation, but instead may underscore biologically neutral passenger alterations. Similarly, mutations within the feline MCT *KIT* gene sequence are not correlated with a worse prognosis. As for the cheetah, the sample size was restricted to four animals rendering statistical evaluation meaningless.

In summary, this research has identified numerous novel *KIT* mutations including many which lie within the enzymatic pocket domain. Knowing the frequency of these mutations will hopefully prove useful for veterinary oncologists considering the use of tyrosine kinase inhibitors for dogs with malignant MCT. This work provides the basis for establishing routine tumour mutation screening tests which can be implemented in diagnostic laboratories. Much remains to be discovered in the initiation and progression of neoplastic mast cells not only in the dog but also in other species including the cat. The contribution of *KIT* to MCT development and the unique predisposition to duplicate at the 3' end in exon 11 of canine *KIT* remains a genetic enigma.

Appendices

Appendix A. World Health Organisation (WHO) Clinical Staging System for canine mast cell tumours. Adapted from Withrow *et al.* (2013).

Stage	Description
0	One tumour incompletely excised from the dermis, identified histologically, without regional lymph node involvement 1. Without systemic signs 2. With systemic signs
I	One tumour confined to the dermis, without regional lymph node involvement 1. Without systemic signs 2. With systemic signs
II	One tumour confined to the dermis, with regional lymph node involvement 1. Without systemic signs 2. With systemic signs
III	Multiple dermal tumours; large, infiltrating tumours with or without regional lymph node involvement 1. Without systemic signs 2. With systemic signs
IV	Any tumour with distant metastasis, including blood or bone marrow involvement

Appendix B. Consumables list and suppliers.

Consumable	Supplier
1 Kb DNA Extension Ladder	Life Sciences, Thermo Fisher Scientific, Waltham, MA, USA
10X Reaction Buffer IV	Thermo Fisher Scientific, Waltham, MA, USA
1X TAE Buffer *0.04 M Tris Acetate, 0.001 M EDTA*	Sigma Aldrich, St. Louis, MO, USA
2X Ion Ampliseq Primer Pool	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
96-well twin.tec® real-time PCR Plate	Eppendorf, Hamburg, Germany
Agarose Low EEO Electrophoresis Grade	Sigma Aldrich, St. Louis, MO, USA
Agencourt AMPure XP Reagent	Beckman-Coulter, Brea, CA, USA
D1000 ScreenTapes	Integrated Sciences, Agilent Technologies, Santa Clara, CA, USA
dNTP Mix	Fisher Biotech Australia, Wembley, WA, Australia
Ion 316™ Chip v2 BC	Thermo Fisher Scientific, Waltham, MA, USA
IonCode Barcode Adapters	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
$MgCl_2$ *25 mM*	Thermo Fisher Scientific, Waltham, MA, USA
MicroAmp Optical Adhesive Film	Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA
Molecular Biology Grade H_2O *DNase and RNase free*	Sigma Aldrich, St. Louis, MO, USA
TE Buffer *10 mM Tris-Cl, pH 8* *1 mM EDTA*	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
ThermoPrime Taq polymerase	Thermo Fisher Scientific, Waltham, MA, USA
Xylene	Thermo Fisher Scientific, Waltham, MA, USA

Research Kits	Supplier
DNA Clean and Concentrator Kit DNA Binding Buffer DNA Wash Buffer DNA Elution Buffer *10 mM Tris-HCl (pH 8.5), 0.1 mM EDTA*	Integrated Sciences, Zymo Research, Irvine, CA, USA
Ion AmpliSeq™ DNA Library Kit 2.0 5X Ion AmpliSeq™ HiFi Mix FuPa Reagent Switch Solution DNA Ligase Ion AmpliSeq™ Adapters Platinum™ PCR SuperMix High Fidelity Library Amplification Primer Mix Low TE Elution Buffer *10 mM Tris-Cl (pH 8), 0.1 mM EDTA*	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
QIAamp DNA FFPE Tissue Kit Buffer ATE *10 mM Tris-Cl (pH 8.3), 0.1 mM EDTA, 0.04% Sodium azide (NaN_3)*	Qiagen, Hilden, Germany
QIAmp AllPrep FFPE Tissue Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QuBit® dsDNA HS Assay Kit	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA
QuBit® dsDNA HS Assay Kit Qubit® dsDNA HS Reagent *200X concentrate in DMSO* Qubit® dsDNA HS Buffer Qubit® dsDNA HS Standard #1 *0 ng/µL in TE buffer** Qubit® dsDNA HS Standard #2 *10 ng/µL in TE buffer**	Ion Torrent, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA

Appendix C. Prevalence of *KIT* exon 11 internal tandem duplications (ITDs) in cutaneous canine mast cell tumours (MCTs) and study pitfalls and biases.

Country	Number of samples	Exon 11 ITD prevalence	Population source	Notes	Reference
USA	11	45.5%	University teaching hospital	Small sample size, only used grade II MCTs	(London *et al.*, 1999)
USA	9	33.3%	-	Small sample size	(Ma *et al.*, 1999)
USA	157	33%	University teaching hospital	Predominant use of grade II and III MCTs	(Downing *et al.*, 2002)
USA and France	124	30.6%	General practice	Recurrent or non-resectable grade II and III MCTs	(Hahn *et al.*, 2008)
USA	147	21%	University VDL and commercial VDL's	Excluded the use of grade II MCTs	(Mochizuki *et al.*, 2017b)
Australia	74	17.6%	University VDL and commercial VDL's	Predominant use of grade II and III MCTs	(Tamlin *et al.*, 2017)
Japan	47	17%	Referral population	-	(Takeuchi *et al.*, 2013)
USA	56	16%	University VDL	-	(Webster *et al.*, 2007)
USA	60	15%	University VDL	-	(Webster *et al.*, 2006b)
USA	28	14%	University teaching hospital	-	(Webster *et al.*, 2008)
USA	88	13.6%	University VDL	Biased population selection	(Zemke *et al.*, 2002)
USA and France	191	13.1%	General practice	Grade I tumours not included in study	(Letard *et al.*, 2008)
Italy	21	10%	-	Reasonably small sample size	(Marconato *et al.*, 2014)
Italy	59	5%	General practice	Predominant use of grade I and II MCTs	(Giantin *et al.*, 2012)
Spain	45	0%	University VDL	-	(Reguera *et al.*, 2002)
Italy	32	0%	-	-	(Riva *et al.*, 2005)

VDL: veterinary diagnostic laboratory.

Appendix D. Genomic location and length in base pairs (bp) of *KIT* exon 11 internal tandem duplication (ITD) mutations identified in cutaneous and subcutaneous canine mast cell tumours (MCTs).

Mutation	Extension into intron 11	Genomic location	mRNA splice variants		Number of cutaneous MCTs
			ENSCAFT00000049830.2	ENSCAFT00000003274.3	
25 bp ITD	No	47,178,595–47,178,619	2,458–2,482	1,692–1,716	1
30 bp ITD	No	47,178,583–47,178,612	2,446–2,475	1,680– 1,709	1
36 bp ITD	No	47,178,579–47,178,614	2,442–2,477	1,676–1,711	1
39 bp ITD	No	47,178,581–47,178,619	2,444–2,482	1,678–1,716	1
39 bp ITD	No	47,178,599–47,178,637	2,462–2,500	1,696–1,734	1
42 bp ITD	No	47,178,581–47,178,622	2,444–2,485	1,678–1,719	1
42 bp ITD	No	47,178,582–47,178,623	2,445–2,486	1,679–1,720	1
45 bp ITD	No	47,178,580–47,178,624	2,443–2,487	1,677–1,721	3
48 bp ITD	Yes	47,178,592–47,178,639	2,455–2,502	1,689–1,736	2
48 bp ITD	Yes	47,178,594–47,178,641	2,457–2,502	1,691–1,736	2
48 bp ITD	Yes	47,178,595–47,178,642	2,458–2,502	1,692–1,736	1
48 bp ITD	Yes	47,178,598–47,178,645	2,461–2,502	1,695–1,736	1
51 bp ITD	No	47,178,581–47,178,631	2,444–2,494	1,678–1,728	1
51 bp ITD	No	47,178,584 - 47,178,634	2,447–2,497	1,681–1,731	1
51 bp ITD	No	47,178,588–47,178,638	2,451–2,501	1,685–1,735	1
54 bp ITD	No	47,178,575–47,178,628	2,438–2,491	1,672–1,725	1 subcutaneous MCT
54 bp ITD	No	47,178,583–47,178,636	2,446–2,499	1,680–1,733	1
54 bp ITD	Yes	47,178,590–47,178,643	2,453–2,502	1,687–1,736	1
60 bp ITD	No	47,178,577–47,178,636	2,440–2,499	1,674–1,733	1
60 bp ITD	Yes	47,178,583–47,178,642	2,446–2,502	1,680–1,736	1
60 bp ITD	Yes	47,178,585–47,178,644	2,448–2,502	1,682–1,736	1
Total					**25**
21 bp deletion	No	47,178,584–47,178,604	2,447–2,467	1,681–1,701	1
C>T variant		47,178,598	2,462	1,695	5 heterozygous C/T, 3 homozygous T/T

Appendix E. Canine *KIT* exon 11 wild-type DNA sequence and internal tandem duplication (ITD)-mutant sequences detected in 25 canine mast cell tumours (MCTs) as well the sequence of a 21 base pair (bp) deletion. The region of the sequence that is duplicated is underlined, the duplication is in red lettering, deleted nucleotides are in green and nucleotides that fall within the intron are represented by lowercase letters.

Wild-type *KIT* (127 bp)

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatga
aacaggggctttcc

21 bp deletion in *KIT*: MCT-ID M29, AmpliSeq Sample 111

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatga
aacaggggctttcc

25 bp ITD in *KIT*: MCT-ID M2

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCTTACGATCACAAATGGGAGTTTCCCAG
AAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

30 bp ITD in *KIT*: MCT-ID M151

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAAACACAGCTTCCTTACGATCACAAATGGGAGTTT
CCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

36 bp ITD in *KIT*: MCT-ID M72

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTACCCAACACAGCTTCCTTACGATCACAAATGG
GAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

39 bp ITD in *KIT*: MCT-ID M169

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCCCAACACAGCTTCCTTACGATCACAAA
TGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

39 bp ITD in *KIT*: MCT-ID M87, AmpliSeq Sample 14

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGATCACAA
ATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

42 bp ITD in *KIT*: MCT-ID M26

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGACCAACACAGCTTCCTTACGATCAC
AAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

42 bp ITD in *KIT*: MCT-ID A110, AmpliSeq Sample 82

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTATGATCACAAATGGGAGTTTCCCAGAACAACACAGCTTCCTTATGATCAC
AAATGGGAGTTTCCCAGAAACAGGCTTGAGCTTTGgtcagtatgaaacaggggctttcc

45 bp ITD in *KIT*: MCT-ID A93, M25 and U4

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACCCAACACAGCTTCCTTACGAT
CACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

48 bp ITD in *KIT*: MCT-ID A10 (AmpliSeq Sample 18) and A11

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTATGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcCTTATG
ATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

48 bp ITD in *KIT*: MCT-ID A40 and M127

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTATGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgTCCTTAT
GATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

48 bp ITD in *KIT*: MCT-ID M108, AmpliSeq Sample 60

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcaTTAC
GATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

48 bp ITD in *KIT*: MCT-ID A75, AmpliSeq Sample 24

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtaCG
ATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

51 bp ITD in *KIT*: MCT-ID A20, AmpliSeq Sample 7

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCACACAGCTTCCT
TACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

51 bp ITD in *KIT*: MCT-ID A91

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGAGCTTCCT
TACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

51 bp ITD in *KIT*: MCT-ID M8, AmpliSeq Sample 54

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGCCAACACAGCTTCCT
TACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

54 bp ITD in *KIT*: MCT-ID M82, subcutaneous MCT

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGATAGACCCAACACAGCT
TCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGTGAGCTTTGgtcagtatgaaacaggggctttcc

54 bp ITD in *KIT*: MCT-ID M37, AmpliSeq Sample 3

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTAACACAGCTT
CCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

54 bp ITD in *KIT*: MCT-ID A26

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagCTTC
CTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctttcc

60 bp ITD in *KIT*: MCT-ID A77, AmpliSeq Sample 6

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTAGACCCAAC
ACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacagggg
ctttcc

60 bp ITD in *KIT*: MCT-ID A9, AmpliSeq Sample 78

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcaAACA
CAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggct
ttcc

60 bp ITD in *KIT*: MCT-ID A79

AAACCCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAG
ACCCAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtCAC
AGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAAACAGGCTGAGCTTTGgtcagtatgaaacaggggctt
tcc

Appendix F. Calculating sample sequencing depth and sample size for AmpliSeq™ Ion Torrent™ Personal Genome Machine next-generation sequencing of canine mast cell tumour-extracted DNA.

Calculating sequencing depth

The binomial distribution equation estimates the probability of detecting a minimum number of variant alleles for a given known or estimated proportion of variant alleles (Jennings *et al.*, 2017). This equation is recommended for determining the minimum sequencing depth of samples when using next-generation sequencing technology. This equation was used to determine the minimum sequencing depth required for sequencing the AmpliSeq libraries using Ion Torrent technology (**Equation 1**).

$$P(x) = \frac{n!}{x!(n-x)!} p^x (1-p)^{n-x} \quad \textbf{(Equation 1)}$$

In **Equation 1**, $P(x)$ is the probability of x variant alleles at a given nucleotide position, x is the number of variant reads, n is the total number of reads, and p is the proportion of mutant alleles in the sample (Jennings *et al.,* 2017).

For the work in this thesis, the binomial distribution equation can be calculated in Excel (2013) was used to estimate the probability of detecting a minimum number of variants (**Equation 2A** and **2B**).

$$BINOM.DIST(number\_s, trials, probability\_s, cumulative) \quad \textbf{(Equation 2A)}$$

In **Equation 2A**, $number\_s$ is the number of successes (x in the binomial equation), $trials$ is the number of reads (n in the binomial equation), $probability\_s$ is the probability of success (p in the binomial equation), and $cumulative$ refers to whether the determination should be the exact probability for a given $x$and n ($FALSE$) or the cumulative probability ($TRUE$).

$$BINOM.DIST(4, 500, 0.024, TRUE) = 0.00710 \quad \textbf{(Equation 2B)}$$

Equation 2B shows the binomial distribution function substituted with parameters for my data. The number of trials (i.e. sequencing depth) was determined by trial and error through substitution a range of values beginning at a depth of 250 and increasing by

increments of 50 until reaching a depth of 600. The most appropriate value was chosen based on the probability of detecting the minimum number of variants (i.e. $number\_s$). The probability of detecting a variant allele ($probability\_s$) was calculated based on a 3% mutant allele frequency and an estimation that neoplastic mast cells contributed 80% of the sample DNA extracted from the tissue sections (3% x 80% = 2.4% = 0.024) (Rathi *et al.*, 2017).

These calculations indicate a 99.29% probability of detecting five or more variants at a sequencing depth of 500X (i.e. 1 minus 0.710% = 99.29%). A sequencing depth of 500X was used in subsequent calculations to determine sample size.

Determining sample size

To determine the maximum number of AmpliSeq DNA libraries per sequencing chip, the following features of the Ion Torrent sequencing chip and the size of the target sequence were considered:

- The Ion 316 sequencing chips (Ion 316™ Chip v2 BC, Thermo Fisher Scientific) produce 2–3 million amplicons per chip.
- The primers were designed to generate amplicons of approximately 150 bp. A small amplicon size was desired because the DNA was expected to be fragmented consequent from formalin fixation.
- The designed AmpliSeq panel aimed to sequence approximately 6,000 bp per sample. The entire *KIT* coding sequence is 3,090 bp and intron 11 is 285 bp, totalling a target size of 3,375. However, due to multiple primers required to amplify a single exon and the overlap of amplicons, a conservative sequencing size of 6,000 bp per sample was estimated.

These data were used to calculate the total number of samples per sequencing chip.

$$2 \times 10^6\ amplicons\ per\ chip \times 150\ bp\ amplicon = 3 \times 10^8\ bp\ per\ chip \qquad \textbf{(Equation 3)}$$

Firstly, **Equation 3** was used to calculate the total number of nucleotides a single Ion 316 sequencing chip could sequence. This was calculated based on the number of reads per chip multiplied by the average amplicon length.

$$6{,}000\ bp \times 500\ read\ depth = 3 \times 10^6 bp\ sample$$ (**Equation 4**)

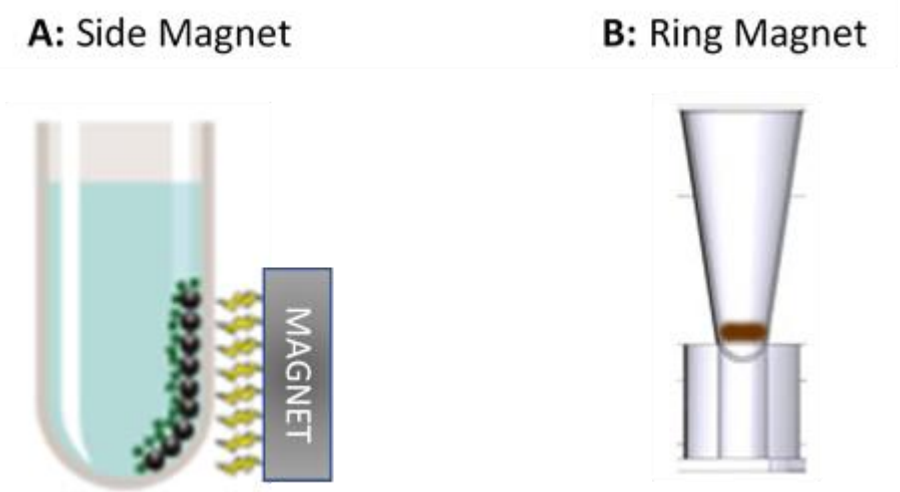
Next, **Equation 4** was used to estimate the amount of sequence data required for each sample by multiplying the size of each DNA library (6,000 bp) by the desired sequencing depth (500X).

$$\frac{3\times10^8 bp\ per\ chip}{3\times10^6 bp\ per\ sample} = 100\ samples\ per\ chip$$ (**Equation 5**)

Finally, the maximum number of AmpliSeq DNA libraries able to be sequenced per chip was calculated using **Equation 5** by dividing the total number of base pairs able to be sequenced on a single chip by the amount of sequence data required for each library.

Based on these calculations, a sequence depth of 500X for 100 AmpliSeq DNA libraries could be achieved on a single Ion 316 sequencing chip. After library construction, it was discovered that the Ion Torrent PGM sequencer requires two sequencing chips per run. Hence, instead of running a blank chip, the 100 DNA libraries were spread across two chips to achieve a greater sequencing depth. This depth consequently increases the sensitivity of this experiment, making it unlikely for a variant to be undetected, even if this variant were present at a low frequency.

Appendix G. Modifications to the published Ion AmpliSeq™ DNA Library Preparation protocol for AmpliSeq Library purification using a Ring Magnet as opposed to a Side Magnet.



The Ion AmpliSeq™ DNA Library Preparation Quick Guide protocol recommends using the DynaMag™-96 Side Magnet for bead capture. Using a Side Magnet facilitates washing of the beads because the magnet is positioned to the side of the microfuge tube and, hence, the beads accumulate to the side. To wash, the plate is rotated on the magnet causing the beads to move from one side of the tube to the other through the ethanol solution and towards the magnet. This effectively washes the beads.

In the current protocol, a Low Elution Magnet Plate was used because it was already available in the laboratory. The Low Elution Magnet Plate collects the beads in a ring near the base of the tube. Rotating the plate from side to side, as the protocol suggests, would not have washed the beads. Instead, the plate was removed from the magnet, ethanol was added, and the solution was mixed by gently pipetting the solution up and down five times. The plate was returned to the Ring Magnet and incubated for two minutes. This was conducted according to the manufacturer's recommendations which are detailed in the full instruction manual: Ion AmpliSeq™ Library Kit 2.0 User Guide, publication number MAN0006735, revision E.0.

