Prognostic and predictive value of BRAF mutation alone and in combination with microsatellite instability in stage III colon cancer

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by

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Abbreviations

ACPS	Australian clinicopathological stage
APC	adenomatous polyposis coli
CIN	chromosomal instability
CIMP	CpG island hypermethylation
CRC	colorectal cancer
EGFR	epidermal growth factor receptor
FFPE	formalin fixed paraffin embedded
5-FU	5-fluorouracil
GCHP	goblet cell hyperplastic polyp
HP	hyperplastic polyp
HR	hazard ratio
IHC	immunohistochemistry
MAPK	mitogen-activated protein kinase pathway
MMR	mismatch repair
MSI	microsatellite instability
MSS	microsatellite stable
MVHP	microvesicular hyperplastic polyp
NCI	National Cancer Institute
OS	overall survival
PFS	progression-free survival
SC	serrated cancer
SSA	sessile serrated adenoma
SA	serrated adenoma

Thesis Abstract

Purpose

The prognostic and predictive role of biomarkers in colorectal cancer is still being defined. The aim of this study was to determine the prognostic value of BRAF mutation alone and in combination with microsatellite instability (MSI), and to determine the interaction between BRAF mutation and MSI status in determining survival benefit after adjuvant 5-FU (5-fluorouracil) based chemotherapy in stage III colon cancer.

Methods

We performed a retrospective cohort study including all curatively resected stage III colon cancer cases over a 33-year period. A clinicopathologic database was collated (adjuvant chemotherapy, age, gender, obstruction, perforation, tumour location, grade, mucin, nodal stage, extramural vascular and perineural invasion). BRAF (V600E) mutation testing was performed and MSI status established by immunohistochemistry for mismatch repair proteins and molecular testing for National Cancer Institute (NCI) panel markers. Patients were categorised into four groups for comparison: MSS and BRAF-ve (termed "*traditional*"), MSI and BRAF-ve (termed "*presumed Lynch*"), MSI and BRAF+ve (termed "*sporadic MSI*") and MSS and BRAF+ve (termed "*other BRAF*"). The primary endpoint was cancer specific survival. Interaction testing was conducted to determine whether there was differential benefit from chemotherapy between groups.

Results

A total of 686 unselected cases met our inclusion criteria and had tissue available, of which 15.7% had BRAF mutation and 13.8% had MSI. In the adjusted analysis, neither BRAF mutation nor MSI mutation were independently prognostic (HR 1.78, 95% CI 0.89-1.79, P = 0.18, and HR 0.49, 95% CI 0.75-1.83, P = 0.48, respectively). On univariate analysis, survival of patients with *presumed Lynch* cancers was similar to those with *traditional* cancers (5-year survival, 62 and 61%, respectively), and while there was no difference in cancer specific survival between *sporadic MSI* and *other BRAF*, these tumours had poorer

outcome when compared to *traditional* or *presumed Lynch* cancers. Adjusted analysis of the four groups, however, showed that none of the subgroups were independently prognostic. Thirty-nine percent received chemotherapy. Overall, adjuvant chemotherapy produced a cancer specific survival benefit (chemotherapy: HR 0.66, 95% CI 0.49-0.88, P < 0.01). On adjusted analysis, neither BRAF nor MSI status were individually predictive of survival benefit. On adjusted analysis specifically of the chemotherapy effect in each subgroup, only patients in the *presumed Lynch* group (HR 0.260, 95% CI 0.09-0.80, P < 0.01) and *other BRAF* groups (HR 0.45, 95% CI 0.23-0.87, P 0.01) had a significant survival benefit from chemotherapy. On interaction testing of subgroups, adjusting for all the clinicopathological parameters, patients in the *presumed Lynch* group (HR 0.28, 95% CI 0.10-0.75, P < 0.01) gained a differentially greater benefit from chemotherapy than other groups.

Conclusions

BRAF mutated cancers demonstrated a trend towards poorer outcomes, however, after adjusting for clinicopathological factors, MSI and chemotherapy, BRAF mutation was not found to be an independent prognostic biomarker in stage III colon cancer, even when combined with MSI. In this historical cohort, MSI testing was predictive of response to adjuvant chemotherapy in stage III colon cancer, but only when results are interpreted in combination with BRAF. This supports the role of routine testing for these biomarkers.

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree in any university and that to the best of my knowledge and belief, contains no material previously written by another person, except where due reference is made in the text. I consent to this thesis being made available for photocopying and loan if applicable and if accepted for the award of the degree.

Hanumant Singh Chouhan

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Podium Presentations

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Title: Prognostic and predictive value of molecular mutations in stage III colon cancer

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Title: Prognostic significance of BRAF mutation in stage III colon cancer

Publications Arising

Tumorigenesis and clinical significance of molecular mutations involved in the serrated tumorigenic pathway to colorectal cancer

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Prognostic value of BRAF mutation alone and in combination with microsatellite instability in stage III colon cancer

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Manuscript under review by the Asia Pacific Journal of Clinical Oncology

The interaction between BRAF mutation and microsatellite instability status in determining survival outcomes after adjuvant 5-FU based chemotherapy in stage III colon cancer

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Chapter 1

Article 1

Tumorigenesis and clinical significance of molecular mutations involved in the serrated tumorigenic pathway to colorectal cancer

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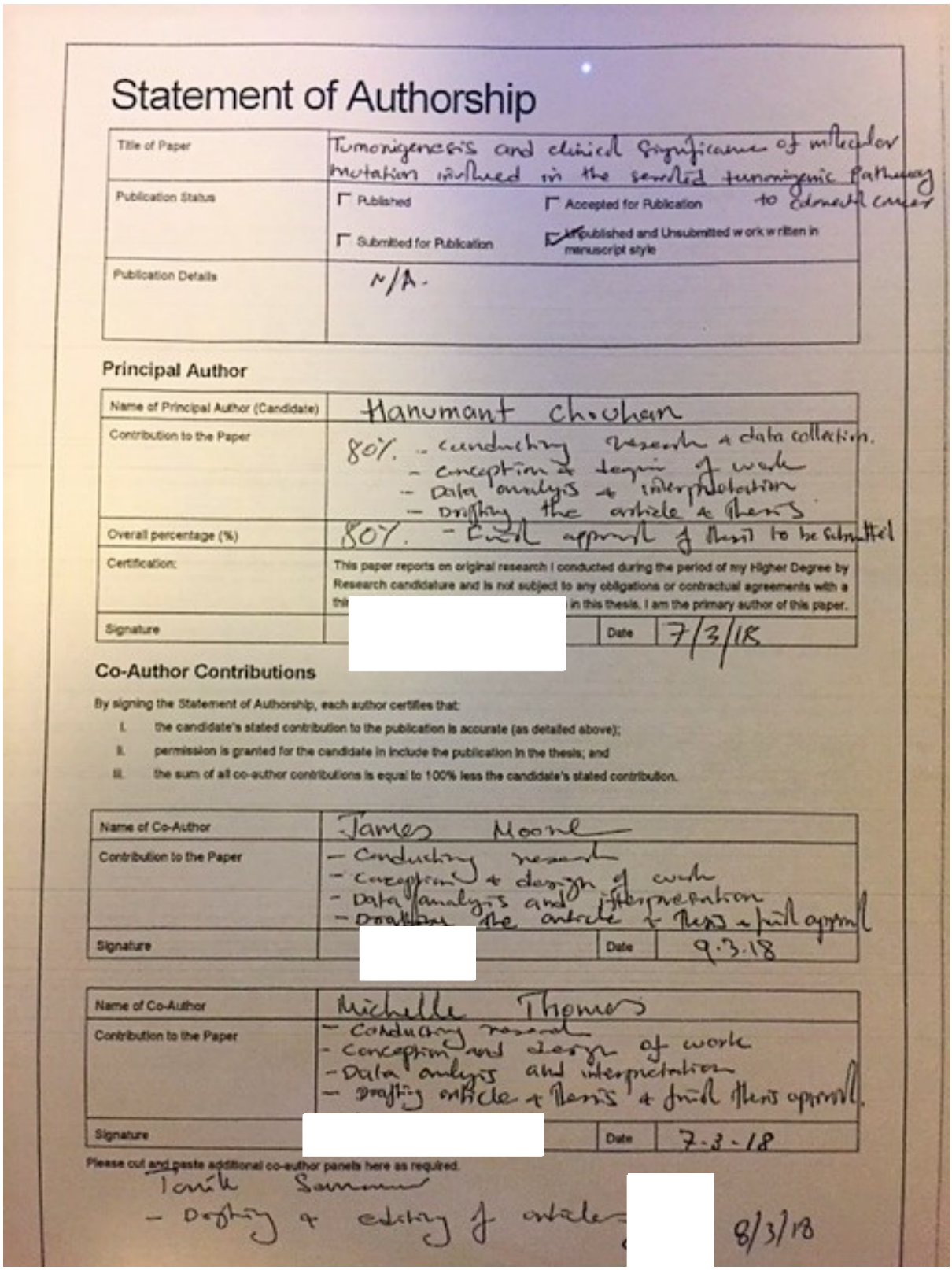