Appendix H. *KIT* exon genomic locations for the KIT-202 splice variant (ENSCAFT00000003274.3).

***KIT* exon**	**Start position**	**End position**	**Exon length (bp)**	**Intron length (bp)**
1	47,144,083	47,144,120	37	326
2	47,144,446	47,144,716	270	2,784
3	47,147,500	47,147,782	282	1,084
4	47,148,866	47,149,003	137	4,579
5	47,153,582	47,153,754	172	3,905
6	47,157,659	47,157,849	190	3,077
7	47,160,926	47,161,042	116	14,015
8	47,175,057	47,175,172	115	1,749
9	47,176,921	47,177,103	182	1,208
10	47,178,311	47,178,418	107	93
11	47,178,511	47,178,638	127	285
12	47,178,923	47,179,028	105	86
13	47,179,114	47,179,225	111	1,190
14	47,180,415	47,180,566	151	1,882
15	47,182,448	47,182,540	92	477
16	47,183,017	47,183,145	128	1,104
17	47,184,249	47,184,372	123	3,490
18	47,187,862	47,187,974	112	113
19	47,188,087	47,188,187	100	353
20	47,188,540	47,188,646	106	1,056
21	47,189,702	47,190,029	327	-
			Total exonic length	3,090
			Total intronic length	42,856
			Total *KIT* length	45,946

Appendix I. Ion AmpliSeq primer design for amplification of *KIT* coding sequence and intron 11.

Exon	Pool	Amplicon ID	Forward primer	Reverse primer	Primer start position	Primer stop position	Designed amplicon length
1	1	AMPL2473456	CCAACCCATGTCATGGTCATGTA	CTGGGTCAAGTTTCCACTCTGA	47,144,026	47,144,146	121
2	2	AMPL2473737	CCTATTTTTATTGTAGAGCGCACAGAAGA	CTGATTTTGCTGGATGGATGGATG	47,144,367	47,144,488	122
	1	AMPL2473738	GCTCTTCTCAACCATCTGTGAGT	GTTGTGTGTGTTCTCATTCAGTTGAC	47,144,470	47,144,593	124
	2	AMPL2473739	CCCAGGATTTGTCAAGTGGACTTT	ATTTACCTCTGACAAACACATAAATGGAC	47,144,583	47,144,693	111
	1	AMPL2473740	ATTACACGTGCACCAACAGAGAT	CTCAGCCATCTCTATGTCATCCATAATTAA	47,144,683	47,144,770	88
3	2	AMPL2473460	GTAGTATTTGGATAAATTCTGTTGCTTGCA	TTTCCCATACAAGGGAAGGTCAAC	47,147,404	47,147,520	117
	1	AMPL2473461	CTGATTTTTGATATGCTTTAGATCCTGCAA	GAACGTCAAGTCCTTGGGAAGA	47,147,510	47,147,627	118
	2	AMPL2473462	CAGAAGTGACCAATTACTCCCTCA	GCAAGCAGAGCCGATGATACTC	47,147,609	47,147,694	86
	1	AMPL2473463	GCTGGCATCACGATCAGAAAC	CCCTCCATCAACAGATGCTCC	47,147,686	47,147,816	131
4	2	AMPL2473464	TTTGAGGAAAAGCAGTAGGTGACAA	ACACATCTTTTATAAAGCACATCACAGAGA	47,148,813	47,148,932	120
	1	AMPL2473741	CAAAACAAGCTCTCTCCTGAAGGA	CCCACAGAGAGAACAATGTATTGAGA	47,148,922	47,149,028	107
5	1	AMPL2473466	CTCTCTTTCCAAAACCAGCAGAC	CGTAACACATGAACACTCCAGAATCATTA	47,153,588	47,153,674	87
	2	AMPL2473467	GAACGTCAGGAAAAGTTGATTATCAGC	CAATTTCTTCTTTCACTACTGCCAGTAATT	47,153,664	47,153,779	116
	2	AMPL2473742	ACAGACTTGTCATGATGCTTTATTATTCTTACT	CTGTGCATTAGTCTGCTGGTTTTG	47,153,456	47,153,573	118
6	1	AMPL2473469	GGTGAAGCTGTGTTTTCTGTTGT	CCGGTTTGGGATATGCCTCATA	47,157,613	47,157,742	130
	2	AMPL2473745	TTGTAAATGATGGAGAGAATGTGGATCTG	CAGACTTGGGATAATCTTCCCATTTATCAG	47,157,734	47,157,800	67
	1	AMPL2473746	GCACCAGCAGTGGATCTATATGAA	GATCTGGTAAGCCAAGGGAAGT	47,157,790	47,157,891	102
7	2	AMPL2473471	GCATGTGTTTCCAGAGAGAAACTG	GGAATTGGACACTTGAAATGTGTAAGTG	47,160,852	47,160,974	123
	1	AMPL2473747	AGTGAACTTCATCTAACCAGATTAAAAGGG	AAGCACGAGCCACAACTTAAAATAATAAAA	47,160,964	47,161,076	113
8	2	AMPL2473473	CTCAGGGTCCTCTTCAAACTCAA	CTGCAACCACACACTGGAGCAT	47,174,969	47,175,098	130
	1	AMPL2473748	ACCAGAAATCCTGACTCATGAAAGTC	GTCCTTCCCTCGTGCACATTAA	47,175,088	47,175,197	110
9	2	AMPL2473476	GAGTTCCATCGATTATAGTGCCTTCA	TATGGCAGGCAGAGCCTAAACAT	47,177,026	47,177,145	120
	2	AMPL2473749	AAGGACATTTCTGATTATGACCTACTCTCT	ACGAGTTTTGCATCTGCACATC	47,176,831	47,176,946	116
	1	AMPL2473750	CTTCCCTCTAGATGTTCTGTCCCTA	TGTAAGCCCTACACTCGACTGT	47,176,936	47,177,036	101
10	2	AMPL2473564	CTGTTCACACCTTTGCTGATTGG	GATCATTATAGCACTTAGGGTAGAGAACAA	47,178,355	47,178,430	76
	1	AMPL2473751	GGGTCAGGTGGTTGTCATGAAG	ACGATAATGCACATCATTCCAGCT	47,178,263	47,178,366	104
11	1	AMPL2473479	CATTCATTTGTTCTCTACCCTAAGTGCT	GCTGTGTTGGGTCTATGTAAACATAATTGT	47,178,452	47,178,560	109
	2	AMPL2473753	GTACAGTGGAAGGTTGTTGAGGA	CATTGTTACACGTACACAAAAAGGTTACA	47,178,550	47,178,663	114
Intron 11	1	AMPL2473643	AACCTTTTTGTGTACGTGTAACAATGAC	GCTAAGGACATTTCTCTATAAGGTGCTTTA	47,178,695	47,178,785	91
	2	AMPL2473644	TTTGTTCTGTTCCAACTGAGACAATAAGTA	GGGTAATTTCAAAGTCAACAATCAAGGAAT	47,178,748	47,178,840	93

12	1	AMPL2473481	TGTTGACTTTGAAATTACCCAGATGCT	AGCATCTTAACGGCAACAGTCAT	47,178,878	47,179,000	123
	2	AMPL2473482	CCACTGCATATGGCCTGATTAAGT	CATTTTAGCAAGAAGCAAATTTGGCAAG	47,178,990	47,179,066	77
13	1	AMPL2473483	CCTCAACATTGGCTTGCCAAAT	CGCTCCAAGAAGATTCACAATATTCATATG	47,179,077	47,179,182	106
	2	AMPL2473484	CTAATGTCTGAGCTCAAAGTCTTGAGTT	CACTGTCGCAATGGTATTAAAATCTCC	47,179,172	47,179,276	105
14	1	AMPL2473754	GCAGGATCGTCTAGAGAGCTCA	CTTCTCCGTGATCTTCCTGCTTT	47,180,384	47,180,500	117
	2	AMPL2473755	TTTTTGCGAAGGAAACGTGATTCA	CCTTGATTGCAAACCCTTTCGA	47,180,490	47,180,612	123
15	2	AMPL2473574	GTTTCTTACGTTGTGCCAACCAA	AGTAATGTATTTCCCAGCAGCATGA	47,182,512	47,182,588	77
	1	AMPL2473756	CCCATTTTCCCATTTTATTCAGTCATAATT	TACCCACCTATTCTCGCAGATCT	47,182,421	47,182,524	104
16	1	AMPL2473757	CTTTGGTATTGTTGTTGTGCTGTCT	ACCTGGTAAGAAAAGCTCAGCA	47,182,962	47,183,089	128
	2	AMPL2473758	TGGAAGATGATGAGTTGGCTCTAGA	TCTAAAATGCGCCACTGGAGTTAG	47,183,079	47,183,197	119
17	1	AMPL2473759	CACCGAAGAGTTTCAAGGCAAA	GATATTTCTAGCAGCCAAGTCTCTGT	47,184,132	47,184,256	125
	2	AMPL2473760	GTTTTCTTTTTCTCTCCCACTCTCCTA	TCACGTTTCCTTTGACCACATAATTAGAAT	47,184,246	47,184,346	101
	1	AMPL2473761	ATTTGTGATTTTGGTCTAGCCAGAGA	AAAGGTAGGATTTGCATTAGGAAACTCATA	47,184,336	47,184,441	106
18	2	AMPL2473762	CTGGAGCGATGTCTGTAGGAAT	TGTACACACAGTTGAAAATGCTCTCA	47,187,784	47,187,891	108
	1	AMPL2473763	TGTTGTGCTTCCATTACAGGCT	AGAAGAGCTCCCACAGAAAAATCC	47,187,866	47,187,944	79
19	2	AMPL2473765	GTTGGGCGAGAGCATCTTCTTTA	CATCCGGAACCCTTCCTTGATC	47,188,025	47,188,136	112
	1	AMPL2473766	TGGGATGCCAGTCGATTCAAAG	CGGTCTTTGAAAAGGAAAATAAAAGGAGAA	47,188,126	47,188,222	97
20	2	AMPL2473494	GAGACTGTCGCATGACACCTTA	GGTGCTATCTGAAATCTGCTTCTCAATTA	47,188,495	47,188,611	117
	1	AMPL2473495	CTGAAAAGGCCGACGTTCAAGCA	GATAGAAGGGATGGGTAACAGAACC	47,188,601	47,188,709	109
21	1	AMPL2473496	GGGACTGGCATATTAACTGTGGA	GCTTCACACATCTTCGTGTACCA	47,189,689	47,189,811	123
	2	AMPL2473617	CTCTGTCCCTCCCTCTTTACGA	TCCCTTGCTCCAAGTCTGTATTATTTTT	47,189,969	47,190,078	110
	2	AMPL2473767	GGGTTTTGGCTGTGAAAGTCTT	GTTCGCGAGGTTGGAATAAATCTG	47,189,579	47,189,699	121
	2	AMPL2473768	CATTCCGTGCGGATCAATTCC	ATGCAAGCCGAAGGAGGACAGAATA	47,189,778	47,189,897	120
	1	AMPL2473769	CCCTGTTCTTTTGGTTCCTATACTGG	ATGACAAAAGTCACTGGCCACTAA	47,189,897	47,189,979	83
				Average amplicon length			108

Appendix J. Sample specific IonCode Barcode Adapters (Thermo Fisher Scientific) AmpliSeq Library Pool allocation, starting FFPE-extracted DNA concentration and final AmpliSeq library concentration of canine mast cell tumour (MCT) DNA samples. Sample concentrations were measured on the Qubit 3.0 Fluorometer using QuBit dsDNA HS Assay kit (Life Technologies).

Sample ID	Library sample	IonCode ID	Starting MCT DNA concentration (ng/µl)	Final AmpliSeq library concentration (ng/µl)	AmpliSeq library Pool
A24	Sample_1	IonCode_0101	19.3	1.68	1
A56	Sample_2	IonCode_0102	18	0.538	1
M37	Sample_3	IonCode_0103	20.4	2.02	1
U9	Sample_4	IonCode_0104	16.8	0.742	1
A77	Sample_6	IonCode_0106	18.1	0.828	1
A20	Sample_7	IonCode_0107	18	2.44	1
A52	Sample_8	IonCode_0108	21.6	1.35	1
M130	Sample_9	IonCode_0109	17.8	0.77	1
A48	Sample_10	IonCode_0110	24	1.64	1
A49	Sample_11	IonCode_0111	17.4	0.936	1
A38	Sample_12	IonCode_0112	20.8	0.758	1
A100	Sample_13	IonCode_0113	21.2	0.896	1
M87	Sample_14	IonCode_0114	22	1.3	1
TESTIS	Sample_15	IonCode_0115	5.64	0.508	1
A106	Sample_17	IonCode_0117	24	0.818	1
A10	Sample_18	IonCode_0118	23.2	0.94	1
A39	Sample_19	IonCode_0119	15.6	0.744	1
A29	Sample_20	IonCode_0120	22.8	0.538	1
A47	Sample_21	IonCode_0121	22	0.938	1
M44	Sample_22	IonCode_0122	16.4	0.75	1
A16	Sample_23	IonCode_0123	16.2	0.578	1
A75	Sample_24	IonCode_0124	15.6	0.174	2
M43	Sample_25	IonCode_0125	15.6	2.46	1
M103	Sample_33	IonCode_0133	15.4	0.456	2
A12	Sample_34	IonCode_0134	14.6	0.244	2
M149	Sample_35	IonCode_0135	14.2	0.238	2
M140	Sample_36	IonCode_0136	12.1	0.142	2
A105	Sample_37	IonCode_0137	12.9	0.105	2
M157	Sample_38	IonCode_0138	12.7	0.12	2
U25	Sample_40	IonCode_0140	11.2	0.162	2
A85	Sample_42	IonCode_0142	10.7	0.632	1
M165	Sample_43	IonCode_0143	10.6	0.244	2
M168	Sample_44	IonCode_0144	10.6	1.05	1
M94	Sample_45	IonCode_0145	8.76	1.01	1
A1	Sample_46	IonCode_0146	8.36	1.22	1
M7	Sample_47	IonCode_0147	8.28	1.59	1
M52	Sample_48	IonCode_0148	8.08	1.62	1
A80	Sample_50	IonCode_0150	21.6	1.84	1
A32	Sample_52	IonCode_0152	20.8	1.35	1
M75	Sample_53	IonCode_0153	20	2.78	1
M8	Sample_54	IonCode_0154	20	1.42	1
A30	Sample_55	IonCode_0155	19	0.676	1
M138	Sample_56	IonCode_0156	17.4	0.446	2
A31	Sample_57	IonCode_0157	22.3	0.544	1
M42	Sample_58	IonCode_0158	16.9	1.17	1
M9	Sample_59	IonCode_0159	14.4	0.738	1
M108	Sample_60	IonCode_0160	14	0.708	1
M68	Sample_61	IonCode_0161	7.12	0.39	2
A51	Sample_62	IonCode_0162	12.3	0.248	2
M105	Sample_63	IonCode_0163	9.88	0.43	2
M119	Sample_64	IonCode_0164	9.68	0.45	2

M160	Sample_65	IonCode_0165	9.4	0.238	2
A37	Sample_66	IonCode_0166	8.92	0.28	2
A22	Sample_67	IonCode_0167	7.92	0.204	2
A94	Sample_68	IonCode_0168	7.84	0.804	1
A58	Sample_69	IonCode_0169	7.6	2.22	1
M147	Sample_70	IonCode_0170	7.04	4.04	1
M133	Sample_71	IonCode_0171	7.3	0.141	2
M55	Sample_72	IonCode_0172	7.28	0.36	2
M172	Sample_73	IonCode_0173	5.84	0.282	2
M139	Sample_74	IonCode_0174	5.52	0.206	2
M41	Sample_75	IonCode_0175	13.4	1.9	1
M186	Sample_76	IonCode_0176	6.56	0.512	2
M48	Sample_77	IonCode_0177	6.12	0.374	2
A9	Sample_78	IonCode_0178	6.11	3.42	1
M115	Sample_79	IonCode_0179	6.08	0.304	2
M126	Sample_80	IonCode_0180	5.96	0.322	2
M84	Sample_81	IonCode_0181	3.29	0.171	2
A110	Sample_82	IonCode_0182	3.28	0.278	2
M61	Sample_83	IonCode_0183	5.84	0.696	1
M143	Sample_84	IonCode_0184	5.48	0.652	1
A95	Sample_85	IonCode_0185	3.24	0.204	2
M104	Sample_86	IonCode_0186	4.92	0.78	1
A107	Sample_87	IonCode_0187	4.84	0.384	2
M123	Sample_88	IonCode_0188	4.6	0.366	2
M92	Sample_89	IonCode_0189	4.28	0.352	2
A88	Sample_90	IonCode_0190	4.24	0.286	2
M109	Sample_91	IonCode_0191	4.16	0.36	2
M107	Sample_92	IonCode_0192	4.16	0.538	1
M113	Sample_93	IonCode_0193	4.08	0.576	1
M93	Sample_94	IonCode_0194	3.54	0.396	2
M117	Sample_95	IonCode_0195	3.52	0.512	2
A108	Sample_96	IonCode_0196	3.32	0.212	2
M150	Sample_99	IonCode_0203	2.89	0.23	2
M80	Sample_100	IonCode_0204	2.79	0.274	2
M11	Sample_101	IonCode_0205	2.63	0.37	2
M33	Sample_102	IonCode_0206	2.62	0.304	2
M178	Sample_103	IonCode_0207	2.54	0.528	2
M32	Sample_104	IonCode_0208	2.44	0.202	2
M77	Sample_105	IonCode_0209	2.36	0.32	2
A86	Sample_107	IonCode_0211	2.15	0.146	2
M27	Sample_108	IonCode_0212	1.78	0.157	2
M156	Sample_109	IonCode_0213	1.74	0.165	2
M116	Sample_110	IonCode_0214	1.64	0.208	2
M29	Sample_111	IonCode_0215	1.54	0.262	2
M31	Sample_112	IonCode_0216	1.4	0.346	2

Appendix K. Patient History Questionnaire sent to primary veterinary practices for the collection of canine mast cell tumour (MCT) patient clinical histories.

Clinic Questionnaire

Please fill in this questionnaire to the best of your ability for each dog with mast cell tumour from your clinic (names of dogs will have been sent to you via e-mail upon agreement to participate).

Name of dog:

Breed:

Birth Date:

Sex: M / F

Neuter status: neutered / Intact

Tumour size (mm):

Tumour anatomical location:

Surgical margins: complete / incomplete

Date of mast cell tumour diagnosis:

Date of mast cell tumour removal:

Lymph node involvement on day of surgery:

Patient history of mast cell tumours:

Post-surgical treatment

- Radiation therapy?
- Chemotherapy?
- Tyrosine kinase inhibitors (e.g. Palladia)?

Metastasis

- If yes, date of metastasis:
- And location:

Development of a second mast cell tumour

- If yes, date of diagnosis:
- And location:

Date of last examination/death:

Health status at last examination/cause of death:

Appendix L. Alignment of the Kit amino acid sequences for 21 species previously diagnosed with mast cell tumour. Sequences were aligned using Clustal Omega Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/; accessed 07/06/2019). The asterisk (*) indicates that the amino acid residue at that position is conserved among all species. A colon (:) denotes conservation between amino acid with strongly similar properties. A period (.) denotes conservation between amino acids with weakly similar properties. A hyphen (–) denotes no amino acid. The blue, orange, green and yellow highlighted sequences are exons 6, 8, 9 and 11, respectively. The unhighlighted regions are the introns or remaining exons. Reference sequence accession numbers are listed in Chapter 6, section 6.2.1**.

```
Axolotl                 ------------------------------------------------------------      0
Burrowing_owl           ------------------------------------------------------------      0
Chicken                 ------------------------------------------------------------      0
Mouse                   ------------------------------------------------------------      0
Ferret                  ------------------------------------------------------------      0
Human                   ------------------------------------------------------------      0
Rhesus_macaque          ------------------------------------------------------------      0
Cynomolgus_macaque      ------------------------------------------------------------      0
Baboon                  ------------------------------------------------------------      0
Horse                   ------------------------------------------------------------      0
Sheep                   ------------------------------------------------------------      0
Cow                     ------------------------------------------------------------      0
Goat                    ------------------------------------------------------------      0
Llama                   ------------------------------------------------------------      0
Pig                     ------------------------------------------------------------      0
Siberian_tiger          ------------------------------------------------------------      0
Cougar                  ------------------------------------------------------------      0
Cat                     ------------------------------------------------------------      0
Cheetah                 MKGLRGQELAARASVPSSSPPAQPPGISTSQEQKPDAARRERDPGGREGGEARRGAEKGR      60
Dog                     ------------------------------------------------------------      0
Pacific_walrus          ------------------------------------------------------------      0
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Axolotl                 ------------------------------------------------------------      0
Burrowing_owl           ----------------------------------------------------------MGMEVPV      7
Chicken                 ---------------------------------------------------------------ME      2
Mouse                   ---------------------------------------------------------------MR      2
Ferret                  ---------------------------------------------------------------MR      2
Human                   ---------------------------------------------------------------MR      2
Rhesus_macaque          ---------------------------------------------------------------MR      2
Cynomolgus_macaque      ---------------------------------------------------------------MR      2
Baboon                  ---------------------------------------------------------------MR      2
Horse                   ---------------------------------------------------------------MR      2
Sheep                   ---------------------------------------------------------------MR      2
Cow                     ---------------------------------------------------------------MR      2
Goat                    ---------------------------------------------------------------MR      2
Llama                   ---------------------------------------------------------------MR      2
Pig                     ---------------------------------------------------------------MR      2
Siberian_tiger          --------------MRL-------KLRKLKLK---EVKSSSSPTPNFTDGWVSVSQYHGD      36
Cougar                  ---------------------------------------------------------------MR      2
Cat                     ---------------------------------------------------------------MR      2
Cheetah                 GRRAGGGRWEEGPLLRARGSGGSALPRSVHLGESRNVERTSDPSA----------ATAMR      110
Dog                     ---------------------------------------------------------------MR      2
Pacific_walrus          -------------------------------------------------MEINIGRNALFR      12


Axolotl                 ----MDALLAPCLVLLLLNVHAA----------CSMPTITPEQDTLVVNSGEEMRFLCKD      46
Burrowing_owl           REGLGKPPGHSQ-LLNLG-H-------------NLGGSLPREESSLVVNKGEEFRLRCNE      52
Chicken                 GAHLAWELAHAV-LLLSL-I-------------PAGGSVPHEESSLVVNKGEELRLKCNE      47
Mouse                   GARGAWDLLC--VLLVLLRGQTATSQPSASPGEPSPPSIHPAQSELIVEAGDTLSLTCID      60
Ferret                  GASGAWDFL--GVLLLLFRVQTGSSQPSVSPGELSLPSIHPAKSELIVSAGDEIRLSCTD      60
Human                   GARGAWDFLC--VLLLLLRVQTGSSQPSVSPGEPSPPSIHPGKSDLIVRVGDEIRLLCTD      60
Rhesus_macaque          GARGAWDFLC--VLLFLLHVQTGSSQPSVSPGEPSPPSIHPAKSELIVRVGNEIRLLCID      60
Cynomolgus_macaque      GARGAWDFLC--VLLFLLHVQTGSSQPSVSPGEPSPPSIHPAKSELIVRVGNEIRLLCID      60
Baboon                  GARGAWDFLC--VLLFLLHVQTGSSQPSVSPGEPSPPSIHPAKSELIVRVGNEIRLLCID      60
Horse                   GARGAWDFLC--VLLLLFRVQTGSSQPSVSPGELSPPSIHPAKSELIVSVGDEIRLLCAD      60
Sheep                   GARGAWDFLF--VLLLLLLVQTGSSQPSVSPGELSLPSIHPAKSELIVSVGDEIRLSCTD      60
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Cow                 GARGAWDFLF--VLLLLLLVQTGSSQPSVSPGELSLPSIHPAKSELIVSVGDEIRLLCTD      60
Goat                GARGAWDFLF--VLLLLLLVQTGSSQPSVSPGELSLPSIHPAKSELIVSVGDEIRLLCTD      60
Llama               GARGAWDFLF--VLLLLLRVHTGLSQPSGSPGGLSPPSIHPAKSELIVSVGDEIRLLCTD      60
Pig                 GARRAWDFLF--VLQLLLRVQTGSSQPSVSPEELSPPSIHPAKSELIVSAGDEIRLFCTD      60
Siberian_tiger      GPYSEWKLDPAGVFSPEKAMASSSSQPSVSPGERSLPSIHPATSELIVSVGDEIRLLCTD      96
Cougar              GARGAWDFLC--VLLLLLRVQTGFSQPSASPGEWSLPSIHPATSELIVSVGDEIRLLCTD      60
Cat                 GARGAWDFLC--VLLLLLRVQTGSSQPSASPGEWSLPSIHPATSELIVSAGDEIRLLCTD      60
Cheetah             GARGAWDFLC--VLLLLLRVQTGSSQPSASPGEWSLPSIHPATSELIVSVGDEIRLLCTD      168
Dog                 GARGAWDFLCVLLLLLLLGVRTGSSQPSVSPGEPSLPSIHPAKSELIVSVGDELRLSCTD      62
Pacific_walrus      QGKRSYS--T-----RFLFFLAGSSQPSVSPGELSLPSIHPAKSELIVSVGDEIRLLCTD      65
                                                    ::    . *:*  *: : : * :

Axolotl             VSPVNWVLQKPGLSRKPKGVPSRPFQGNRNEQEVKVAKALTKDTGRYTCINKETSSNRSI      106
Burrowing_owl       DGPVTWNFQNSDPSAKAR---------SSNEKEWYTKNATVRDIGKYICKSKGI-IVTSF      102
Chicken             EGPVTWNFQNSDPSAKTR---------ISNEKEWHTKNATIRDIGRYECKSKGS-IVNSF      97
Mouse               PDFVRWTFKTYFNE--MV---------ENKKNEWIQEKAEATRTGTYTCSNSNG-LTSSI      108
Ferret              PGFVKWTFETL-GQ--LK---------EDAHNEWVTEKAEAANTGNYTCTNRDG-LSRSI      107
Human               PGFVKWTFEIL-DE--TN---------ENKQNEWITEKAEATNTGKYTCTNKHG-LSNSI      107
Rhesus_macaque      PGFVKWTFEIL-DE--TN---------ENKQNEWITEKAEATNTGKYTCTNKHG-LSSSI      107
Cynomolgus_macaque  PGFVKWTFEIL-DE--TN---------ENKQNEWITEKAEATNTGKYTCTNKHG-LSSSI      107
Baboon              PGFVKWTFEIL-DE--TN---------ENKQNEWITEKAEATNTGKYTCTNKHG-LSSSI      107
Horse               PGFVKWTFETW-GQ--LS---------ENTHKEWVTEKAEATNTGSYTCTNEGG-LSSSI      107
Sheep               PGFVKWTFEIL-GQ--LS---------EKTNPEWITEKAEATNTGNYTCTNKGG-LSSSI      107
Cow                 PGFVKWTFEIL-GQ--LS---------EKTNPEWITEKAEATNTGNYTCTNKGG-LSSSI      107
Goat                PGFVKWTFEIL-GQ--LS---------EKTNPEWITEKAEATNTGNYTCTNKGG-LSSSI      107
Llama               PGFVKWTFETL-GQ--LS---------ENRHAEWIMEKAEATNTGNYTCTNKGG-FSTST      107
Pig                 PGSVKWTFETL-GQ--LS---------ENTHAEWIVEKAEAMNTGNYTCTNEGG-LSSSI      107
Siberian_tiger      PGFVKWTFETL-GQ--SS---------EITHNEWITEKAEATNTGNYTCTNGGG-LSSSI      143
Cougar              PGFVKWTFETL-GQ--SS---------EITHNEWITEKAEATNTGNYTCTNGGG-LSSSI      107
Cat                 PGFVKWTFETL-GQ--SS---------EITHNEWITEKAEATNTGNYTCTNGGG-LSSSI      107
Cheetah             PGFVKWTFETL-GQ--SS---------EITHNEWITEKAEATNTGNYTCTNGGG-LSSSI      215
Dog                 PGFVKWTFETL-GQ--LN---------ENTHNEWITEKAEAGHTGNYTCTNRDG-LSRSI      109
Pacific_walrus      PGFVKWTFETP-GQ--LS---------ENTRNEWITEKAEATNTGNYTCTNRDG-LSRSI      112
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Axolotl                 YVFVKDSQTPFLASFSASVDGEEGRDVVVMCAASDPTISNFTLKKCDGTAPPEGMTFIPD      166
Burrowing_owl           YVFVKDPNVLFLVD--SLIYGKEDSDIVLVCPLTDPDVSNFTLRKCDGKRLPKNMTFIPD      160
Chicken                 YVFVKDPNVLFLVD--SLIYGKEDSDILLVCPLTDPDVLNFTLRKCDGKPLPKNMTFIPN      155
Mouse                   YVFVRDPAKLFLVG--LPLFGKEDSDALVRCPLTDPQVSNYSLIECDGKSLPTDLTFVPN      166
Ferret                  YVFVRDPARLFLVD--IRLYGKEDNDTLVRCPLTDPDVTNYSLTGCEGKPLPKDLTFVAD      165
Human                   YVFVRDPAKLFLVD--RSLYGKEDNDTLVRCPLTDPEVTNYSLKGCQGKPLPKDLRFIPD      165
Rhesus_macaque          YVFVRDPAKLFLVD--RSLYGKEDNDTLVRCPLTDPEVTSYSLKGCQGKPLPKDLRFVPD      165
Cynomolgus_macaque      YVFVRDPAKLFLVD--RSLYGKEDNDTLVRCPLTDPEVTSYSLKGCQGKPLPKDLRFVPD      165
Baboon                  YVFVRDPAKLFLVD--RSLYGKEDNDTLVRCPLTDPEVTSYSLKGCQGKPLPKDLRFVPD      165
Horse                   YVFVRDPAKLFLFD--PSLYGKEGSDTLVRCPLTDPEVTNYSLMACEGKSLPKDLTFVAD      165
Sheep                   YVFVRDPEKLFLID--LPLYGKEENDTLVRCPLTDPEVTNYSLTGCEGKPLPKDLTFVAD      165
Cow                     YVFVRDPEKLFLID--LPLYGKEENDTLVRCPLTDPEVTNYSLTGCEGKPLPKDLTFVAD      165
Goat                    YVFVRDPEKLFLID--LPLYGKEENDTLVRCPLTDPEVTNYSLTGCEGKPLPKDLTFVAD      165
Llama                   YVFVRDPEKLFLVF--LPLYGKEDSDTLVRCPLTDPEVTHYSLTGCEGKPLPKDLTFVAD      165
Pig                     YVFVRDPEKLFLVD--PPLYGKEDNDALVRCPLTDPEVTNYSLTGCEGKPLPKDLTFVAD      165
Siberian_tiger          YVFVRDPAKLFLVD--LPLYGKEDHDTLVRCPLTDPEVTNYSLRGCEGKPLPKDLTFVTD      201
Cougar                  YVFVRDPAKLFLVD--LPLYGKEDHDTLVRCPLTDPEVTNYSLRGCEGKPLPKDLTFVTD      165
Cat                     YVFVRDPAKLFLVD--LPLYGKEDHDTLVRCPLTDPEVTNYSLRGCEGKPLPKDLTFVTD      165
Cheetah                 YVFVRDPAKLFLVD--LPLYGKEDHDTLVRCPLTDPEVTNYSLRGCEGKPLPKDLTFVTD      273
Dog                     YVFVRDPAKLFLVD--LPLYGKEGNDTLVRCPLTDPEVTNYSLRGCEGKPLPKDLTFVAD      167
Pacific_walrus          YVFVRDPAQLFLVD--LRLYGKEDNDTLVRCPLTDPEVTNYSLKGCEGKPLPKDLTFVAD      170
                        ****:*    **      : *:*  * :: *  :** :  ::*  *:*.  * .: *: :

Axolotl                 LLQGITLKNVQRAFE-GCYLCSALQDGQLKTSQQYTLKVKPVHKVIPTIFLFQKQSTYLK      225
Burrowing_owl           PQKGIIIKNVQRSFK-GCYQCLARHNGVDKISEHIFLNVRPVHKTLPVITL-SKSYELLK      218
Chicken                 PQKGIIIKNVQRSFK-GCYQCLAKHNGVEKISEHIFLNVRPVHKALPVITL-SKSYELLK      213
Mouse                   PKAGITIKNVKRAYHRLCVRCAAQRDGTWLHSDKFTLKVRAAIKAIPVVSV-PETSHLLK      225
Ferret                  PKAGITIRNVKREYHRLCLHCSADQKGKSVLSTKFTLKVRAAIRAVPVVSV-SKASSLLR      224
Human                   PKAGIMIKSVKRAYHRLCLHCSVDQEGKSVLSEKFILKVRPAFKAVPVVSV-SKASYLLR      224
Rhesus_macaque          PKAGITIKSVKRAYHRLCLHCSADQEGKSVLSDKFILKVRPAFKAVPVVSV-SKASYLLR      224
Cynomolgus_macaque      PKAGITIKSVKRAYHRLCLHCSADQEGKSVLSDKFILKVRPAFKAVPVVSV-SKASYLLR      224
Baboon                  PKAGIMIKSVKRAYHRLCLHCSADQEGKSVLSDKFILKVRPAFKAVPVVSV-SKASYLLR      224
Horse                   PKAGITIRNVKREYHRLCLRCSADKDGKSVLSNKFTLKVRAAIRAVPVVSV-SKASYLLR      224
Sheep                   PKAGITIRNVKREYHRLCLHCSANQKGKSILSKKFTLKVRAAIKAVPVVSV-SKTSYLLR      224
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Cow                 PKAGITIRNVKREYHRLCLHCSANQRGKSMLSKKFTLKVRAAIKAVPVVSV-SKTSYLLR      224
Goat                PKAGITIRNVKREYHRLCLHCSANQKGKSMLSKKFTLKVRAAIKAVPVVSV-SKTSYLLR      224
Llama               PKAGITIRNVKREYHRLCLHCSANQGGKSVLSQKFTLKVRAAIRAVPLVSV-SKASFLLR      224
Pig                 PKAGITIKNVKREYHRLCLHCSANQGGKSVLSKKFTLKVRAAIRAVPVVAV-SKASYLLR      224
Siberian_tiger      PKAGITIRNVKREYHRLCLHCSADRKGKSVLSKKFTLKVRAAIRAVPVVSV-SKASHLLR      260
Cougar              PKAGITIRNVKREYHRLCLHCSADRKGKSVLSKKFTLKVRAAIRAVPVVSV-SKASHLLR      224
Cat                 PKAGITIRNVKREYHRLCLHCSADRKGKSVLSKKFTLKVRAAIRAVPVVSV-SKASHLLR      224
Cheetah             PKAGITIRNVKREYHRLCLHCSADRKGKSVLSKKFTLKVRAAIRAVPVVSV-SKASHLLR      332
Dog                 PKAGITIRNVKREYHRLCLHCSADQKGRTVLSKKFTLKVRAAIRAVPVVSV-SKTSSLLK      226
Pacific_walrus      PKAGITIRHVKREYHRLCLHCSADQKGKSVLSMKFTLKVRAAIRAVPVVSV-SKASSLLR      229
                       ** :: *:* :.  *  * . : *    * :  *:*: . :.:* : :  :    *:

Axolotl             QGEKFEICCTIRSQHGRSL----K----GSSEHAVASKSKYPSDYSYELNLTLSSSSVEV      277
Burrowing_owl       EGEEFEVTCIITDVDSSVQASWISQKN-----GIVTSKSRNLGDYGYERKLTLNIRSVGV      273
Chicken             EGEEFEVTCIITDVDSSVKASWISYKS-----AIVTSKSRNLGDYGYERKLTLNIRSVGV      268
Mouse               KGDTFTVVCTIKDVSTSVNSMWLKMNPQPQHIAQVKHNSWHRGDFNYERQETLTISSARV      285
Ferret              EGEEFSVMCLVKDVSSSVDSMWIKENS-QQTNAQTQSNSWHQGDFNFVRQEKLTIRSARV      283
Human               EGEEFTVTCTIKDVSSSVYSTWKRENS--QTKLQEKYNSWHHGDFNYERQATLTISSARV      282
Rhesus_macaque      EGEEFTVTCTIKDVSSSVYSTWKRENS--QTKLQEKYNSWHHGDFNYERQATLTISSARV      282
Cynomolgus_macaque  EGEEFTVTCTIKDVSSSVYSTWKRENS-QQTKLQEKYNSWHHGDFNYERQATLTISSARV      283
Baboon              EGEEFTVTCTIKDVSSSVYSTWKRENS-QQTKLQEKYNSWHHGDFNYERQATLTISSARV      283
Horse               EGEEFSVTCLIKDVSSSVDSMWIRENS-RT-KEQVKSSSSHQGDFNFVRQERLTISPARV      282
Sheep               EGEEFAVTCLIKDVSSSVDSMWIKENS-QQSKAQTKKNSWHQGDFSYLRQERLTISSARV      283
Cow                 EGEEFAVTCLIKDVSSSVDSMWIKENS-QQTKAQMKKNSWHQGDFSYLRQERLTISSARV      283
Goat                EGEEFAVTCLIKDVSSSVDSMWIKENS-QQSKAQTKKNSWHQGDFSYLRQERLTISSARV      283
Llama               EGEEFTVTCLIKDVSSSVDSMWIRENS-Q-TKAQVKRNSWHQGDFNFLRQERLTVSSARV      282
Pig                 EGEEFAVMCLIKDVSSSVDSMWIRENS-Q-TKAQVKRNSWHQGDFNFLRQEKLTISSARV      282
Siberian_tiger      EGEEFSVMCLIKDVSSSVDSMWIKENS-PQTNAQPQSNSWHQGDFNFVRQERLTISSARV      319
Cougar              EGEEFSVMCLIKDVSSSVDSMWIKENS-PQTNAQPQSNSWHQGDFNFVRQERLTISSARV      283
Cat                 EGEEFSVMCLIKDVSSSVDSMWIKENS-PQTNAQPQSNSWHQGDFNFVRQERLTISSARV      283
Cheetah             EGEEFSVMCLIKDVSSSVDSMWIKENS-PQTNAQPQSNSWHQGDFNFVRQERLTISSARV      391
Dog                 EGEAFSVMCFIKDVSSFVDSMWIKENS-QQTNAQTQSNSWHHGDFNFERQEKLIISSARV      285
Pacific_walrus      EGEEFSVMCSVKDVSSSVDSMWIKENS-QQTNAQTQSNSWHQGDFNFVRQERLTISSARV      288
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Axolotl                 NDWGIFTCQARNSVGTSHASILLNVIEAGFINLFPVENASIEVHAGESLVLRVVEYEAYP      337
Burrowing_owl           NDSGEFTCQAENPFGKTNATVTLKALAKGFVRLFATMNTTIDINAGQNGNL-TVEYEAYP      332
Chicken                 NDSGEFTCQAENPFGKTNATVTLKALAKGFVRLFATMNTTIDINAGQNGNL-TVEYEAYP      327
Mouse                   DDSGVFMCYANNTFGSANVTTTLKVVEKGFINISPVKNTTVFVTDGENVDL-VVEYEAYP      344
Ferret                  SDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMNTTIFVNDGENVDL-IVEYETYP      342
Human                   NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTVFVNDGENVDL-IVEYEAFP      341
Rhesus_macaque          NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTVFVNDGENVDL-IVEYEAFP      341
Cynomolgus_macaque      NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTVFVNDGENVDL-IVEYEAFP      342
Baboon                  NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTLFVNDGENVDL-IVEYEAFP      342
Horse                   NDSGVFMCYANNTFGSANVTTTLEVVDKGFINVFPMMNTTVFVNDGENVDL-IVEYESYP      341
Sheep                   NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMNTTVFVNDGENVDL-VVEYEAYP      342
Cow                     NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMNTTVFVNDGENVDL-VVEYEAYP      342
Goat                    NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMNTTVFVNDGENVDL-VVEYEAYP      342
Llama                   NDSGVFTCYANNTFGSANVTTTLEVVDKGFINIFPTMNTTVFVNDGENVDL-VVEYEAYP      341
Pig                     NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMNTTVFVNDGEDVDL-IVEYEAYP      341
Siberian_tiger          NDSGVFMCYANNTFGSANVTTTLEVVAKGFIHIFPMMNTTIFVNDGENVDL-IVEYEAYP      378
Cougar                  NDSGVFMCYANNTFGSANVTTTLEVVAKGFINIFPMMNTTIFVNDGENVDL-IVEYEAYP      342
Cat                     NDSGVFMCYANNTFGSANVTTTLEVVAKGFINIFPMMNTTIFVNDGENVDL-IVEYEAYP      342
Cheetah                 NDSGVFMCYANNTFGSANVTTTLEVVAKGFINIFPMMNTTIFVNDGENVDL-IVEYEAYP      450
Dog                     NDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMMSTTIFVNDGQNVDL-IVEYEAYP      344
Pacific_walrus          NDSGVFMCYANNTFGSANVTTTLEVVDRGFINIFPMMNTTIFVNDGENVDL-IVEYEAYP      347
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Axolotl                 KPEEEFWTYMNDTVLNTSDHFVRFREEGNSRYISELHLIRLKGAERGIYTFHVDNSDDSA      397
Burrowing_owl           KPKEEVWMYMNETLQNSSDHYVKFKTVGNNSYTSELHLTRLKGTEGGIYTFFVSNSDASS      392
Chicken                 KPKEEVWMYMNETLQNSSDHYVKFKTVGNNSYTSELHLTRLKGTEGGIYTFFVSNSDASS      387
Mouse                   KPEHQQWIYMNRTSANKGKDYVKSDNKSNIRYVNQLRLTRLKGTEGGTYTFLVSNSDASA      404
Ferret                  RPEHQQWIYMNRTFTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNADVNS      402
Human                   KPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNA      401
Rhesus_macaque          KPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNA      401
Cynomolgus_macaque      KPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNA      402
Baboon                  KPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNA      402
Horse                   KPEHQQWIYMNRTSTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVDS      401
Sheep                   KPEHRQWIYMNRTSTDKWDDYPKSENESNIRYVNELHLTRLKGTEGGTYTFHVSNSDVNS      402
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Cow                 KPVHRQWIYMNRTSTDKWDDYPKSENESNIRYVNELHLTRLKGTEGGTYTFHVSNSDVNS      402
Goat                KPEHRQWIYMNRTSTDKWDDYPKSENESNIRYVNELHLTRLKGTEGGTYTFHVSNSDVNS      402
Llama               KPEHRQWVYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVSS      401
Pig                 KPEHRQWIYMNRTATDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNADVNS      401
Siberian_tiger      KPEHQRWVYMNRTLTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNSDVNS      438
Cougar              KPEHQRWVYMNRTLTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNSDVNS      402
Cat                 KPEHQRWVYMNRTLTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNSDVNS      402
Cheetah             KPEHQRWVYMNRTLTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNSDVNS      510
Dog                 KPEHQQWIYMNRTFTDKWEDYPKSDNESNIRYVSELHLTRLKGNEGGTYTFQVSNSDVNS      404
Pacific_walrus      KPEHQQWIYMNRTFTDKWEDYPKSDNESNIRYVSELHLTRLKGDEGGTYTFQVSNSDVNS      407
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Axolotl             LMIFNILVKTKPEILSSERLDNGMLQCVAAGFPAPTIDWYFCPGAEQGCTDSPAIPAEDV      457