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Abstract

The development of colorectal cancer (CRC) is a multi-step process, with a complex interplay of factors that are still being elucidated. Molecular mechanisms are important but so far there has been limited success in using biomarkers to stratify patients with colorectal cancer for treatment, targeted therapy and prognostication. Of the various major pathways in CRC, the serrated pathway of colorectal cancer is the most recently described and thus least well understood. In this review of the literature, we evaluate the serrated pathway in colorectal cancer and explore further its molecular basis.

Introduction

Despite advances in management of colorectal cancer (CRC) over the last couple of decades there is significant cancer-related mortality associated with this disease (1). Historically, CRC has been managed as a single entity (2), but it is now recognised that multiple distinct pathways exist in colorectal cancer (3). There is wide variation in the incidence of colorectal cancer by geographical location and gender (1, 2), possible reflecting significant heterogeneity in pathogenesis of these cancers. Despite recognising morphological subtypes of CRC, such as mucinous carcinoma (4) for decades, it has only become apparent recently that CRC is not a single disease, but rather a heterogenous disorder including multiple pathways with diverse molecular backgrounds and clinicopathological manifestations. About a third of colorectal cancers are now thought to develop through an alternative pathway to the adenoma to carcinoma sequence as described by Vogelstein et al. (3, 5). It is important that with the growing knowledge of colorectal cancer tumorigenesis, due attention is given to new findings related to histological assessment, and molecular characterisation, prognostic and predictive information, to improve the cancer-related mortality seen with this disease.

Genetic instability in different pathways

Historically, the progression of colorectal cancer was thought to follow the adenoma to carcinoma sequence, with driver APC (adenomatous polyposis coli) activation in adenoma, with additional KRAS mutation and p53 inactivation in carcinoma. This pathway, also

described as the chromosomal instability (CIN) pathway or traditional adenoma to carcinoma sequence is well established in colorectal tumorigenesis (5). However, the genetic and epigenetic alterations seen in colorectal cancer tumorigenesis are complex and difficult to understand. The CIN pathway only accounts for about 60-70% of CRC. The notion that an independent alternative pathway exists in sporadic CRC was slow to develop, and a second model of genetic instability was only accepted when adenomas in Lynch syndrome (a type of hereditary colorectal cancer) patient were found to accumulate a number of mutations which specifically target repetitive sequences of DNA (6). This form of genetic instability is caused by inactivation of the DNA mismatch repair (MMR) system and microsatellite instability (MSI) is the biomarker (7-9). The existence of two different pathways (CIN-traditional and MSI-alternative) led to further research to understand the distinct pathological and molecular characteristics of these pathways, and to characterise their precursor lesions and clinical manifestations.

Alternative pathway or serrated cancers

Jass and Smith first described pathological characteristics of alternative pathway cancers in 1992 (10). These alternative pathway cancers may have epithelial serrations, eosinophilic and abundant cytoplasm with vesicular nuclei, chromatin condensation and lack of necrosis, mucin production, and presence of cell balls and rods (10, 11). Given their distinct morphological characteristics they were described as serrated cancers (SC). However, only a small number of alternative pathway cancers show these characteristic histopathological features, and histologically distinguishing serrated cancers from common cancers (arising from the CIN pathway) is difficult (11-13). Mäkinen et al. first described the relationship between a distinctive polyp that histologically showed epithelial serration (serrated architecture) and serrated cancer (11). These polyps were later defined as serrated polyps or serrated adenomas (14), and due to their serrated architecture were thought to be precursor lesions to alternative pathway cancers. The presence of serrated polyps in the periphery of infiltrating carcinoma is another very important histological finding that helps distinguish serrated cancers (12). However, histological distinction of these cancers can still be quite difficult, and it is hypothesised that this may be due to loss of histological

characteristics as the lesion progresses. Therefore, identification of these serrated cancers must rely on molecular characteristics.

Molecular characteristics of serrated cancers

Research over the last two decades has improved recognition of serrated lesions, both endoscopically and on pathology (15). Despite a continually growing understanding of underlying serrated cancer tumorigenesis (3, 15), the molecular profile of these tumours is heterogenous and less well characterised. The mitogen-activated protein kinase (MAPK) cascade is implicated to serrated cancer tumorigenesis due to high frequency of both BRAF and KRAS in serrated adenomas (16), and serrated cancers (9). Both BRAF and KRAS encode kinases within the MAPK cascade that mediate cellular signalling for cell proliferation, apoptosis and differentiation (17). Activation of either of these two mutations leads to activation of the MAPK pathway. Interestingly, despite being part of same pathway, BRAF and KRAS mutations are mutually exclusive (9, 18), suggesting that tumours derived from these mutations may have different biology and natural history. It is also unclear which of the two mutations, if either, is the dominant oncogene driving tumour proliferation in serrated cancers. Stefanius et al. (9) studied the incidence of BRAF and KRAS mutations in non-serrated (CIN pathway) and serrated cancers, histologically confirmed by two independent pathologists. This study found that BRAF mutation was exclusively found in serrated cancers and is a likely diagnostic mutation for serrated cancers. Interestingly, KRAS mutation was found in both serrated and non-serrated cancers, suggesting KRAS mutation may be part of the tumorigenic process in these cancers, thus challenging the long-held Vogelstein theory. Precursor serrated lesions rarely demonstrate MSI (16, 19-23), and only about 20% of serrated cancers are MSI (9). Therefore, high frequency of BRAF and KRAS mutations, and the relatively low frequency of MSI in precursor serrated lesions, suggests that the MAPK cascade is central in initiating serrated cancer tumorigenesis. BRAF and KRAS are early driver mutations of this pathway, initiating tumorigenesis while mismatch repair dysfunction is likely a late event. This perhaps clarifies early and late events in serrated cancer, but the progression of tumorigenesis in these cancers is unclear.

It is hypothesised that epigenetic silencing (by DNA hypermethylation) of key genes may explain progression from dysplasia (in precursor serrated lesion) to cancer in alternative pathway tumours (24), but this remains to be validated. Issa et al. first introduced the concept of epigenetic silencing of CpG islands and defined it as the CpG island methylator phenotype (CIMP) (25). CpG islands are located in the promotor region of multiple genes (70%) and in normal cells these promotor CpG island loci are protected and scarcely methylated (26). However, aberrant hypermethylation of CpG islands leads to silencing of key genes such as MLH1, MGMT and development of either microsatellite stable or unstable cancer depending on which genes are silenced (25, 27). CIMP is thought to be an acquired change, mainly because of a gradual increase of CIMP cancer incidence with advancing age (28, 29). This suggests that the ageing right colon becomes prone to hypermethylation, possibly secondary to potential causes such as smoking, obesity, diet and sedentary lifestyle. However, the exact cause of CIMP is not yet known (30), and there is still controversy regarding the clinical utility of this category (30).

BRAF mutation is common in sporadic MSI CRC and CIMP, but not in Lynch syndrome (18, 25, 30). This led researchers to speculate that progression of BRAF mutated (26, 31-33) sporadic MSI tumours could be due to silencing of key genes (MLH1, MGMT) by hypermethylation (30), and that BRAF mutation may contribute to the development of CIMP (34, 35). The hypothesis is that BRAF mutation activates the MAPK cascade, predisposing to promoter hypermethylation (34-36).