Burrowing_owl       SVTFNVYVKTKPEILTLDILSNGILQCVAAGFPAPTIYWYFCPGTEQRCFDSPTISPMDV      452
Chicken             SVTFNVYVKTKPEILTLDMLGNDILQCVATGFPAPTIYWYFCPGTEQRCLDSPTISPMDV      447
Mouse               SVTFNVYVNTKPEILTYDRLINGMLQCVAEGFPEPTIDWYFCTGAEQRCTTP--VSPVDV      462
Ferret              SVTFNVYVNTKPEILSHESLVNGMLQCVVSGFPEPTVDWYFCPGTEPRCSLP--VGPMDV      460
Human               AIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSAS--VLPVDV      459
Rhesus_macaque      SIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSAS--VLPVDV      459
Cynomolgus_macaque  SIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSAS--VLPVDV      460
Baboon              SIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSAS--VLPVDV      460
Horse               SVTFNVYVNTKPEILTRDRLMNGMLQCVAAGFPEPTIDWYFCPGTEQRCSIP--VGPVDV      459
Sheep               SVTFNVYVNTKPEILTHDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSVP--VGPVDV      460
Cow                 SVTFNVYVNTKPEILTHDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSVP--VGPVDV      460
Goat                SVTFNVNVNTKPEILTHDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSVP--VGPVDV      460
Llama               SMTFNVYVNTKPEILTHDRLANGMLQCVAAGFPEPTIDWYFCPGTEQRCSVP--IGPVDV      459
Pig                 SVTFNVYVNTKPEILTHDRLMNGMLQCVAAGFPEPTIDWYFCPGTEQRCSVP--VGPVDV      459
Siberian_tiger      SVTLNVYVNTKPEILTHESLVSGILQCLVAGFPEPTVDWYFCPGTEQRCPVP--VGPLDV      496
Cougar              SVTLNVYVNTKPEILTHESLVSGILQCLVAGFPEPTVDWYFCPGTEQRCPVP--VGPLDV      460
Cat                 SVTLNVYVNTKPEILTHESLVSGILQCLVAGFPEPTVDWYFCPGAEQRCPVP--VGPLDV      460
Cheetah             SVTLNVYVNTKPEILTHESLVSGILQCLVAGFPEPTVDWYFCPGTEQRCPVP--VGPLDV      568
Dog                 SVTFNVYVNTKPEILTHESLTNGMLQCVVAGFPEPAVGWYFCPGAEQRCSVP--IGPMDV      462
Pacific_walrus      SVTFNVYVNTKPEILTHESLVNGMLQCVVAGFPEPTVDWYFCPGTEQRCSVP--IGPMDV      465
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Axolotl                 KQRPENSSFASIGRIVVESTIDVSAFKKNGTVQCIASNTVESTSSVFSFAI----EEKIS      513
Burrowing_owl           KISYTNSSVPSFERILVESTINASMFRSTGTVCCEASSNGDRSSAFFNFAI----KEQIR      508
Chicken                 KVSYTNSSVPSFERILVESTVNASMFKSTGTICCEASSNGDKSSVFFNFAI----KEQIR      503
Mouse                   --QVQNVSVSPFGKLVVQSSIDSSVFRHNGTVECKASNDVGKSSAFFNFAFKGNNKEQIQ      520
Ferret                  --QLQNSSVSPSGKLVVQSSIDYSAFKHNGTVECRAYNNVGRSSAFFNFAFKGNSKEQIH      518
Human                   --QTLNSSGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKGNNKEQIH      517
Rhesus_macaque          --QTLNASGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKGNNKEQIH      517
Cynomolgus_macaque      --QTLNASGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKGNNKEQIH      518
Baboon                  --QTLNASGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKGNNKEQIH      518
Horse                   --KIQNSSVSPFGKLVVQSSIDYSAFKHNGTVECRAYNDVGKSSAFFNFAFK----EQIH      513
Sheep                   --QIQNSSVPPFGKLVVYSTIDDSTFKHNGTVECRAYNDVGKSSASFNFAFK----EQIH      514
Cow                     --QIQNSSVSPFGKLVVYSTIDDSTFKHNGTVECRAYNDVGKSSASFNFAFKGNSKEQIH      518
Goat                    --QIQNSSVSPFGKLVVYSTIDDSTFKHNGTVECRAYNDVGKSSASFNFAFKGNNKEQIH      518
Llama                   --QIQNSSVSPFGKLVIHSSIDYSAFKHNGTVECRAHNDVGKSSAFFNFAFK----EQIQ      513
Pig                     --QIQNSSVSPFGKLVIHSSIDYSAFKHNGTVECRAYNDVGKSSAFFNFAFK----EQIH      513
Siberian_tiger          --QMQNSSVSPSGKLVVQSSIDYSAFKHNGTVECRASNNVGKTSAFFNFAFK----EQMH      550
Cougar                  --QMQNSSVSPSGKLVVQSSIDYSAFKHNGTVECRASNNVGKTSAFFNFAFKGNSKEQMH      518
Cat                     --QMQNSSVSPSGKLVVQSSIDYSAFKHNGTVECRASNNVGKTSAFFNFAFKGNSKEQMH      518
Cheetah                 --QMQNSSVSPSGKLVVQSSIDYSAFKHNGTVECRASNNVGKTSAFFNFAFKGNSKEQMH      626
Dog                     --QMQNSSLSPSGKLVVQSSIDYSAFKHNGTVECRAYNNVGRSSAFFNFAFK----EQIH      516
Pacific_walrus          --QMQNSSVSPSGKLVVQSSIDYSTFKHNGTVECRAYNNVGRSSAFFNFAFKGNSKEQIH      523
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Axolotl                 THTLFTPLLIGFIVAAGLMCIAVAVLVYKYIQKPKYEDQWKVVDEINGNNYVYIDPTQLP      573
Burrowing_owl           THTLFTPLLIAFGVAAGLMCIIVMILVYVYLQKPKYEVQWKVVEEINGNNYVYIDPTQLP      568
Chicken                 THTLFTPLLIAFGVAAGLMCIIVMILVYIYLQKPKYEVQWKVVEEINGNNYVYIDPTQLP      563
Mouse                   AHTLFTPLLIGFVVAAGAMGIIVMVLTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      580
Ferret                  PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Human                   PHTLFTPLLIGFVIVAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      577
Rhesus_macaque          PHTLFTPLLIGFVIVAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      577
Cynomolgus_macaque      PHTLFTPLLIGFVIVAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Baboon                  PHTLFTPLLIGFVIVAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Horse                   PHTLFTPLLIGFVVAAGMMCVIVMVLTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      573
Sheep                   AHTLFTPLLIGFVIAAGLMCIFVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      574
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Cow                 AHTLFTPLLIGFVIAAGLMCIFVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Goat                AHTLFTPLLIGFVIAAGLMCIFVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Llama               AHTLFTPLLIGFVIAAGIMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      573
Pig                 AHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      573
Siberian_tiger      PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      610
Cougar              PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Cat                 PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      578
Cheetah             PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      686
Dog                 PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      576
Pacific_walrus      PHTLFTPLLIGFVIAAGMMCIIVMILTYKYLQKPMYEVQWKVVEEINGNNYVYIDPTQLP      583
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Axolotl             YDHKWEFPRDRLSFGKILGAGAFGRVVEATAYGLFKPDTTITVAVKMLKPSAHSTEREAL      633
Burrowing_owl       YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLFKSDAAMTVAVKMLKTSAHLTEREAL      628
Chicken             YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLFKSDAAMTVAVKMLKPSAHLTEREAL      623
Mouse               YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      640
Ferret              YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Human               YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      637
Rhesus_macaque      YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      637
Cynomolgus_macaque  YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Baboon              YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Horse               YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      633
Sheep               YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      634
Cow                 YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Goat                YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Llama               YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      633
Pig                 YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      633
Siberian_tiger      YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      670
Cougar              YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Cat                 YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      638
Cheetah             YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      746
Dog                 YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      636
Pacific_walrus      YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL      643
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Axolotl                 MSELKVLSYLGQHINIVNLLGACTVGGPTLVITEYCCYGDLLNYLRRKRDSFICPKIEDL      693
Burrowing_owl           MSELKVLSYLGNHINIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICPKHEEH      688
Chicken                 MSELKVLSYLGNHINIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICPKHEEH      683
Mouse                   MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFIFSKQEEQ      700
Ferret                  MSELKVLSYLGNHMNIVNLLGACTVGGKTVTKLTFTIFQII----K--RGEILI------      686
Human                   MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      697
Rhesus_macaque          MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      697
Cynomolgus_macaque      MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Baboon                  MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Horse                   MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKHDSFICSKQEDH      693
Sheep                   MSELKVLSYLGNHMNIVNLLGACTIGGPTLAITEYCCYGDLLNFLRRKRDSFICSKQEDH      694
Cow                     MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Goat                    MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Llama                   MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      693
Pig                     MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      693
Siberian_tiger          MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      730
Cougar                  MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Cat                     MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      698
Cheetah                 MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      806
Dog                     MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      696
Pacific_walrus          MSELKVLSYLGNHMNIVNLLGACTVGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDH      703
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Axolotl                 AEMALYKNLLNPKELLCN-TNSEYMDMKLSGQHLRSFVQVGEVPGSAEPCMTRMGYGSGA      752
Burrowing_owl           AETAVYENLLHQAEPAA-DVANEYMDMKPGVSYAVPPKA-D------------------      727
Chicken                 AEAAVYENLLHQAEPTA-DAVNEYMDMKPGVSYAVPPKA-D------------------      722
Mouse                   AEAALYKNLLHSTEPSCD-SSNEYMDMKPGVSYVVPTKT-D------------------      739
Ferret                  ------------------------------------------------------------      686
Human                   AEAALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      737
Rhesus_macaque          AESALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      737
Cynomolgus_macaque      AESALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      738
Baboon                  AETALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      738
Horse                   AEAALYKNLLHSKESSCNDSTNEYMDMKPGVSYVVPTKT-D------------------      733
Sheep                   AEVALYKNLLHSKESSCNDSTNEYMDMKPGVSYVVPTKAAD------------------      735
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Cow                 AEVALYKNLLHSKESSCNDSTNEYMDMKPGVSYVVPTKA-D------------------      738
Goat                AEVALYKNLLHSKESSCNDSTNEYMDMKPGVSYVVPTKAAD------------------      739
Llama               AEAALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      733
Pig                 AEAALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      733
Siberian_tiger      AEVALYKNLLQSKESSCNDSTNEYMDMKPGVSYVVPTKA-D------------------      770
Cougar              AEVALYKNLLQSKESSCNDSTNEYMDMKPGVSYVVPSKA-D------------------      738
Cat                 AEVALYKNLLQSKESSCNDSTNEYMDMKPGVSYVVPTKA-D------------------      738
Cheetah             AEVALYKNLLQSKESSCNDSTNEYMDMKPGVSYVVPTKA-D------------------      846
Dog                 GEVALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKA-D------------------      736
Pacific_walrus      AEVALYKNLLHSKESSCNDSTNEYMDMKPGVSYVVPTKA-D------------------      743


Axolotl             PCGLKSSPRRAPRRLRVRAAPTVAKSSERPSCLWP-WLFSLTLLAAICLLESPIE-PQTL      810
Burrowing_owl       ----KKRPAKSGSYTDQDVTLSMSEDDELALDVEDLLSFSYQVAKGMSFLASKNCIHRDL      783
Chicken             ----KKRPVKSGSYTDQDVTLSMLEDDELALDVEDLLSFSYQVAKGMSFLASKNCIHRDL      778
Mouse               ----KRRSARIDSYIERDVTPAIMEDDELALDLDDLLSFSYQVAKGMAFLASKNCIHRDL      795
Ferret              ------------------------------------------------------------      686
Human               ----KRRSVRIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      793
Rhesus_macaque      ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      793
Cynomolgus_macaque  ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      794
Baboon              ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      794
Horse               ----KRRAARIGSYIERDVAPSIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      789
Sheep               ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      791
Cow                 ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      794
Goat                ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      795
Llama               ----KRRPARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      789
Pig                 ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      789
Siberian_tiger      ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      826
Cougar              ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      794
Cat                 ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      794
Cheetah             ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      902
Dog                 ----KRRSARIGSYIERDVTPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      792
Pacific_walrus      ----KRRSARIGSYIERDATPAIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDL      799
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Axolotl                NLQEPPLMTGVLEP--------------NLKLRQAVRLYEDQLVGPESIANIGDVLFTGT      856
Burrowing_owl          AARNILLTHGRITKICDFGLARDIRNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      842
Chicken                AARNILLTHGRITKICDFGLARDIRNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      837
Mouse                  AARNILLTHGRITKICDFGLARDIRNDSNYVVKGNARL-PVKWMAPESIFSCVYTFESDV      854
Ferret                 ------------------------------------------------------------      686
Human                  AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      852
Rhesus_macaque         AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      852
Cynomolgus_macaque     AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      853
Baboon                 AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      853
Horse                  AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      848
Sheep                  AARNILLTHGRITKICDFGLARDTKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      850
Cow                    AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      853
Goat                   AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      854
Llama                  AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      848
Pig                    AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      848
Siberian_tiger         AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      885
Cougar                 AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      853
Cat                    AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      853
Cheetah                AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      961
Dog                    AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      851
Pacific_walrus         AARNILLTHGRITKICDFGLARDIKNDSNYVVKGNARL-PVKWMAPESIFNCVYTFESDV      858


Axolotl                ADGQILKIEDGNIHTVARLGKLPCGTREYEPTCGRPLGIRVGPNGTLFVSDAYMGIFEVN      916
Burrowing_owl          WSYGIL------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGYRMFS      881
Chicken                WSYGIL------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGYRMFS      876
Mouse                  WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMVS      893
Ferret                 ------------------------------------------------------------      686
Human                  WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      891
Rhesus_macaque         WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      891
Cynomolgus_macaque     WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      892
Baboon                 WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      892
Horse                  WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      887
Sheep                  WSYGIF------LWELFPLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      889
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Cow                 WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      892
Goat                WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      893
Llama               WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      887
Pig                 WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      887
Siberian_tiger      WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      924
Cougar              WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      892
Cat                 WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      892
Cheetah             WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      1000
Dog                 WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      890
Pacific_walrus      WSYGIF------LWELFSLGSSP--------YPGMPVDS-------KFYKMIKEGFRMLS      897


Axolotl             PVTGEAVMLIDSQKPIQGKKMSFVNDLVLNSGWEENVFHRFQL-----------------      959
Burrowing_owl       PECAPP-----------------EMYDIMKSCWDADPLQRPTFKQIVQLIEQQLSDNAPR      924
Chicken             PECSPP-----------------EMYDIMKSCWDADPLQRPTFKQIVQLIEQQLSDNAPR      919
Mouse               PEHAPA-----------------EMYDVMKTCWDADPLKRPTFKQVVQLIEKQISDSTKH      936
Ferret              ------------------------------------------------------------      686
Human               PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      934
Rhesus_macaque      PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      934
Cynomolgus_macaque  PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      935
Baboon              PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      935
Horse               PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      930
Sheep               PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      932
Cow                 PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      935
Goat                PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      936
Llama               PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      930
Pig                 PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISESTNH      930
Siberian_tiger      PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      967
Cougar              PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      935
Cat                 PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      935
Cheetah             PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      1043
Dog                 PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      933
Pacific_walrus      PEHAPA-----------------EMYDIMKTCWDADPLKRPTFKQIVQLIEKQISDSTNH      940
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Axolotl                 -------------------------------------------  959
Burrowing_owl           VYANFSS--PPSS---APDHSVRINSVGSSASSTQPLLVREDV  962
Chicken                 VYANFST--PPSTQGNATDHSVRINSVGSSASSTQPLLVREDV  960
Mouse                   IYSNLANCNPNPENPVVVDHSVRVNSVGSSASSTQPLLVHEDA  979
Ferret                  -------------------------------------------  686
Human                   IYSNLANCSPNRQKP-VVDHSVRINSVGSTASSSQPLLVHDDV  976
Rhesus_macaque          IYSNLANCSPNRQKP-VVDHSVRINSVGSTASSSQPLLVHDDV  976
Cynomolgus_macaque      IYSNLANCSPNRQKP-VVDHSVRINSVGSTASSSQPLLVHDDV  977
Baboon                  IYSNLANCSPNRQKP-VVDHSVRINSVGSTASSSQPLLVHDDV  977
Horse                   IYSNLANCSPRQENS-AVDHSVRINSVGSSASSTQPLLVHEDV  972
Sheep                   IYSNLANCSPHRENP-AVDHSVRINSVGSSASSTQPLLVHEDV  974
Cow                     IYSNLANCSPHRENP-AVDHSVRINSVGSSASSTQPLLVHEDV  977
Goat                    IYSNLANCSPHRENP-AVDHSVRINSVGSSASSTQPLLVHEDV  978
Llama                   IYSNLANGSPHRENP-VLDHSVRINSVGSSASSTQPLLVHEDV  972
Pig                     IYSNLANCSPHRENP-AVDHSVRINSVGSSASSTQPLLVHEDV  972
Siberian_tiger          IYSNLANCSPNRERPTAVDHCVRINSVGSSTSSTQPLLVHEDA  1010
Cougar                  IYSNLANCSPNRERPTAVDHCVRINSVGSSTSSTQPLLVHEDA  978
Cat                     IYSNLANCSPNRERPTAVDHCVRINSVGSSTSSTQPLLVHEDA  978
Cheetah                 IYSNLANCSPNRERPTAVDHCVRINSVGSSTSSTQPLLVHEDA  1086
Dog                     IYSNLANCSPNPERPV-VDHSVRINSVGSSASSTQPLLVHEDV  975
Pacific_walrus          IYSNLANCSPHQERPV-VDHSVRINSVGSSASSTQPLLVHEDV  982
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Appendix M. Canine long interspersed nuclear element (LINE)* and short interspersed nuclear element (SINE) accession numbers.

Minnick et al. (1992)
X57357.1

Bentolila et al. (1999)
AJ239530
AJ239531
AJ239532
AJ239533
AJ239534
AJ239535
AJ239537
AJ239538
AJ239539
AJ239541
AJ239542
AJ239543
AJ239544
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AJ239546
AJ239548
AJ239549
AJ239523
AJ239524*
AJ239525*
AJ239527*
AJ239528*
AJ239529*
AJ239522*

Kirkness *et al.* (2003)
AY339975*
AY339976*
AY339977*
AY339978*
AY339979*
AY339980*

Wang and Kirkness (2005)
AACN010000032
AACN010000041
AACN010000048
AACN010000171
AACN010000350
AACN010000711
AACN010000888
AACN010001026
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AACN010683013
AACN010685309
AACN010615139

AACN010008345	AACN010517091	AACN010552240	AACN010613395	AACN010604946
AACN010403899	AACN010520006	AACN010561657	AACN010597081	AACN010569879
AACN010403926	AACN010521063	AACN010563043	CE694886	CE719177
AACN010404060	AACN010522349	AACN010567230	CE695237	CE743721
AACN010404485	AACN010524646	AACN010567315	CE695838	CE752542
AACN010407788	AACN010525453	AACN010866926	CE697836	CE758969
AACN010409647	AACN010526828	AACN011032133	CE707376	CE762272
AACN010413324	AACN010873127	AACN011032471	CE123000	CE494965
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AACN010430666	AACN010882591	AACN011037828	CE137310	CE498897
AACN010434560	AACN010886674	AACN011067159	CE157185	CE498897
AACN010435994	AACN010889640	CE005952	CE158380	CE516621
AACN010437334	AACN010892008	CE016254	CE160133	CE524190
AACN010443236	AACN010892458	CE016707	CE176861	CE541328
AACN010446235	AACN010900730	CE041222	CE176911	CE545726
AACN010449371	AACN010900878	CE051313	CE181015	CE555654
AACN010463324	AACN010910112	CE057335	CE184726	CE578312
AACN010464056	AACN010914664	CE059563	CE201344	CE594363
AACN010464806	AACN010915927	CE061319	CE205266	CE595586
AACN010475839	AACN010920901	CE061577	CE227231	CE602613
AACN010483878	AACN010923113	CE067619	CE232551	CE606978
AACN010729548	AACN010926587	CE071774	CE234305	CE619096
AACN010747443	AACN010932185	CE079206	CE337313	CE620862
AACN010751616	AACN010948676	CE084427	CE344767	CE626511
AACN010764425	AACN010953599	CE108269	CE359789	CE632378
AACN010769860	AACN010954028	CE114214	CE360169	CE648617
AACN010773429	AACN010954554	CE122499	CE361432	CE655613
AACN010774301	AACN010958351	CE291529	CE374584	CE660400
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AACN010791906	AACN010980694	CE304737	CE386511	CE674906
AACN010792342	AACN010981731	CE311266	CE407132	CE679881
AACN010799124	AACN010991539	CE317453	CE413488	CE683273
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AACN010816480	AACN010704356	CE239560	CE438859	CE794709
AACN010818065	AACN010723076	CE255934	CE454004	CE796209
AACN010822764	AACN010728733	CE259533	CE455441	CE801347
AACN010823869	AACN010616134	CE284134	CE462846	CE802676
AACN010825803	AACN010630191	CE287176	CE470055	CE810849
AACN010828778	AACN010632750	CE479555	CE472427	CE850622
AACN010840197	AACN010569502	CE821184	CE822459	CE718891
AACN010844982	AACN010687948	CE006163	CE817227	CE839728
AACN010845439	AACN010691086	CE008814	CE837422	CE686385
AACN010850231	AACN011075541	CE010799	CE005161	

Appendix N. Canine endogenous retrovirus (ERV) accession numbers.

Tarlinton *et al.* (2013)
(Reference: CanFam2.0 Chr 13, NC_006595)
CanERV.34_chr13.fa_3024405_3025636

Jo *et al.* (2012)
(Reference: CanFam2.0 Chr 13, NC_006595)
HM460338
HM460339
HM460340
HM460341
Der13_2 CfERV β7 11845810 – 11851339
Der13_1 CfERV γ10 64185988 – 64181275
DerL 13_1 58741780 – 58742681
DerL 13_2 19257607 – 19258471

Besmer *et al.* (1986)
LC462187.1
MH116005.1
MH116004.1

Appendix O. Method for predicting canine, human and feline *KIT* genomic DNA secondary structure**.**

Mfold Web Server DNA Folding Form was used to predict the secondary structure of canine, human and feline *KIT* exon 11 – intron 11 DNA sequences. The software predicted four structures for the canine sequence. Between the four structures, the folding pattern of the exon 11 sequence was conserved and it was only the intron 11 sequence folding pattern that was unique between the four predictions. The first predicted structure was chosen as a representative for species comparison. Respectively, two and three structures for the human and feline *KIT* sequences were predicted. The human and feline structures which differed the most from the canine structure were chosen for comparison. The most unique structures were chosen to emphasise any major differences between the canine, human and feline sequences.

Appendix P. Alignment of *KIT* and *FLT3* gDNA, mRNA and amino acid sequences using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

	Canine *KIT*	**Human *FLT3***	**Sequence identity (%)**
Whole gene and amino acid sequences, Accession Number (position)			
gDNA	NC_006595.3 (47,108,442–47,190,935)	NC_000013.11 (28,003,274–28,100,587)	NSS
mRNA	NM_001003181.1	NM_004119.2	NSS
Amino acid	NP_001003181.1	NP_004110.2	Kit amino acids 188–938 Flt3 amino acids 232–958 37% identity
Exon 11 sequences only, Accession Number (position)			
gDNA	NC_006595.3 (47,178,511–47,178,638)	NC_000013.11 (28,036,043–28,035,935)	NSS
mRNA	NM_001003181.1 (1,673–1,801)	NM_004119.2 (1,392–1,500)	NSS
Amino acid	NP_001003181.1 (553–595)	NP_004110.2 (438–472)	NSS

NSS: no significant similarity.

Supplementary Material

Supplementary Material 1

Tamlin, V.S., Bottema, C. D. K., Peaston, A. E., Comparative aspects of mast cell neoplasia in animals and the role of *KIT* in prognosis and treatment. *Veterinary Medicine and Science.* [published online 24/10/2019]. doi: 10.1002/vms3.201.

Statement of Authorship

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REVIEW

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Comparative aspects of mast cell neoplasia in animals and the role of *KIT* in prognosis and treatment

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Abstract

Mast cell neoplasia clinical presentation and biological behaviour vary considerably across mammalian species, ranging from a solitary benign mass to an aggressive systemic malignancy. Mutations in the *KIT Proto-Oncogene Receptor Tyrosine Kinase (KIT)* gene are common molecular abnormalities involved in mast cell tumorigenesis. *KIT* mutations often occur in dog, cat and human neoplastic mast cells and result in altered Kit protein structure and function. In dogs, certain *KIT* mutations are associated with more malignant and lethal disease. In contrast, *KIT* mutations in feline and human mast cell neoplasms are not correlated with prognosis, but are of value in diagnosis and treatment planning in humans. *KIT* genetic abnormalities have not been well investigated in other species, although aberrant cytoplasmic Kit protein staining detected in neoplasms of the ferret, horse and cow resembles aberrant Kit staining patterns detected in neoplastic mast cells of dogs, cats and humans. Mutations within *KIT* are classified as either regulatory-type or enzymatic pocket-type mutations according to their location within the *KIT Proto-Oncogene*. Mutations within the enzymatic pocket domain confer tumour resistance to tyrosine kinase inhibitors (TKIs). Hence, knowledge of tumour *KIT* mutation status adds valuable information for optimizing patient treatment strategies. The use of TKIs in combination with conventional chemotherapeutics has opened a new treatment avenue for patients unresponsive to existing drugs. This review highlights the similarities and differences of mast cell neoplasia in mammals with a special focus on the involvement of *KIT* in the canine and feline forms in comparison to human mast cell neoplasia.

KEYWORDS
animals, humans, mast cells, mastocytosis, mutation, proto-oncogene

1 | MAST CELL FUNCTION AND MALIGNANCY

Mast cells are derived from pluripotent CD34+ haematopoietic progenitor cells and are normally found throughout the body in bone marrow, connective tissue, the skin, the gastrointestinal tract and the respiratory tract (Siebenhaar, Redegeld, Bischoff, Gibbs, & Maurer, 2018). Mast cells are normal components in both innate and adaptive immune responses to bacterial and parasitic infections and they are notorious for the role they play in allergic reactions (Siebenhaar et al., 2018). Mast cell activation in response to antigens causes mast cell degranulation and release of various cytokines and chemokines into the blood, thereby facilitating host immune responses (Siebenhaar et al., 2018).

Mast cell neoplasia is characterized by abnormal mast cell proliferation and accumulation (Bodemer et al., 2010; Siebenhaar et al.,

2018). Neoplastic mast cell masses can spontaneously degranulate, releasing bioactive molecules which can exert serious and potentially fatal effects, such as anaphylaxis, gastric and duodenal ulceration or perforation, glomerular disease and haemorrhage.

In dogs, mast cell tumours (MCTs) are a common skin cancer and have extremely varied clinical appearance and biological behaviour. In other species, such as cats, MCTs are less common and have less variable biological behaviour. In humans, mast cell neoplasia, termed mastocytosis, often presents as a systemic disease rather than a defined mass and varies from benign disease in infants to aggressive malignancies in adults (Kiszewski, Duran-Mckinster, Orozco-Covarrubias, Gutierrez-Castrellon, & Ruiz-Maldonado, 2004). Despite these distinct differences in behaviour and clinical manifestation, an underlying molecular abnormality shared by neoplastic mast cells in different species is mutation of the *KIT Proto-Oncogene Receptor Tyrosine Kinase* gene.

2 | ROLE OF *KIT* IN MAST CELL TUMOURIGENESIS

The *KIT* gene was first discovered in the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) (London et al., 1999). This acute transforming retrovirus causes fibrosarcomas in cats. The transforming activity of HZ4-FeSV is carried by the oncogene *V-KIT* which is thought to have arisen through truncation and transduction of feline *KIT* sequences in the feline leukaemia virus (Yarden et al., 1987).

The *KIT* gene encodes Kit, a transmembrane tyrosine kinase receptor (TKR) protein that is involved in the development, proliferation and function of mast cells, melanocytes, interstitial cells of Cajal and haematopoietic stem cells (Lennartsson, Jelacic, Linnekin, & Shivakrupa, 2005). Kit protein structure comprises an extracellular region including five immunoglobulin-like domains, a transmembrane domain and an intracellular region. The intracellular region includes the juxtamembrane domain and two tyrosine kinase domains separated by a kinase insert (Lennartsson et al., 2005; Ma et al., 1999). Kit protein activation by haematopoietic stem cell factor triggers distinct downstream signalling cascades within the cell inducing mast cell development, survival, proliferation, secretory function and chemotaxis (Lennartsson et al., 2005; Letard et al., 2008).

Mutations within *KIT* are common in several human cancers including mastocytoma, gastrointestinal stromal tumours, melanoma and acute myeloid leukaemia (Longley, Reguera, & Ma, 2001). Point mutations, initially discovered in HMC-1 cells derived from a human patient with mast cell leukaemia and then later in mouse and rat mastocytomas, suggested that mutations in *KIT* play a role in mast cell oncogenesis (Furitsu et al., 1993; Tsujimura et al., 1994, 1995). Comparison of the whole canine Kit protein sequence with the sequence from mice and humans shows 82% and 88% homology, respectively (London et al., 1999; Ma et al., 1999). High protein sequence conservation across species raised speculation about whether *KIT* mutations could be found also in MCTs of other species. Indeed, at least 51 unique gain-of-function mutations in the *KIT* gene have been identified in mast cell neoplasms of humans, cats and dogs (Downing, Chien, Kass, Moore, & London, 2002; Giantin et al., 2012; Haenisch, Nothen, & Molderings, 2012; Hahn et al., 2008; Isotani et al., 2010; Letard et al., 2008; London et al., 1999; Ma et al., 1999; Marconato et al., 2014; Nakano et al., 2014; Riva, Brizzola, Stefanello, Crema, & Turin, 2005; Takeuchi et al., 2013; Webster et al., 2006; Zemke, Yamini, & Yuzbasiyan-Gurkan, 2002). Studies also document over 80 other nucleotide substitutions and frameshift mutations in neoplastic mast cells from these species, although these have not been confirmed to be Kit activating. While some identified nucleotide substitutions are silent or species-specific, many result in non-conservative amino acid changes and hence, may contribute to mast cell malignancy (Giantin et al., 2012; Haenisch et al., 2012; Hahn et al., 2008; Isotani et al., 2006; Letard et al., 2008; Marconato et al., 2014; Nakano, Kobayashi, Bonkobara, & Takanosu, 2017; Sabattini et al., 2017; Takeuchi et al., 2013; Zemke et al., 2002).

Gain-of-function mutations within the *KIT* gene disrupt normal Kit protein function, leading to constitutive Kit activation in the absence of ligand binding (Furitsu et al., 1993; Letard et al., 2008; Nakano et al., 2017). These mutations can be classified into two groups according to their location within the gene: regulatory-type mutations or enzymatic pocket-type mutations (Longley et al., 2001).

In humans, cats and dogs, Kit protein regulatory regions commonly affected by mutations include the extracellular ligand-binding fifth immunoglobulin-like domain, encoded by exons 8 and 9, and the juxtamembrane domain, encoded by exon 11, that regulates inhibition of Kit activation (Lennartsson et al., 2005; Longley et al., 2001). *KIT* mutations occurring in exons 13–21, encoding the intracellular kinase domains of the Kit protein, are termed enzymatic pocket-type mutations.

2.1 | Tyrosine kinase inhibitors

Receptor tyrosine kinases, such as Kit, are candidates for molecular targeted therapy. Tyrosine kinase inhibitors (TKIs) are used in human and veterinary medicine to treat mast cell neoplasms harbouring *KIT* mutations. TKIs bind directly to the ATP-binding site in tyrosine kinase proteins, including Kit, blocking TKR autophosphorylation, preventing activation caused by regulatory-type mutations and thereby preventing initiation of downstream signalling cascades (London, 2009). Consequently, neoplastic mast cell proliferation and tumour growth are inhibited.

The change in Kit protein structure resulting from mutations in the enzymatic pocket domain leads to decreased affinity of TKIs for the binding site. Neoplastic mast cells harbouring this mutation type are resistant to most TKI therapeutics (Nakano et al., 2017; Verstovsek et al., 2006). Hence, the location of *KIT* mutations within the gene influences whether the tumour will be responsive or resistant to treatment with TKIs, and therefore, the mutation location is of prognostic importance.

3 | CANINE MCTS

In dogs (*Canis lupus familiaris*), cutaneous MCTs have been reported to occur in 335 per 100,000 animals and account for 10%–21% of all skin neoplasms (Leidinger, Freeman, Kirtz, Hooijberg, & Sick, 2014; Withrow, MacEwen, Vail, & Page, 2013). Canine prognosis and treatment options are affected by patient signalment, clinical signs, and tumour anatomical location, growth rate, size, gross appearance (e.g. ulceration), metastasis, post-surgical recurrence, clinical stage and tumour histological grade (Blackwood et al., 2012; Mullins et al., 2006). The presence of multiple lesions should be considered in prognosis but does not necessarily indicate more aggressive disease (Kiupel, Webster, Miller, & Kaneene, 2005; Murphy, Sparkes, Blunden, Brearley, & Smith, 2006). Unlike human patients, there is no unequivocal evidence of familial MCT inheritance in dogs, although boxers and other breeds of bulldog descent appear to be more predisposed to MCT development (Leidinger et al., 2014; Mochizuki, Motsinger-Reif, Bettini, Moroff, & Breen, 2017).

3.1 | Canine MCT prognosis

Histological grade is currently the single most powerful prognostic factor for dogs with cutaneous MCTs and has been described in depth (Table 1) (Blackwood et al., 2012; Kiupel & Camus, 2019; Kiupel et al., 2011; Patnaik, Ehler, & MacEwen, 1984). The two established grading schemes, Patnaik and Kiupel, are not applicable to subcutaneous, visceral or mucosal MCTs or MCTs of other species due to differences in tumour biology and histological features. Clinical staging according to the World Health Organisation (WHO) Clinical Staging System for canine MCTs is regarded by some as an important prognostic indicator, with non-metastatic lower clinical stages (stages 0 and I) having a better prognosis than higher stages, with an exception outlined below. Clinical stage II is defined as a single tumour with regional lymph node metastasis. Assessment of regional nodes is most accurately accomplished by node excision and histopathology (Ferrari et al., 2018; Weishaar, Thamm, Worley, & Kamstock, 2014). A worse outcome for dogs with clinical stage II than with clinical stage 0/I MCT has been documented (Krick, Billings, Shofer, Watanabe, & Sorenmo, 2009; Murphy et al., 2006; Weishaar et al., 2014). Furthermore, dogs with clinical stage II tumours derive a survival benefit from excision of the metastatic nodes (Marconato et al., 2018). Clinical stage III includes multiple dermal tumours or one large infiltrating tumour with or without regional lymph node involvement. In a retrospective study, the median survival time of eight dogs with stage II disease was 431 days, whereas the median survival time for 50 dogs with stage III or stage I disease was not reached (Murphy et al., 2006). In this study, most dogs included in stage III had multiple tumours without lymph node metastasis. Evidence that dogs with multiple tumours either synchronously or in series do not have a worse prognosis than dogs with single tumours indicates that the WHO staging system needs revision to improve prognostic acumen (Horta et al., 2018; Mullins et al., 2006; Murphy et al., 2006). Amendments to the current WHO clinical staging criterion have been proposed (Horta et al., 2018).