This hypothesis is a plausible explanation of serrated pathway tumorigenesis, but it remains to be validated. However, Carragher et al. demonstrated that unidentified factors apart from BRAF mutation are required to generate epigenetic changes (34). Similarly, Hinoue et al. found that transfection of BRAF mutant to colonic tumour lines did not lead to aberrant DNA hypermethylation (35). There are no data on the effect of KRAS mutation on CIMP. Therefore, the exact cause of CIMP and progression of serrated polyps to serrated cancer remain unknown.

Precursor lesions to serrated cancer

Historically, hyperplastic polyps (HP) were considered benign with no malignant potential. In 1996, Torlakovic et al. demonstrated histological differences between adenomas and hyperplastic polyps (37). Subsequently atypical histological features were identified in HPs, and CRC arising in HPs (38). These findings suggested that another precursor lesion may give rise to CRC separate from the Vogelstein pathway. HPs with atypical features and dysplasia (epithelial serration) were labelled as sessile serrated adenoma (SSA) or serrated adenoma (SA) (37). However, which HPs led to SSA was debated, and this led pathologists to look at histological similarities between of the histology of HPs and molecular characteristics of these lesions. Based on histological features (15), HPs can be divided into two main subtypes: microvesicular hyperplastic polyps (MVHP) and goblet cell hyperplastic polyps (GCHP) (16, 20). Based on histological and molecular similarities between certain HPs, SSA and serrated cancer (cancer with serrated architecture, when present), it does appear that they are a continuum from polyp to cancer (15).

The high incidence of driver mutations such as BRAF and KRAS in HPs add weight to their being precursor lesions to serrated pathway cancers (12, 16, 20). KRAS mutations were found to be more common in GCHP (20). Whereas, BRAF mutation was seen more commonly in MVHPs (16), again reflecting the mutually exclusive nature of these mutations within the MAPK pathway, and demonstrating the multiple pathways leading to cancers within the serrated pathway. Interestingly, these precursor HPs have rarely demonstrated a high level of MSI (16, 19-23).

Molecular classification of colorectal cancer

The main aim of classifying CRC and studying prognostic and predictive value of different biomarkers is to aid clinical practice and help in treatment decision making and genetic counselling. Based on molecular differences in CRC, Jass et al proposed a molecular classification according to clinicopathological characteristics (3).

Group A: MSI (BRAF mutated/KRAS wild type/CIMP+ve)

Group A accounts for 10-12% (3) of CRCs. Tumours in this group have propensity to occur in older women, are found more often in the proximal colon, present at high tumour (T-stage) stage and are considered common tumours derived from the serrated pathway (3, 39). Histologically, this group of cancers may show features characteristic of serrated cancers (8) (epithelial serrations, eosinophilic and abundant cytoplasm, chromatin condensation and lack of necrosis and mucin production) and the most common precursor lesions seen are MVHPs (16, 20) or SSA (39). Molecularly, this group of cancers have BRAF mutation and show aberrant DNA hypermethylation leading to silencing of key genes, such as MLH1, which leads to development of dysplasia and microsatellite instability (3, 30, 39). These tumours are usually higher grade but have a favourable prognosis when compared with stage-matched controls (40-43).

Group B: MSS (BRAF mutated/KRAS wild type/CIMP+ve)

Group B accounts for 8% (3) of CRCs and similarly have a predilection for proximal colon (3), are often poorly differentiated, exhibit mucinous histology (44), contain signet ring cells (45), have a higher rate of lymphatic, vascular and perineural invasion and lymph node metastases compared than other CRCs (44). Similar to group A, the histology of this group of cancers may reveal features characteristic for SC (3). The most common precursor lesion for this group of cancer is SSA (39). Molecularly, this group of tumours also has BRAF mutation but CIMP in this group leads to hypermethylation of either MGMT or P16, which then leads to dysplasia, but not MLH1, so they do not show microsatellite instability (3, 39). In most series, they have a relatively poorer prognosis (44, 46, 47).

Jass suggested that group B might be a fusion pathway with overlapping features from the two major colorectal cancer pathways, conventional (CIN) and serrated. In these pathways, MGMT serves as a crossover point; fusion of these two pathways may generate lesions with enhanced aggressiveness (48). Recently, Bond et al. (49) found significantly increased levels of overall CIN with advanced stages of presentation, higher number of lymph node involvement and metastases in BRAF mutant/MSS cancer, leading to a hypothesis that CIN

may contribute to progression of this type of cancer. Based on this evidence (49), it can be hypothesised that MAPK initiates tumorigenesis in this group of CRC and further progression is via CIN. The compounding effects of these two different pathways could contribute to the aggressive phenotype and unfavourable outcomes observed in this group of patients.

Group C: MSS (serrated KRAS mutated/BRAF wild type/CIMP+ve)

Group C accounts for 20% (3) of CRCs. The most common precursor lesion is the serrated adenoma (31) Tumours in this group have not been clearly defined due to current difficulty in differentiating KRAS mutated cancers in the serrated pathway from KRAS mutated CIN cancers (3), Hence, there are limited specific clinicopathological data relating to these tumours (27, 50).

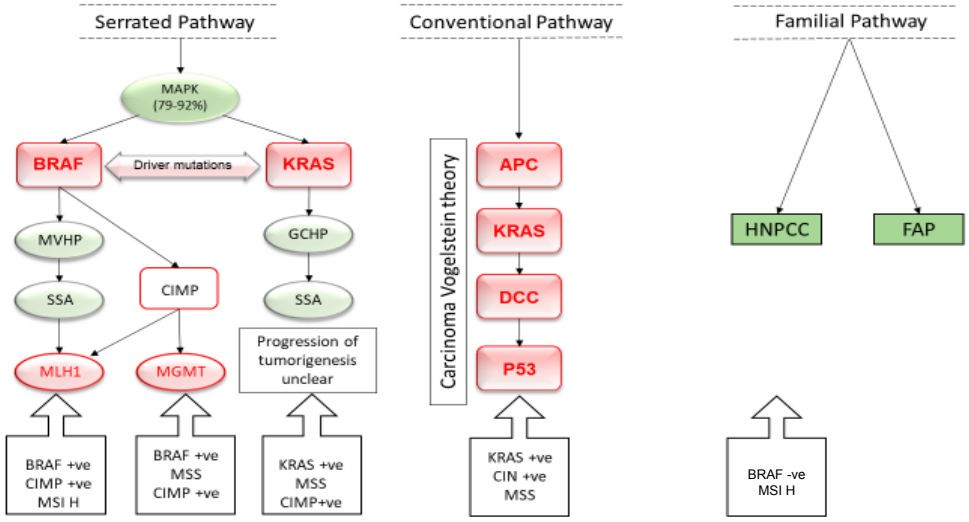
Group D: MSS (CIN; KRAS mutated/BRAF wild type/CIMP-ve)

Group D accounts for the majority of CRCs at 57% (3). They have been well described by the adenoma to carcinoma sequence (3, 5). Most are sporadic but this group includes cancers associated with FAP (familial adenomatous polyposis) (3).

Group E: MSI (BRAF wild type/CIMP-ve)

Group E accounts for 3-5% of CRCs (3) and are Lynch syndrome tumours. They are due to germline mutation of DNA mismatch repair gene and demonstrate MSI (3). Tumours in this group are MSI-H but do not have BRAF V600E mutation (3). BRAF mutation is a surrogate marker for hypermethylation (CIMP). The presence of BRAF mutation virtually excludes Lynch syndrome (51, 52). Histology of this group of cancers may reveal features characteristic for SC (8), tend to affect a younger population (51, 53) and have good prognosis when compared with stage-matched controls (54).

Figure 1 – Colorectal tumorigenesis and subgroups



This classification system of Jass et al. has changed the understanding and perspectives of the serrated cancer pathway. However, there are still gaps in knowledge in the tumorigenic process in SC. The main cause of epigenetic silencing of key genes remain unclear even though the BRAF theory is commonly accepted. There are no molecular markers with which to identify different KRAS mutated cancers, and future studies to completely profile the genome with may help understand these missing links.