3.2 | Canine *KIT* mutations

Between 8% and 29% of canine MCTs carry a regulatory-type mutation in exon 8, 9 or 11 (Giantin et al., 2012; Hahn et al., 2008; Horta et al., 2018; Letard et al., 2008; Marconato et al., 2014; Mochizuki, Thomas, Moroff, & Breen, 2017). Exon 11 internal tandem duplications (ITDs) comprise 60%–74% of these mutations and are prevalent in 18% of tumours (Giantin et al., 2012; Hahn et al., 2008; Letard et al., 2008; Marconato et al., 2014; Mochizuki, Thomas, et al., 2017; Webster et al., 2006). *KIT* mutation frequency increases with increasing tumour histological grade and exon 11 ITDs are associated with decreased survival times and the increased chance of tumour recurrence and metastasis (Table 1) (Downing et al., 2002; Horta et al., 2018; Letard et al., 2008; Tamlin et al., 2017). Although less frequent, ITDs in exon 8 as well as insertions, deletions and nucleotide substitutions in exons 8, 9, 11, 14 and 17 are of functional significance (Giantin et al., 2012; Hahn et al., 2008; Letard et al., 2008; Mochizuki, Thomas, et al., 2017; Nakano et al., 2017; Zemke et al., 2002).

In one study, the homozygous deletion of the entire intron 11 of canine *KIT* was detected in 49% of MCT samples compared to 13% in control, non-cancerous tissue (Reguera, Ferrer, & Rabanal, 2002). The intron 11 deletion was correlated with higher grade, more aggressive MCTs. However, this large deletion has not been reported in any other molecular studies evaluating genomic DNA. The presence of a *KIT*-derived pseudogene was suspected because of the apparent intron 11 deletion. However, we aligned the canine *KIT* gene coding sequence (GenBank accession number AF044249, nucleotide bases 1737-1899) to the canine reference genome (CanFam3.1; GCF_000002285.3) using National Center for Biotechnology Information Basic Local Alignment Search Tool and found no alignments outside the annotated *KIT* gene, indicating that a *KIT* pseudogene is unlikely to exist (data not shown). Sample contamination with RNA may explain homozygous intron 11 deletion. The described primers produce a wild-type, genomic DNA amplicon of 448 base pairs and an RNA amplicon of 165 base pairs (Reguera et al., 2002). Differing concentrations of contaminating RNA between MCT samples could lead to favourable PCR amplification of the smaller PCR product, resulting in apparent homozygous intron 11 deletion in some samples. This is speculative and further research is necessary for clarification.

3.3 | Other genetic mechanisms implicated in canine mast cell malignancy

Cellular proliferative markers AgNOR, Ki-67, PCNA and Kit protein have been investigated by immunohistochemical evalutation for their use as independent or supplementary prognostic markers to histological grade. The extent of their use in prognosis has been well summarized previously (Blackwood et al., 2012; Kiupel & Camus, 2019).

TABLE 1 Canine mast cell tumour prognosis, incidence and *KIT* mutation prevalence according to the Patnaik and Kiupel histological grading schemes

Histological grade	Histological grade frequency[a]	*KIT* mutation prevalence[a,b]	Prognosis
Patnaik grade I	8%–53%	0%–6%	• Generally benign with low chance of recurrence. • Surgery alone is often curative. • Predictably good long-term prognosis. • 12-month survival probability up to 100%.
Patnaik grade II	59%–76%	6%–35%	• Unpredictable biological behaviour. • Unclear treatment recommendations. • Discordance among pathologist when grading. • 12-month survival probability 87%–92%.
Patnaik grade III	5%–26%	33%–71%	• Biologically aggressive with high probability of local recurrence and metastasis. • Requires aggressive therapeutic management. • Poor long-term prognosis. • 12-month survival probability 16%–46%.
Kiupel low-grade	59%–89%	4%–13%	• Good long-term prognosis. • Median survival times of more than 2 years. • 12-month survival probability 95%.
Kiupel high-grade	11%–41%	14%–52%	• Increased chance of metastasis or recurrence. • Poor long-term prognosis. • Median survival times of less than 4 months. • 12-month survival probability 24%.
References	Giantin et al. (2012), Leidinger et al. (2014), Mochizuki, Motsinger-Reif, et al. (2017), Patnaik et al. (1984), Kiupel et al. (2011), Mochizuki, Thomas, et al. (2017), Tamlin et al. (2017), Sabattini, Scarpa, Berlato, and Bettini (2015), Murphy, Sparkes, Smith, Blunden, and Brearley (2004)	Downing et al. (2002), Giantin et al. (2012), Webster et al. (2006), Zemke et al. (2002), Mochizuki, Thomas, et al. (2017), Tamlin et al. (2017)	Kiupel et al. (2011), Sabattini et al. (2015), Murphy et al. (2004)

[a]Studies with less than 49 tumour samples were omitted from this Table as deemed too small to be an accurate representation of true grade incidence/mutation prevalence according to the sample size calculation equation described by Naing, Winn, and Rusli (2006). A 95% level of confidence and precision of 10% was used in the calculations based on the prevalence determined by Webster et al. (2006).
[b]Prevalence includes mutations in *KIT* exons 8, 9 and 11.

Some genes known to be mutated in human mast cell diseases have been evaluated in MCTs from dogs (Zorzan, Hanssens, Giantin, Dacasto, & Dubreuil, 2015). Mutations in the *TET2* gene are present in up to 27% of all human systemic mastocytosis (SM) patients but only 2.7% of canine MCTs harbour *TET2* genetic aberrations (Damaj et al., 2014; Zorzan et al., 2015). No other genes known to be frequently mutated in human SM cases have been shown to be mutated in canine MCT samples (Zorzan et al., 2015).

However, single-nucleotide substitutions in the *GNAI2* gene and in various hyaluronidase genes on CFA14 and CFA20 are significantly associated with increased risk of MCT development in golden retriever dogs (Arendt et al., 2015). The *GNAI2* and hyaluronidase genes are involved in cellular signalling and cancer metastasis, respectively, and are associated with various types of human cancers. The exact involvement of these genes in canine cancers, and more specifically in canine MCT pathogenesis, has not been investigated.

Transcriptomic and proteomic analyses comparing low-grade MCTs with good patient prognosis and high-grade tumours with poor patient prognosis have identified a variety of differentially expressed genes and proteins potentially involved in mast cell aetiology and pathogenesis in dogs (Giantin et al., 2016; Schlieben et al., 2012). The roles of these various genes and proteins have not been further evaluated.

Global DNA methylation is a known heritable epigenetic modulator of gene expression. Hypomethylation of proto-oncogenes up-regulates gene expression, potentially favouring carcinogenesis and a more aggressive tumour type. DNA hypomethylation predominates in poorly differentiated, high-grade canine MCTs and may represent a novel target for epigenetic therapy (Morimoto et al., 2017).

3.4 | Treatment of canine MCTs

Clinical treatment of MCTs is based on tumour size, location, histological grade and evidence of metastasis (Blackwood et al., 2012).

Common MCT treatment recommendations and conventional therapies have been outlined elsewhere (Blackwood et al., 2012). The use of TKIs is the subject of ongoing research.

Toceranib, the most commonly used TKI in veterinary medicine, is licensed to treat non-resectable or recurrent grade II and III canine cutaneous MCTs in the USA (Anon, 2009), EU (Anon, 2019) and Australia (Agricultural & Veterinary Chemicals, 2011). Toceranib was originally developed as an antiangiogenic agent but was later found to possess potent anti-tumour characteristics by inhibiting Kit autophosphorylation and mast cell proliferation (Halsey et al., 2014). Early studies reported successful use of toceranib in treatment of recurrent MCTs which failed standard therapies, with tumours more likely to respond if harbouring a *KIT* exon 11 ITD (London et al., 2003, 2009). Canine overall survival time, time to tumour progression and the duration of tumour response to toceranib were not influenced by tumour ITD mutation status (London et al., 2003, 2009). Furthermore, in these studies not all animals possessing an ITD responded to treatment. It is possible that unresponsive animals had MCTs which harboured a secondary mutation in the enzymatic domain of *KIT*, inducing tumour TKI resistance (Halsey et al., 2014; Nakano et al., 2017). In a more recent study, an objective response rate (ORR: complete response or partial response) of 46% to toceranib therapy was observed (Weishaar et al., 2018), mimicking earlier observations (ORR = 37%–50%) (London et al., 2003, 2009). The difference in ORR between dogs with ITD-mutant and non-ITD mutant MCTs was not statistically analysed in this study, although, overall canine survival was not influenced by tumour ITD status (Weishaar et al., 2018). In the studies by London and colleagues, progression free survival times were significantly increased in dogs with non-mutant MCTs compared to dogs with ITD-mutant MCTs, however, this did not retain significance in a multivariable model (London et al., 2003, 2009). In the study by Weishaar and colleagues, a higher proportion of dogs with MCTs responded to treatment with toceranib than to treatment with vinblastine (46% vs. 30%, respectively), but this difference was not statistically significant (Weishaar et al., 2018). Given the greater expense and potentially more frequent and severe adverse events accompanying toceranib therapy, vinblastine is likely to remain a primary therapeutic option, regardless of tumour mutation status, with toceranib as a rescue therapy.

The TKI masitinib mesylate is not currently licensed for use in the USA (Anon, 2018) but is used in the EU (Anon, 2013) to treat non-resectable grade II and III cutaneous canine MCTs with confirmed mutated *KIT*. It is not licensed for use in Australia. Masitinib significantly improves overall survival of dogs with recurrent or non-resectable grade II or III MCTs harbouring a regulatory-type *KIT* mutation compared with results for placebo-treated dogs (Hahn et al., 2010, 2008). Time to tumour progression is also increased in masitinib-treated dogs compared to placebo-treated animals, regardless of tumour mutation status (Hahn et al., 2010, 2008). Tumour response to masitinib treatment was more pronounced in dogs with no prior chemotherapeutic treatment, suggesting that chemotherapy may select for growth of TKI-resistant neoplastic cells, limiting the effectiveness of masitinib treatment (Hahn et al., 2008). Complete tumour response, partial tumour response or stable disease at 6 months after masitinib treatment initiation has a high predictive value for 12- and 24-month survival, whereas tumour response at 6 weeks does not provide predictive value (Hahn et al., 2010). As the majority of studies evaluated patient response to TKIs for less than 6 months, the results must be interpreted carefully when estimating long-term health benefits for dogs receiving TKI therapeutics.

Tyrosine kinase inhibitor-resistant tumours harbouring enzymatic pocket-type mutations may still be susceptible to combination therapy involving the use of TKIs with conventional chemotherapeutic agents. A phase I/II study evaluating combination therapy of toceranib with lomustine in 41 dogs determined the maximally tolerated dose of the drugs in a pulse delivery setting, and concluded that the combined therapy was well tolerated and had value in the treatment of some dogs with high-grade unresectable or metastatic MCTs (Burton et al., 2015). Tumour *KIT* mutation status did not influence response to the treatment (Burton et al., 2015). Complete response for more than 1 year was observed in 2 of 10 dogs in a study using the same drug combination with a different administration schedule that was, however, not well tolerated by the dogs (Bavcar et al., 2017).

In a retrospective study of 40 dogs with grade II or III MCTs, toceranib in combination with vinblastine was used as adjuvant or neoadjuvant therapy with surgery or as medical palliative therapy alone. Overall, the treatment was reasonably well tolerated, and in 29 patients with measureable disease, initial response rates (complete response and partial response) of 90% were observed (Olsen, Thomson, O'Connell, & Wyatt, 2018).

Tyrosine kinase inhibitor-independent means of canine MCT therapy are currently under investigation for use against MCTs resistant to TKIs and conventional chemotherapeutic agents.

4 | FELINE MCTS

The clinical manifestations of mast cell neoplasia in domestic cats (*Felis catus*) include visceral (splenic, intestinal) disease as well as cutaneous MCT. Cutaneous MCTs are the second most common type of skin cancer in the cat, representing up to 21% of all cutaneous feline neoplasms in the USA (Withrow et al., 2013). Clinical understanding of feline MCT biological behaviour is relatively poor and most prognostic markers have a relatively weak correlation with survival. Mitotic index is probably the strongest prognostic indicator for cats with cutaneous MCT, with high index associated with worse clinical outcome although there is considerable variability (Blackwood, 2015; Sabattini et al., 2013). Ki-67 score and staining of aberrant cytoplasmic Kit protein localization are correlated with mitotic index but add no supplementary prognostic value to that achieved by mitotic index evaluation alone (Blackwood, 2015). The Patnaik and Kiupel histological grading systems provide no

prognostically useful information for feline cutaneous MCTs. Instead, tumours have been histologically classified based on cellular and nuclear morphology as either atypical or mastocytic type tumours (Blackwood, 2015). Mastocytic tumours can be further divided into well-differentiated or poorly differentiated tumours, both sub-types of which can present with pleomorphic cells (Blackwood, 2015). However, there are no official guidelines for feline cutaneous MCT histological classification and, hence, there are inconsistencies in the definitions of histological sub-types between published reports. These discrepancies in histological classifications and correlations with prognosis have been described previously (Blackwood, 2015). To overcome this, a recent study of 25 cats suggests a two-tier histological classification of feline cutaneous MCTs (Sabattini & Bettini, 2019). High-grade tumours are categorized by the presence of >5 mitotic figures per 10 high-power fields and at least two of the following three criteria: tumour diameter >1.5 cm, irregular nuclear shape and nucleolar prominence/chromatin clusters. According to this grading scheme, cats with high-grade tumours had a significantly shorter median overall survival (349 days) compared to cats with low-grade tumours (not reached, $p < .001$).

MCTs are the most common cause of splenic disease in cats. Affected cats are usually older than 10 or 11 years and there is no sex predilection (Evans, O'Brien, Allstadt, Gregor, & Sorenmo, 2018; Sabattini et al., 2017). The disease frequently involves multiple other viscera and bone marrow. There is no consensus on which factors significantly influence prognosis (Evans et al., 2018).

MCTs of the gastrointestinal tract are rare, but rank as the third most common intestinal tumour in cats, following lymphoma and adenocarcinoma, and have been previously viewed as an aggressive form of feline MCT disease (Barrett et al., 2018). Cats with poorly differentiated intestinal MCTs, fitting a description of feline intestinal sclerosing MCT, survive 2–30 days compared with 28–538 days for cats with well- or moderately differentiated tumours (Sabattini et al., 2016). Feline intestinal sclerosing MCT is seldom reported, but the short survival for cats with this variant is agreed, although there is disagreement as to whether the tumours are characterized by a low or high mitotic index (Halsey, Powers, & Kamstock, 2010; Sabattini et al., 2016). Intestinal sclerosing MCTs reportedly exhibit different histological appearance to gastrointestinal MCTs, and histological guidelines for diagnosis are yet to be developed (Halsey et al., 2010; Sabattini et al., 2016).

4.1 | Feline *KIT* mutations

In cats, 56%–68% of cutaneous and splenic MCTs harbour *KIT* mutations primarily in exons 8 and 9, but mutations in exons 6 and 11 also exist (Isotani et al., 2006, 2010; Sabattini et al., 2017, 2013). ITDs in exon 8 are the most prevalent and are Kit protein activating (Hadzijusufovic et al., 2009). Cases of tumours with two simultaneous *KIT* mutations have been recorded but significant correlations between mutation status and prognosis have not been documented (Isotani et al., 2010; Sabattini et al., 2017, 2013). Gain-of-function mutations in feline intestinal MCTs have not been documented (Sabattini et al., 2016).

4.2 | Treatment of feline MCT

Treatment of feline MCTs has been reviewed recently.(Blackwood, 2015; Blackwood et al., 2012) Surgical excision is standard of care for cutaneous atypical MCTs, which are generally thought to be benign (Blackwood, 2015). Spontaneous tumour regression has been reported in younger cats, reminiscent of paediatric mastocytosis in humans (Chastain, Turk, & O'Brien, 1988; Wilcock, Yager, & Zink, 1986). However, over the last three decades there have been no additional published cases and, hence, the existence of the spontaneously regressing variant in cats is uncertain. Cats with pleomorphic poorly differentiated mastocytic MCTs are at risk of unfavourable outcome, hence, post-operative adjuvant radiation therapy and/or chemotherapy has been recommended for these cats (Blackwood, 2015).

Data using TKIs for treatment of MCTs in cats are scant, and prospective studies are lacking. An objective response was recorded in a cat with systemic and cutaneous MCT after treatment with imatinib (Isotani et al., 2006). A follow-up investigation demonstrated an objective response in five of eight cats with *KIT*-mutated MCTs and in one of two cats without *KIT* mutations (Isotani et al., 2010). Low animal numbers, previous therapeutic drug administration and short follow-up times limit the interpretation of this study. Retrospectively, toceranib phosphate was assessed as well tolerated by cats. Complete or partial responses were documented in 35 of 50 cats treated for cutaneous, visceral and gastrointestinal MCTs with toceranib alone or in combination with corticosteroids (Berger et al., 2017). Other TKIs, including midostaurin, nilotinib and dasatinib, show dose-dependent growth-inhibitory effects on exon 8 ITD *KIT* mutant neoplastic feline mast cells in vitro (Hadzijusufovic et al., 2009). Mutations in the enzymatic pocket domain of *KIT* have not been documented in feline MCTs, making TKIs an attractive therapeutic option.

Splenectomy is the standard therapy for cats with splenic mastocytosis and provides longer disease-free survival than chemotherapy alone (856 days vs. 342 days, respectively) (Evans et al., 2018). Cats with intestinal MCT historically carry a guarded prognosis and metastasis to mesenteric lymph nodes is common (Barrett et al., 2018; Morrice, Polton, & Beck, 2019; Sabattini et al., 2016). However, recent literature describes variable biologic behaviour of feline gastrointestinal MCT with the overall median survival time of 31 cats to be 531 days in one study (Barrett et al., 2018) and 35% of cats surviving more than 1-year post-diagnosis in a different study which included a case where no treatment or surgery was received (Sabattini et al., 2016).