Prognostic value of individual molecular mutations in serrated cancer

Given the varying molecular pathways leading to CRC (3), it could be predicted that prognoses of patients will vary according to presence or absence of different molecular mutations. Thus, knowing the molecular characteristics of a patient's tumour aids personalised cancer treatment, increasing the ability to prognosticate accurately and to guide treatment effectively.

The individual markers of the serrated pathway (BRAF, KRAS and MSI) have been studied for their prognostic value but this is yet to fully established for stage III CRC. Most studies have shown that patients with early stage MSI CRC have a better 5-year survival compared to microsatellite stable (MSS) cases, with about half the risk of death (55, 56). A meta-

analysis evaluating MSI status reported a more favourable prognosis across all stages of CRC (43). However, more recent studies have found no difference in prognosis based on MSI status alone especially in stage III disease (55-57).

Several studies have shown that patients with BRAF mutated cancer do poorly (58-60). The presence of BRAF mutation in MSI colorectal cancer distinguishes sporadic MSI cases from Lynch syndrome (52, 61). However, few studies have examined the prognostic value of BRAF in combination with MSI. Interestingly, even though there is high incidence of BRAF mutation in sporadic MSI cancer (range 13-78%) (47, 62, 63) in comparison to MSS tumour (<10%) (47, 62, 63), the poor prognosis of BRAF mutation in MSS CRCs does not appear to have the same effect in MSI CRCs (64). The molecular mechanisms underlying this disparity in prognosis are currently unknown.

Two studies that evaluated the prognostic role of KRAS mutations found no prognostic impact in non-metastatic CRCs patients (65, 66). However, in a recent Korean study KRAS mutation was found to have an adverse prognostic impact on stage II or III CRC patients (67). However, these studies included all KRAS mutated cancers. It is postulated that KRAS mutation occurs in the serrated pathway as well as in the CIN pathway, and no one has attempted to differentiate them prognostically due to difficulty in differentiating KRAS mutated cancers formed via the serrated pathway from KRAS mutated cancers formed via the CIN pathway.

According to the Jass classification, molecular combination may signify different pathways and, therefore, BRAF, KRAS and MSI mutations may provide greater prognostic information when assessed in combination. However, it is not known if this information will have impact on routine clinical and surgical practice.

Predictive role of individual molecular mutations in serrated cancer

While 5-FU based adjuvant chemotherapy has been shown to reduce the risk of recurrence (68, 69) in CRC, it only leads to 8-10% improvement in overall 5-year survival in stage III CRC (70, 71), and even less in stage II CRC (55). Given cancer biology influences disease progression, it may also influence response to chemotherapy (3, 72, 73). This gives rise to

the question of whether individualised therapy for the patient based on a better understanding of CRC tumour biology may avoid the toxicity, cost and inconvenience associated with over treatment of patients who may get no additional benefit from adjuvant chemotherapy.

While most recent research has tried to identify factors other than stage to predict who will gain benefit, a consistent molecular marker has not been identified. There is equivocal evidence in the literature regarding the predictive value of MSI. Evidence from earlier in vitro studies showed that inactivation of the MMR system can result in resistance, or rather, tolerance to 5-FU treatment (74-76). However, the mechanism underlying this effect is unclear. Some early non-randomised retrospective studies actually showed greater chemotherapy benefit in MSI-H cases (40, 41, 77) but may have only highlighted the better prognosis seen in MSI-H cases (78, 79). In 2003, Ribic et al. used retrospectively analysed data from five randomised controlled trials (RCTs) and found a trend towards MSI showing less benefit from 5-FU based chemotherapy (42). This led to a change in practice, with tumours showing MSI not always offered adjuvant 5-FU chemotherapy. Subsequently in 2010, Sargent et al. pooled data from the Ribic et al. study with the addition of 457 further cases and found significant results (42, 80). In addition, the Sargent group found that stage II MMR deficient (MSI) cases had a decreased overall survival in the chemotherapy group relative to the MSS group, however, no detrimental effect was observed in stage III cases (80). In contrast to the above two studies, reanalysis of data from two large RCT and other work found MSI status does not affect chemotherapy responsiveness (57, 72, 81). These highly heterogeneous studies with conflicting results have contributed to confusion about MSI status. Importantly, the use of MSI as a single molecular marker does not independently explain the heterogeneity seen in SC.