5 | MAST CELL NEOPLASIA IN OTHER ANIMALS

Mast cell cancer is well documented in humans, cats and dogs but is also a frequent skin cancer in the ferret and has been documented in various other species including domesticated ungulates, non-human

primates, birds and reptiles (Table 2). As with cats, the histological grading schemes that are useful for canine cutaneous MCTs cannot be applied to MCTs of other animals due to interspecies differences in clinical and histological disease manifestation.

5.1 | Ferret (Mustela putorius furo)

Mast cell tumours represent up to 44% of all cutaneous and subcutaneous neoplasms in ferrets (Avallone et al., 2016). The tumours are typically benign and neither local recurrence after surgical excision nor metastatic disease has been reported (Vilalta et al., 2016). Histologically, most ferret MCTs consist of well-differentiated mast cells where mitotic figures are rare and, similar to cats, few eosinophils are present (Vilalta et al., 2016). In one study of 15 tumours from 10 ferrets, neither haematoxylin and eosin nor Toluidine blue staining detected mast cell granules in histologic sections (Vilalta et al., 2016). However, in cytologic preparations of 12 tumours stained with Toluidine blue or Wright's–Giemsa stains, metachromatic granules were visualized in all cases, but not in any case stained with Modified Wright's stain (Vilalta et al., 2016). The discrepancy in Toluidine blue staining between cytology and histology in ferret MCTs is confusing and could lead to misdiagnosis. In the same study, all tumours had either cytoplasmic or membrane immunostaining with Kit (Vilalta et al., 2016). As would be expected from the lack of reported tumour recurrence or metastasis, there was no relationship between Kit immunostaining and prognosis (Vilalta et al., 2016).

5.2 | Horse (Equus ferus caballus)

Mast cell tumours comprise 3.4% of all cutaneous equine tumours, at least in the Pacific Northwest of the USA (Valentine, 2006). Equine MCTs appear most frequently on the head as a single nodule; multiple lesions are not indicative of malignant disease and may occur in any anatomical area of the skin (Ressel, Ward, & Kipar, 2015). Arabian horses appear to be more at risk compared to other breeds but no sex predilection has been described (Mair & Krudewig, 2008; Ressel et al., 2015). Similar to MCTs in ferrets, equine MCTs follow a benign disease course and appear histologically well differentiated with a low mitotic rate. Aberrant cytoplasmic Kit staining is detected by immunohistochemistry in 15% of cases but is not correlated to malignant disease or a worse prognosis, albeit numbers were low (n = 9) (Ressel et al., 2015). Metastatic MCT behaviour and MCT-related death in horses are seldom documented and the necessity for treatment is questionable, although some owners opt for therapy for the cosmetic appearance of the horse. Surgery alone is curative in the majority of cases and other therapies including surgical laser ablation and intralesional injection with methylprednisone acetate appear to be effective, non-invasive treatment options (Mair & Krudewig, 2008).

5.3 | Cow (Bos taurus)

In cattle, MCTs comprise less than 1% of all bovine neoplasms (Hill, Langheinrich, & Kelley, 1991). Reports range from multiple MCTs randomly distributed over the entire body with or without organ involvement to single visceral lesions (Hill et al., 1991; Khodakaram-Tafti, Eshraghi, Geramizadeh, Shaterzadeh-Yazdi, & Taghipur-Bazargani, 2015; Perez et al., 1999). Published data predominantly describe MCTs affecting Holstein cattle, however, statistical evidence of breed predisposition does not exist (Hill et al., 1991; Khodakaram-Tafti et al., 2015; Perez et al., 1999). Curiously, neoplastic mast cells appear well differentiated in cattle, even in cases with metastasis (Hill et al., 1991). Whether the tumours progress to lethal disease is unknown because individuals are often euthanized due to poor carcass quality. The most recent record of a bovine MCT was of a 4-year-old female Holstein with multiple cutaneous lesions (Khodakaram-Tafti et al., 2015). The authors were able to demonstrate Kit protein immunohistochemistry in a cow for the first time (Khodakaram-Tafti et al., 2015).

5.4 | Pig (Sus scrofa domesticus)

Mast cell neoplasia in pigs is rarely reported and can range from well differentiated, benign lesions to malignant and metastatic cancer (Bundza & Dukes, 1982; Newman & Rohrbach, 2012). In one study, multiple cutaneous and visceral MCTs were reported in three pigs, two of which were simultaneously diagnosed with eperythrozoonosis (Bundza & Dukes, 1982). In a recent case report, staining with Toluidine Blue or Giemsa was inconclusive and the diagnosis of MCT was based on positive Kit and tryptase immunohistochemistry (Williams, Annetti, & Nagy, 2018). Rare cases of mast cell leukaemia in miniature pigs have been reported with a poor outcome (Sipos, Hirschberger, Breuer, Zenker, & Elicker, 2010).

5.5 | Other species

Mast cell neoplasia is reported in a variety of species and ranges from solitary, benign tumours to malignant, systemic disease. Case reports of visceral MCT have been documented in a number of the big cat species, perhaps resembling the disease in the domestic cat and reflecting the close genetic relation between these species (Table 2).

Mast cell tumour is occasionally reported in non-human primates (Table 2). Three macaques and a baboon were observed with benign MCT with similar histological appearance to that of indolent SM in humans (Colgin & Moeller, 1996; Jones, MacKenzie, & Robinson, 1974; Seibold & Wolf, 1973; Zoller & Kaspareit, 2010). A fourth macaque harboured subcutaneous MCT metastatic to the local lymph nodes and internal organs (Tsugo et al., 2017). Immunohistochemical staining of membranous Kit protein was comparable between humans and this macaque case (Tsugo et al., 2017).

6 | MASTOCYTOSIS IN HUMANS

Similar to the disease in dogs and cats, mastocytosis in humans can range from a spontaneously regressing skin lesion to a highly

TABLE 2 Documented cases of vertebrates infrequently diagnosed with mast cell neoplasia

Species	Age (years)	Sex	MCT Type	Affected organs	Outcome
Big cats					
Cheetah (*Acinonyx jubatus*)	13	F	Visceral (spleen)	Metastatic (larynx)	Non-MCT-related death (Owston, Ramsay, & Rotstein, 2008)
Cougar (*Puma concolor*)	9 months	M	Visceral	Stomach	Alive at end of study (Martin, Lewis, Lin, & Jacobson, 1985)
Indian lion (*Panthera leo*)	16	F	Cutaneous	Multiple skin tumours (>20)	Euthanized (Stolte & Welle, 1995)
Jaguar (*Panthera onca*)	26	F	Visceral (jejunum)	Metastatic (liver, kidneys)	Died during anaesthesia (Castro, Werther, Godoy, Borges, & Alessi, 2003)
Tiger (*Panthera tigris*)	6	M	Visceral (spleen)	Metastatic (liver, lymphoid, kidney, pulmonary organs)	Died during anaesthesia (Graille, Huyghe, & Nicolier, 2013)
	14	M	Visceral	Metastatic (LN, lung, liver)	Euthanized due to other causes (Owston et al., 2008)
Miscellaneous mammals					
African hedgehog (*Atelerix albiventris*)	~1	F	Subcutaneous	Metastatic (lymph node)	Euthanized (Raymond, White, & Janovitz, 1997)
	>1	F	Cutaneous	Confined to skin	U (Raymond & Garner, 2001)
	>1	U	Cutaneous	Confined to skin	U (Raymond & Garner, 2001)
	3	U	Cutaneous	Metastatic (lymph)	U (Raymond & Garner, 2001)
Llama (*Lama glama*)	9	F	Cutaneous	Multiple cutaneous tumours	Alive at end of study (Lin, Hamberg, Pentecost, Wellman, & Stromberg, 2010)
Nubian goat (*Capra aegagrus hircus*)	4	F	Systemic mastocytosis	Heart, lung, liver, spleen, lymph, bone marrow	MCT-related death (Khan, Sagartz, Koenig, & Tanaka, 1995)
	6 weeks	F	Cutaneous (ear)	Confined to skin	Alive at end of study (Allison & Fritz, 2001)
Pacific Walrus (*Odobenus rosmarus divergens*)	>15	F	Visceral (lung)	Non-metastatic	Discovered at slaughter (Seguel, Stimmelmayr, Howerth, & Gottdenker, 2016)
Richardson's ground squirrel (*Spermophilus richardsonii*)	4	F	Cutaneous	Metastatic (lymph)	U (He et al., 2009)
Sheep (*Ovis aries*)	U	U	Visceral (liver)	Metastasis (lymph)	Discovered at slaughter (Johnstone, 1972)
	U	U	Visceral (liver)	Metastasis (hepatic)	Discovered at slaughter (Johnstone, 1972)
Non-human primates					
Baboon (*Papio sp.*)	Young	U	Cutaneous (neck)	Confined to skin	Discovered at necropsy (Jones et al., 1974)
Cynomolgus macaque (*Macaca fascicularis*)	3	M	Systemic mastocytosis	Liver, caecum	Non-MCT-related euthanasia (Zoller & Kaspareit, 2010)
Japanese macaque (*Macaca fuscata*)	19	F	Subcutaneous	Metastatic (lymph, kidney, peritoneum, mammary lobule)	MCT-related death (Tsugo et al., 2017)

(Continues)

TABLE 2 (Continued)

Species	Age (years)	Sex	MCT Type	Affected organs	Outcome
Rhesus macaque (*Macaca mulatta*)	7	M	Cutaneous (thorax)	Confined to skin	Alive at end of study (Colgin & Moeller, 1996)
	Adult	F	Subcutaneous (thigh)	-	U (Seibold & Wolf, 1973)
Birds					
Burrowing owl (*Speotyto cunicularia*)	U	U	Cutaneous (oral)	Confined to skin	Released, lost to follow-up (Schmidt & Okimoto, 1992)
Chicken (*Gallus gallus domesticus*)	5	F	Cutaneous (multiple)	Metastasis (lung)	Discovered at slaughter (Hafner & Latimer, 1997)
	1.5	M	Cutaneous (eyelid)	Confined to skin	Alive at end of study (Patnaik & Mohanty, 1970)
	Adult	F	Multiple, cutaneous	Confined to skin	Discovered at slaughter (Hall, Mosier, & Degraw, 1994)
Great horned owl (*Bubo virginianus*)	Adult	M	Cutaneous	Multiple (eye, ear)	Dead (Swayne & Weisbrode, 1990)
Lovebird (*Agapornis personata*)	12	F	Cutaneous	Metastatic (kidney, liver, spleen, periovarian, bone marrow)	MCT-related death (Dallwig, Whittington, Terio, & Barger, 2012)
Pueo (*Asio flammeus sandwichensis*)	U	U	Cutaneous (upper eyelid)	Confined to skin	MCT-related euthanasia (Schmidt & Okimoto, 1992)
Reptiles					
African fat-tailed gecko (*Hemitheconyx caudicinctus*)	3	F	Systemic	Liver, kidneys, skeletal muscle, bones, spleen, uterus, ovaries and lungs	MCT-related death (Rovira, Holzer, & Credille, 2014)
Boa constrictor (*Boa constrictor constrictor*)	U	U	Malignant	Cutaneous	U (Frye, 1994)
Desert tortoise (Xerobates agassizii)	U	U	Cutaneous	Cutaneous	U (Frye, 1994)
Eastern kingsnake (*Lampropeltis getulus getulus*)	16	M	Cutaneous	Metastatic (liver, heart, lung, kidney, spleen)	MCT-related death (Schumacher et al., 1998)
Giant Galapagos tortoise (*Geochelone nigra vicina*)	Subadult	F	Cutaneous	Confined to skin	Healthy 11 months post-surgery (Santoro et al., 2008)
Green iguana (*Iguana iguana*)	Adult	F	Cutaneous	Mastocytosis of periphery	Euthanized (Reavill et al.,)
Amphibians					
Axolotl (*Ambystoma mexicanum*)	11–17 (18 animals)	U	Cutaneous	Single or multiple lesions, some metastatic (skeletal muscle)	Dead (Harshbarger, Chang, DeLanney, Rose, & Green, 1999)
Tiger salamander (*Ambystoma tigrinum*)	Neotenic (6 animals)	U	Cutaneous	Single or multiple lesions, some metastatic (skeletal muscle)	Dead (Harshbarger et al., 1999)

Note: male (M), female (F), unknown (U).
Abbreviation: MCT, mast cell tumour.

aggressive, multisystem malignancy. Mastocytosis is divided into two main groups based on the presence or absence of extracutaneous organ involvement: cutaneous mastocytosis (CM) and SM. While CM and SM present differently, the disorders exhibit common genetic abnormalities in the *KIT* gene (Bodemer et al., 2010; Valent, Akin, & Metcalfe, 2017).

6.1 | Human CM

Cutaneous mastocytosis, also referred to as urticaria pigmentosa, is isolated to the skin, with no other organ involvement and represents 80% of all mastocytosis cases in humans (Siebenhaar et al., 2018). CM is more common in infants but can occur at any age and is more frequent in males than in females with a ratio of up to 1.8:1 (Bodemer et al., 2010; Kiszewski et al., 2004). Paediatric CM describes the disease in infants and has a favourable prognosis, often spontaneously regressing once the individual reaches adolescence (Kiszewski et al., 2004). In a minority of cases, paediatric CM progresses into mast cell activation syndrome, a multisystem inflammatory disorder of chronic mast cell hyper-reactivity, or SM, a proliferative and accumulative neoplastic mast cell disease of one or multiple organs (Valent et al., 2017). Both are malignant forms of the disease.

6.2 | Human SM

Classification of SM is based on mast cell morphology and immunohistochemistry and includes five sub-variants according to the WHO classification system (Valent et al., 2017). With increasing severity, these sub-classifications are: indolent SM, smouldering SM, SM with associated haematologic non-mast cell lineage disease neoplasm, aggressive SM and mast cell leukaemia. All sub-variants are characterized by extracutaneous organ involvement and gain-of-function mutations in the *KIT* gene.

6.3 | Familial mastocytosis

Familial predisposition to mastocytosis is evident in up to 13% of human cases but the exact mode of inheritance is unclear (Bodemer et al., 2010). Mastocytosis can occur in the presence or absence of *KIT* mutations and there is no apparent relationship between a patient's genotype and familial or spontaneous disease development (Bodemer et al., 2010). Familial mastocytosis is known to pass through generations from parent to offspring, although it appears that *KIT* mutations are not inherited (Wohrl et al., 2013). Instead the mutations arise somatically, potentially as a secondary event resulting from a common germline abnormality predisposing individuals to familial disease (Bodemer et al., 2010; Jawhar et al., 2015).

6.4 | *KIT* mutations in human patients

In contrast to dogs and cats, mutations are more frequent in the enzymatic domain of *KIT* in human SM patients. Approximately 44% of paediatric mastocytosis patients and less than 5% of adult SM patients harbour a regulatory-type *KIT* mutation, in either exon 8, 9 or 11 (Bodemer et al., 2010; Haenisch et al., 2012). Comparatively, enzymatic-type *KIT* mutations are detected in mastocytoma or bone marrow biopsies in over 80% of adult and paediatric human patients with mastocytosis (Bodemer et al., 2010; DeAngelo et al., 2017). The commonest *KIT* mutation is a nucleotide substitution occurring in exon 17 at codon 816 where aspartic acid is replaced with valine (D816V). Unlike in dogs, tumour *KIT* mutation status in humans is not correlated with survival or disease progression (Bodemer et al., 2010). However, mutation status is a minor criterion for mastocytosis diagnosis and is clinically relevant when deciding optimal treatment regimens for mastocytosis patients.

6.5 | Other genetic mutations involved in human mastocytosis

Mutations in various genes encoding splicing factors *SRSF2*, *SF3B1* and *U2AF1*, epigenetic regulators *ASXL1*, *DNMT3A* and *TET2*, the transcription factor *RUNX1* and signalling molecules *JAK2*, *CBL*, *KRAS* and *NRAS* are frequently identified and co-exist with *KIT* mutations in SM patients (Damaj et al., 2014; DeAngelo et al., 2017; Jawhar et al., 2015; Jawhar, Schwaab, et al., 2016; Traina et al., 2012). Mutations in *ASXL1*, *DNMT3A*, *RUNX1* and *SRSF2* are predictive of overall patient survival and aberrations in other genes predict response to particular treatment types (Jawhar, Schwaab, et al., 2016; Traina et al., 2012). Mutations in these genes have not been investigated in other species in relation to MCTs.

6.6 | Treatment of mastocytosis

Therapy is seldom sought for human patients with CM due to its benign clinical course, however, some CM cases may require antihistamines and corticosteroids to stabilize symptoms (Kiszewski et al., 2004). Conversely, patients with mast cell leukaemia experience average survival times of less than 12 months even with the best treatment available (Valent et al., 2017).

Various TKIs have been investigated for therapeutic use against mastocytosis. Imatinib and masitinib mesylate show efficacy for patients with wild-type *KIT* mast cell neoplasia (Lortholary et al., 2017). However, they are not a suitable treatment option for many patients due to the high prevalence of the D816V mutation which confers drug resistance (Siebenhaar et al., 2018; Valent et al., 2017).

The TKI nilotinib exhibits potent growth-inhibitory and pro-apoptotic activity against mast cells harbouring the regulatory-type *KIT* mutation D560G (Verstovsek et al., 2006) There is conflicting evidence regarding the activity of nilotinib against wild-type and D816V mutant mast cells (Hochhaus et al., 2015; Verstovsek et al., 2006).

Tyrosine kinase inhibitors that demonstrate growth-inhibitory activity against both wild-type and *KIT* D816V mutant neoplastic mast cells include dasatinib, midostaurin and ponatinib (Gleixner et al., 2007, 2013). Combination therapies evaluating in vitro cooperative antineoplastic effects of dasatinib with cladribine, midostaurin and ponatinib show synergistic, anti-proliferative and apoptotic

effects against D816V neoplastic mast cells (Gleixner et al., 2007, 2013). Ponatinib also down-regulates phosphorylation of the tyrosine kinase protein LYN and the transcription factor STAT5, suggesting that ponatinib may contribute to neoplastic mast cell growth inhibition in a Kit-independent manner (Gleixner et al., 2013).

A compelling new therapy for SM patients with D816V mutation is the use of BLU-285, a potent and selective inhibitor of the Kit activation loop. In vitro, BLU-285 blocks autophosphorylation of D816V mutant Kit as well as phosphorylation of downstream Kit signalling proteins (AKT and STAT3), thereby inducing cellular apoptosis (DeAngelo et al., 2017; Drummond et al., 2016; Jawhar, Naumann, et al., 2016). In phase I human clinical trials, BLU-285 was well tolerated by patients and mast cell burden in peripheral blood, bone marrow and organs decreased (DeAngelo et al., 2017; Drummond et al., 2016). Moreover, BLU-285 shows efficacy in D816V SM patients resistant to the TKI midostaurin (Drummond et al., 2016; Jawhar, Naumann, et al., 2016). Such therapies may be of value in the veterinary setting as well.

7 | CONCLUSIONS AND PROSPECTS

Mast cell neoplasia is most often described in dogs but has been observed in numerous other mammals, birds and reptiles. Despite differences in the clinical presentation and biological behaviour documented across species, a common genetic anomaly exists within the *KIT Proto-Oncogene* of neoplastic mast cells. Mutations in *KIT* are regularly identified in neoplastic mast cells of dogs, cats and humans and contribute to mast cell carcinogenesis. The *KIT* gene is also likely to be implicated in the disease within other species as determined by aberrant cytoplasmic Kit protein staining in ferrets, horses and cattle.

The dog is particularly susceptible to MCT disease, but *KIT* is not the sole genetic contributor to tumour development. Genetic alterations frequently detected in human mastocytosis patients are not common in the dog and few other genes have been explored as potential causative factors in dogs. Thus, the exact molecular events and genetic predispositions contributing to the establishment and growth of canine MCTs remain unknown.

Mast cell neoplasia is generally well managed in most feline and human cases, however, presents a therapeutic problem in the dog due to its varied biological nature. TKIs are approved for use in veterinary medicine for the treatment of unresponsive or recurrent high-grade canine MCTs harbouring a regulatory-type *KIT* mutation. The place of TKIs in canine MCT treatment is not entirely clear but they appear to provide a useful addition to standard therapies. Combination therapies supplementing the use of conventional chemotherapeutic agents with TKIs are being explored and hold promise as new treatment opportunities for canine MCT patients.

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Name of Principal Author (Candidate)	Vanessa S Tamlin
Contribution to the Paper	Involved in the conception and design of the experiment. Collected the samples, conducted wet-lab experiments, interpreted the results. Drafted and edited the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 10/10/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Anne E Peaston
Contribution to the Paper	Involved in the conception and design of the experiment. Assisted in data interpretation and manuscript drafting/editing.
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Signature	Date 17/10/19.

Name of Co-Author	Lucy Woolford		
Contribution to the Paper	Pathological diagnosed the tumour samples and critically revised the manuscript.		
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DNA purification increases PCR-amplifiable DNA extracted from formalin-fixed, paraffin-embedded canine mast cell tumors for routine *KIT* mutation detection

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Abstract. DNA amplification by PCR detects *KIT* exon 11 internal tandem duplications in canine mast cell tumors (MCTs). Tissue-specific inhibitors often contaminate DNA extracted from formalin-fixed, paraffin-embedded (FFPE) canine MCTs, blocking PCR amplification and, consequently, preventing mutation detection. We used a commercial kit to extract DNA from FFPE canine MCTs. Two independent PCR assays, each with one primer set, were used to amplify target genes (*HPRT* and *KIT*) directly after FFPE DNA extraction. PCR amplification failed with at least one primer set in 153 of 280 samples (54.6%, 95% CI: 48.8–60.5%). One or 2 DNA washing steps were required to remove PCR inhibitors in 130 of 280 (46.4%) and 23 of 280 (8.2%) of these cases, respectively. DNA concentration and quality (A_{260}/A_{280} and A_{260}/A_{230}) either pre- or post-washing were not associated with ability of the samples to be amplified by PCR using both *HPRT* and *KIT* primer sets. Low-grade and subcutaneous MCTs were less likely to amplify directly after DNA extraction and without any washing steps compared to high-grade MCTs using *KIT* gene primers.

Keywords: dogs; internal tandem duplication; *KIT* proto-oncogene; mast cell tumor; PCR inhibition.

Dogs have a unique risk of developing cutaneous mast cell tumors (MCTs). Internal tandem duplications (ITDs) in exon 11 of the *KIT* proto-oncogene cause constitutive activity of the Kit protein,[10] contribute to mast cell malignancy, and are prevalent in up to 50% of high-grade MCTs.[5,16] Veterinary diagnostic laboratories use PCR and gel electrophoresis to test DNA from high-grade MCTs for exon 11 ITDs. Dogs who harbor a *KIT* exon 11 ITD in their MCT, and are unresponsive to conventional chemotherapeutic treatments, or whose MCT is not amenable to wide surgical excision, are suitable candidates for treatment with tyrosine kinase inhibitors. Tyrosine kinase inhibitors block the abnormal autophosphorylating activity of the Kit protein caused by ITDs in exon 11 of *KIT*.[11,14]

Formalin-fixed, paraffin-embedded (FFPE) tissue archives are a valuable resource for retrospective studies of a plethora of diseases given the economic and space-efficient storage requirements of blocks in comparison to frozen materials. However, PCR amplification of DNA extracted from FFPE tissues can be problematic because of the variable and often high level of DNA degradation induced by formalin fixation. This limits amplification for most FFPE samples to small fragments of <250 base pairs (bp).[1,4] Various techniques have been investigated to optimize extracted DNA integrity and quality for downstream molecular analysis. Type of fixative, length of fixation, extraction method, length of enzymatic tissue digestion, and age of block impact the quality of the DNA end product, and all have been targets for optimization.[1,6,8,15,17] Moreover, extraction of high-quality DNA from either FFPE or frozen tissue samples has been reported as peculiarly problematic for MCTs in comparison to other tumor types and normal tissues, most likely as a result of the co-extraction of unknown cell-specific PCR inhibitors.[6,16,17]

We investigated poor DNA amplification from 280 FFPE canine MCTs. FFPE canine MCT tissue blocks were sourced from the Veterinary Diagnostic Laboratory at the University of Adelaide (Roseworthy, SA, Australia; $n = 16$) and from Gribbles Veterinary Pathology commercial laboratories in Clayton (VIC, Australia; $n = 175$) and Glenside (SA, Australia; $n = 89$). A commercial kit (QIAamp DNA FFPE tissue kit; Qiagen, Hilden, Germany), which has been previously determined to yield good quality FFPE-extracted DNA in comparison to other techniques,[7] was used to extract DNA

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Table 1. Nucleotide sequences of primers used in PCR assays of canine mast cell tumors.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
HPRT	HPRT2FRD2 GCGAGAGAGAACCTTGTGTG	HPRT2REV2 GGGACTTTGGGGAACTGAC	163
KIT exon 11[5]	PE1 CCCATGTATGAAGTACAGTGGAAG	PE2 GTTCCCTAAAGTCATTGTTACACG	Wild-type: 190; ITD mutant: >190

Table 2. Number of canine mast cell tumor samples that required a wash to obtain a successful PCR amplicon when using the *HPRT* and *KIT* gene primers.

	HPRT primers		
KIT primers	No wash required	Wash required	Total
No wash required	127	0	127
Wash required	52	101	153
Total	179	101	280

from a 20-μm thick section of each FFPE MCT block. Xylene was used for deparaffinization of FFPE tissue sections, and DNA was extracted according to the manufacturer's instructions, eluted in 60 μL of a 0.1 mM EDTA buffer supplied in the kit, and stored at 4°C for later use. The DNA was extracted between 2015 and 2016, and the DNA quantity (concentration) and quality (A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios) were measured (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). At the time of DNA extraction, 68 blocks were ≤1 y old, 189 blocks were 1–2 y old, and 23 blocks were 2–4 y old. Sample fixation times are unknown.

Each sample underwent PCR amplification using 2 different primer sets (Table 1). Primers for the reference gene *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) were used to determine whether the DNA was of sufficient quality to generate a small PCR product. Previously published primers were used to identify ITDs in exon 11 of the *KIT* gene.[5] PCR conditions were identical for both sets of primers, and reactions were performed as described previously.[16] Approximately 10–50 ng of sample DNA was added to each 20-μL PCR reaction. DNA extracted from frozen normal canine testis and water were used as positive and negative controls for PCR amplification, respectively. Resulting PCR products were analyzed on 2% agarose gel by electrophoresis.

DNA that failed to amplify by PCR using either one or both primer sets underwent a "washing" step (DNA clean and concentrator kit; Zymo Research, Irvine, CA) per the manufacturer's instructions. In brief, DNA samples were mixed with DNA binding buffer at a ratio of 5:1 and then transferred to a spin column in a collection tube, before centrifugation at 13,000 × *g* for 30 s. The columns were washed twice with 200 μL of DNA wash buffer, and each wash was followed by 13,000 × *g* centrifugation for 30 s. DNA was eluted in 40 μL of the 0.1 mM EDTA elution buffer supplied in the Zymo kit and the concentration measured on the NanoDrop. The samples then underwent a second PCR amplification test. Samples that still failed to produce a PCR amplicon underwent a second DNA washing procedure, a direct repeat of the first wash, followed by a third PCR test.

Using the *HPRT* primers, 179 of 280 (63.9%) samples were successfully amplified directly after DNA extraction. Eighty-six (30.7%) samples required a single wash, and 15 (5.4%) samples required 2 washes before amplifiable DNA was obtained. Using the *KIT* primers, 127 of 280 (45.4%) samples required no washing, 130 (46.4%) samples required a single wash, and 23 (8.2%) samples required 2 washes in order for amplifiable DNA to be obtained. Overall, 153 of 280 (54.6%, 95% CI: 48.8–60.5%) MCT DNA samples required at least 1 wash for successful PCR of both *HPRT* and *KIT* (Table 2).

Data were analyzed using SPSS statistical software (v.25; IBM, Armonk, NY). As determined by Pearson chi-square test, MCT DNA samples that required washing for successful PCR amplification by one primer set were predictably more likely to require washing for PCR amplification success when using the other primer set (χ^2 [1, n = 280] = 131, p <0.001). The association between *HPRT* with *KIT* primer pairs for washing requirements was strong ($\phi = 0.684$). There was no significant relationship between age of block and washing requirements when using either the *KIT* primers ($p = 0.074$) or *HPRT* primers ($p = 0.262$) as determined by a Pearson chi-square test.

Post-washing DNA concentrations and the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios varied from being more diluted to more concentrated than the original sample (Supplementary Table 1). There was no statistically significant relationship between ability to amplify the DNA using both *HPRT* and *KIT* primer sets and DNA quantity (concentration) or quality (absorbance ratios; $p = 0.700$), as determined by binary logistic regression analysis fitted with a Hosmer–Lemeshow model.

To further investigate the possibility that co-extraction of PCR inhibitors interfered with DNA amplification, we conducted *HPRT* PCR reactions on MCT DNA samples that required at least one wash for successful PCR amplification. Two PCR assays were prepared from a single master mix solution. The first assay was conducted according to the standard *HPRT* protocol in which 1 μL of 10–50 ng of MCT DNA was added to each reaction. The second assay was identical to the first, but each reaction was "spiked" with 1

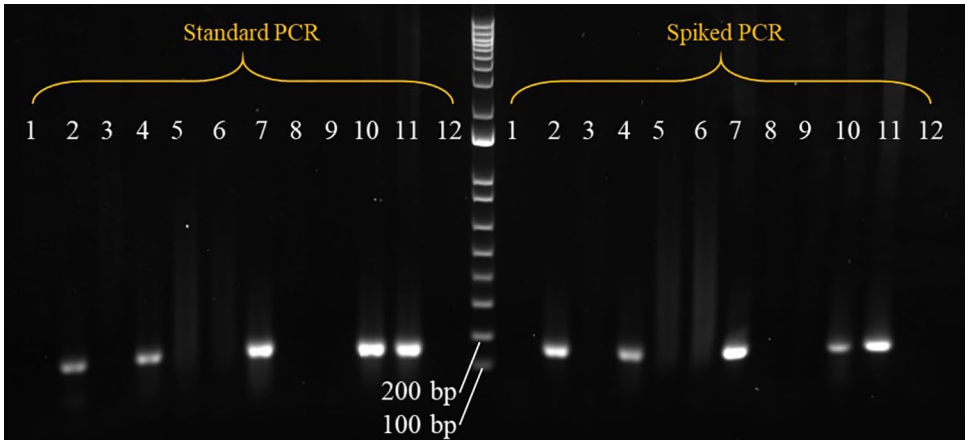

Figure 1. Agarose gel electrophoresis of genomic DNA extracted from 4 formalin-fixed, paraffin-embedded canine mast cell tumor (MCT) blocks, amplified by PCR using *HPRT* reference gene primers and run against a 1-kb DNA ladder marker. Expected product size is 163 bp. Two PCR assays were performed from a single master mix solution. The first assay amplified canine MCT DNA according to the "standard" PCR protocol. The second assay was exactly the same as the first except that each reaction, excluding the negative control, was "spiked" with wild-type canine testis positive control DNA in addition to the tumor sample DNA. Lanes 1, 2 = sample A86 with no wash and after one wash, respectively. Lanes 3, 4 = sample M186 with no wash and after one wash, respectively. Lanes 5–7 = sample M127 with no wash, after 1 wash, and after 2 washes, respectively. Lanes 8–10 = sample U47 with no wash, after 1 wash, and after 2 washes, respectively. Lane 11 = positive control (wild-type canine testis DNA). Lane 12 = negative control (water). Sample identifiers as per Supplementary Table 1.

µL of canine control DNA (concentration of 12 ng/µL) in addition to the 1 µL of MCT sample DNA. PCR products were analyzed on 2% agarose gel by electrophoresis (Fig. 1). Amplification of the positive control was inhibited in the unwashed but not the washed DNA. This supports a conclusion that contaminants existing in the MCT DNA solutions inhibit amplification by PCR but are successfully removed using the DNA washing method described herein.

The 280 FFPE tumor specimens were histologically classified as subcutaneous (n = 41, 14.6%) or cutaneous (n = 239, 85.4%) MCT by 2 board-certified veterinary pathologists (EC Dobson and L Woolford). The cutaneous tumors were graded according to 2 systems widely used in veterinary pathology.[9,13] According to the Patnaik grading scheme, the cutaneous MCTs included 13 grade I (5.4%), 181 grade II (75.7%), and 45 grade III (18.8%) tumors. All Patnaik grade I and III tumors were graded as Kiupel low- and high-grade, respectively. Of the Patnaik grade II tumors, 171 of 181 (94.5%) were classified as Kiupel low-grade and the remaining 10 (5.5%) as Kiupel high-grade. Given the low numbers representing the Patnaik grade I class, only the Kiupel grading scheme was investigated in the subsequent statistical analyses.

A *KIT* exon 11 ITD was observed in 22 of 55 (40%) high-grade tumors and 2 of 184 (1%) low-grade tumors, with an overall prevalence in cutaneous MCTs of 10% (24 of 239; 95% CI: 6.2–13.9%). Additionally, 1 cutaneous low-grade tumor harbored a 21-bp exon 11 deletion (Fig. 2), and one of the 41 subcutaneous MCTs carried an ITD. A *KIT* exon 11 ITD mutation in a subcutaneous MCT has not been reported previously, to our knowledge. ITDs ranged from 25 to 60 bp

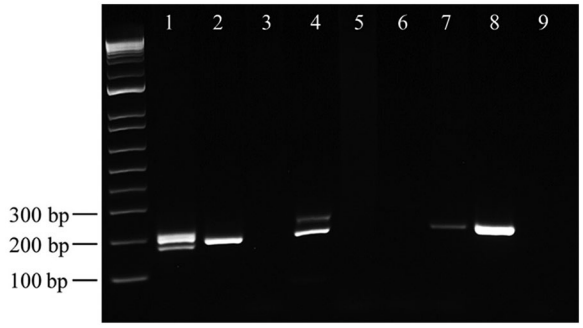

Figure 2. Agarose gel electrophoresis of genomic DNA extracted from 4 formalin-fixed, paraffin-embedded canine mast cell tumor blocks amplified by PCR using *KIT* gene primers and run against a 1-kb DNA ladder marker. Wild-type PCR product is 190 bp, internal tandem duplication PCR products are larger (represented by the upper bands in lane 4), and deletion mutant product is smaller (represented by the lower band in lane 1). Lane 1 = sample M29; 21-bp deletion. Lane 2 = sample A1; wild-type *KIT*. Lanes 3, 4 = sample M25 with no wash and after one wash, respectively; ITD mutant. Lane 5–7 = sample M107 with no wash, after 1 wash, and after 2 washes, respectively; wild-type *KIT*. Lane 8 = positive control (wild-type canine testis DNA). Lane 9 = negative control (water). Sample identifiers as per Supplementary Table 1.

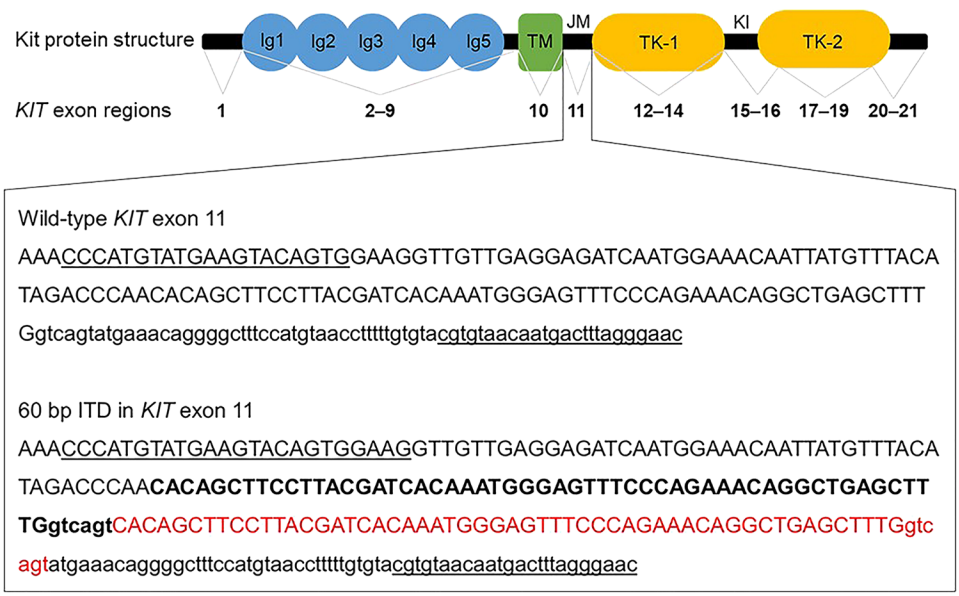

Figure 3. Structure of canine Kit protein and corresponding *KIT* gene exon regions. Noted are the immunoglobulin-like domains (Ig1–5), transmembrane domain (TM), juxtamembrane domain (JM), tyrosine kinase domains (TK-1, TK-2), and the kinase insert (KI). The zoomed-in textbox represents the wild-type DNA sequence of exon 11 (uppercase text) and part of intron 11 (lowercase text) from canine *KIT* gene. Primer sequences are underlined. Also shown is the DNA sequence of a 60-bp internal tandem duplication (ITD) detected in a mast cell tumor. The region of the *KIT* sequence that is duplicated is in boldface, and the duplication is in red.

duplicates and resided in the 3′-end of exon 11, with 9 ITDs extending into intron 11 (Fig. 3). The largest ITD extended 35 nucleotides 5′ of the reverse primer and, therefore, ITD mutations are not suspected to impede primer ability to amplify the DNA.

When using *HPRT* primers, 17 of 41 (42%) subcutaneous MCTs, 71 of 184 (39%) cutaneous low-grade MCTs, and 13 of 55 (24%) cutaneous high-grade MCTs required at least 1 wash to obtain amplifiable DNA (Supplementary Table 1). A generalized linear logistic model with binary logistic regression fitted with a Wald chi-square analysis suggested no significant relationship between washing requirements for *HPRT* primers with tumor type or grade ($p = 0.060$). For *KIT* primer pair, 27 of 41 (66%) subcutaneous MCTs, 105 of 184 (57%) cutaneous low-grade MCTs, and 20 of 55 (36%) cutaneous high-grade MCTs required at least 1 wash to obtain amplifiable DNA. In pairwise comparisons, cutaneous low-grade ($p = 0.005$) or subcutaneous ($p = 0.001$) MCTs were significantly more likely than cutaneous high-grade MCTs to require at least one wash to obtain amplifiable DNA when using the *KIT* primer pair. Washing requirements for cutaneous low-grade MCTs and subcutaneous MCTs were not significantly different from each other ($p = 0.167$).

High-grade MCTs are commonly affected by local inflammation, ulceration, and tissue necrosis associated with spontaneous degranulation by tumor cells of bioactive molecules such as histamine, serotonin, serine proteases, eicosanoids, and cytokines.[3] It has been hypothesized that high-grade MCTs would experience greater PCR inhibition as a result of these factors.[2] However, our results do not support this hypothesis and instead show that the DNA from the more benign MCTs (i.e., cutaneous low-grade and subcutaneous MCTs) was more likely to require washing to enable successful PCR amplification of *KIT*. There is no clear explanation for this result. One possible explanation is related to the copy number of the *KIT* gene in high-grade tumors. MCTs with *KIT* mutations have been reported to have increased copies of the *KIT* locus on CFA 13.[12] An increased quantity of initial DNA templates might offset the effect of tissue inhibitors in some PCR reactions. However, this does not explain why *HPRT* but not *KIT* was amplified from 44 low-grade or subcutaneous samples without washing (Supplementary Table 1). In these cases, primer annealing properties or primary DNA structural properties in concert with PCR inhibitors may have contributed to the finding. For samples successfully amplified by *HPRT* primers directly after DNA extraction, 2-sample *t*-tests determined statistically significant differences in the mean DNA concentration ($p = 0.016$) and A_{260}/A_{280} ($p < 0.001$) and A_{260}/A_{230} ($p < 0.001$) absorbance ratios for samples requiring and not requiring a wash for successful *KIT* primer amplification. It is unclear whether post-washing DNA concentrations were the sole contributor to successful PCR amplification in these cases or if removing inhibitors also had an effect on outcome.

Our study is limited by lack of data regarding tissue sample processing. Time delay between surgical excision of the

tumor and placement in formalin is unknown, and standard operating procedures regarding tissue processing likely varied between veterinary pathology laboratories. Discrepancies in these processes may have affected downstream molecular analyses. The thickness of FFPE sections taken for DNA extraction may have also contributed to the co-extraction of PCR inhibitors, although this has not been investigated. Nonetheless, the washing method described herein is effective in resolving this issue.

MCTs are the most common skin tumors in dogs, and high-grade tumors have a guarded prognosis given their aggressive biologic behavior. Detection of *KIT* gene mutants can guide therapy. Failure of PCR amplification using DNA from canine MCTs occurred in 54.6% of cases, presumably because of the combined effects of *KIT* gene copy numbers and the co-extraction of tissue-specific contaminants. This problem can be solved readily by our method described herein. The exact biological contaminants interfering with the PCR remain unknown, but our results suggest that they are more prominent in cutaneous low-grade MCTs and subcutaneous MCTs.

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Supplementary material

Supplementary material for this article is available online.

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