Furthermore, cancers associated with sporadic MSI and MSI associated with Lynch syndrome behave differently. Lynch syndrome patients usually have early-onset colon cancer (51, 53). This has been suggested by recent work, with Sinicrope et al. reanalysing data from previous RCTs with patients from the Ribic et al. and Sargent et al. studies (57, 72, 81, 82). MSI cancers were found to benefit from chemotherapy, though, the benefit was

limited to the presumed germline cases (82). However, Sinicrope et al. did not have molecular confirmation of Lynch syndrome in all patients. BRAF V600E was confirmed in some, while others were only assumed to be Lynch if there was MLH1 loss in patients under 55 years at diagnosis. Based on these findings, it can be hypothesised that BRAF mutation may impact on the responsiveness of MSI-H sporadic cancer to chemotherapy. This however, requires further validation.

Only a few studies have examined the impact of BRAF mutation alone on the efficacy of adjuvant chemotherapy for CRC (72, 73, 83). Hutchins et al. assessed BRAF mutation in predicting the recurrence and benefit from chemotherapy in stage II CRC from the QUASAR trial (n = 1584). In this trial patients were randomly assigned to chemotherapy (5-FU and folinic acid) and no chemotherapy groups. BRAF mutation was found not to be predictive of chemotherapy benefit (recurrence used as an outcome measure) (72). However, this study had limited statistical power due to fewer recurrences in BRAF mutated cancers (risk ratio = 0.86, 95% CI 0.57-1.22, P = 0.36) (72).

Ogino et al. found no significant trend for better survival with combinations of fluorouracil or leucovorin with irinotecan in stage III CRC patients with BRAF V600E mutation, compared to patients without BRAF mutation (HR = 0.52, 95% CI 0.25-1.10) (83).

The MRC FOCUS trial examined the impact of BRAF mutation on overall survival (OS) and progression-free survival (PFS) with use of 5-FU, either with oxaliplatin or irinotecan in advanced colorectal cancer. This study found that BRAF mutation status does not affect the impact of irinotecan or oxaliplatin on PFS or OS and concluded that BRAF mutation is not a predictive biomarker of differential benefit (73). However, the low frequency of BRAF mutation this study reduced its power to detect or exclude any BRAF specific effect. Given the lack of evidence for an adjuvant predictive role of BRAF mutation in CRC, further studies are required to establish its role as a predictive biomarker.

KRAS mutation predicts resistance to anti-epidermal growth factor receptor (EGFR) therapy in the metastatic setting (84, 85). The role of anti-EGFR therapy in adjuvant setting has not been established due to high toxicity and lack of benefit reported in various studies (65, 66).

The literature on the predictive role of KRAS mutation in non-metastatic CRC to 5-FU is conflicting (86, 87). Results from an CALGB 89803 adjuvant chemotherapy trial (5-fluorouracil or leucovorin with or without irinotecan) found that the effect of adjuvant chemotherapy did not differ according to KRAS mutation status, adjusting for all clinicopathological parameters and chemotherapy (65). In contrast to CALGB study, a single centre retrospective study, assessing the predictive role of KRAS mutation to adjuvant 5-FU chemotherapy, found adjuvant chemotherapy improved 3-year disease-free survival only among patients with KRAS mutant tumours (with and without chemotherapy, 78.0 and 69.2%, respectively) (88). These results remained significant even on multivariate analysis (HR 0.454, 95% CI 0.229-0.901, P = 0.024) (88). There are no studies, however, that attempted to differentiate KRAS mutated tumours based on its pathway of origin (CIN pathway or from KRAS serrated pathway), and as previously stated differentiating these groups is difficult.

Conclusions

Most colorectal cancer biomarkers still have limited utility in guiding adjuvant therapy. Thus far, only tumour stage and associated validated pathological prognostic parameters have been shown to be useful in guiding treatment strategy. It is still hoped that further research, possibly using molecular combinations, may provide useful markers to guide therapy.

Chapter 2

Article 2

Prognostic value of BRAF mutation alone and in combination with microsatellite instability in stage III colon cancer

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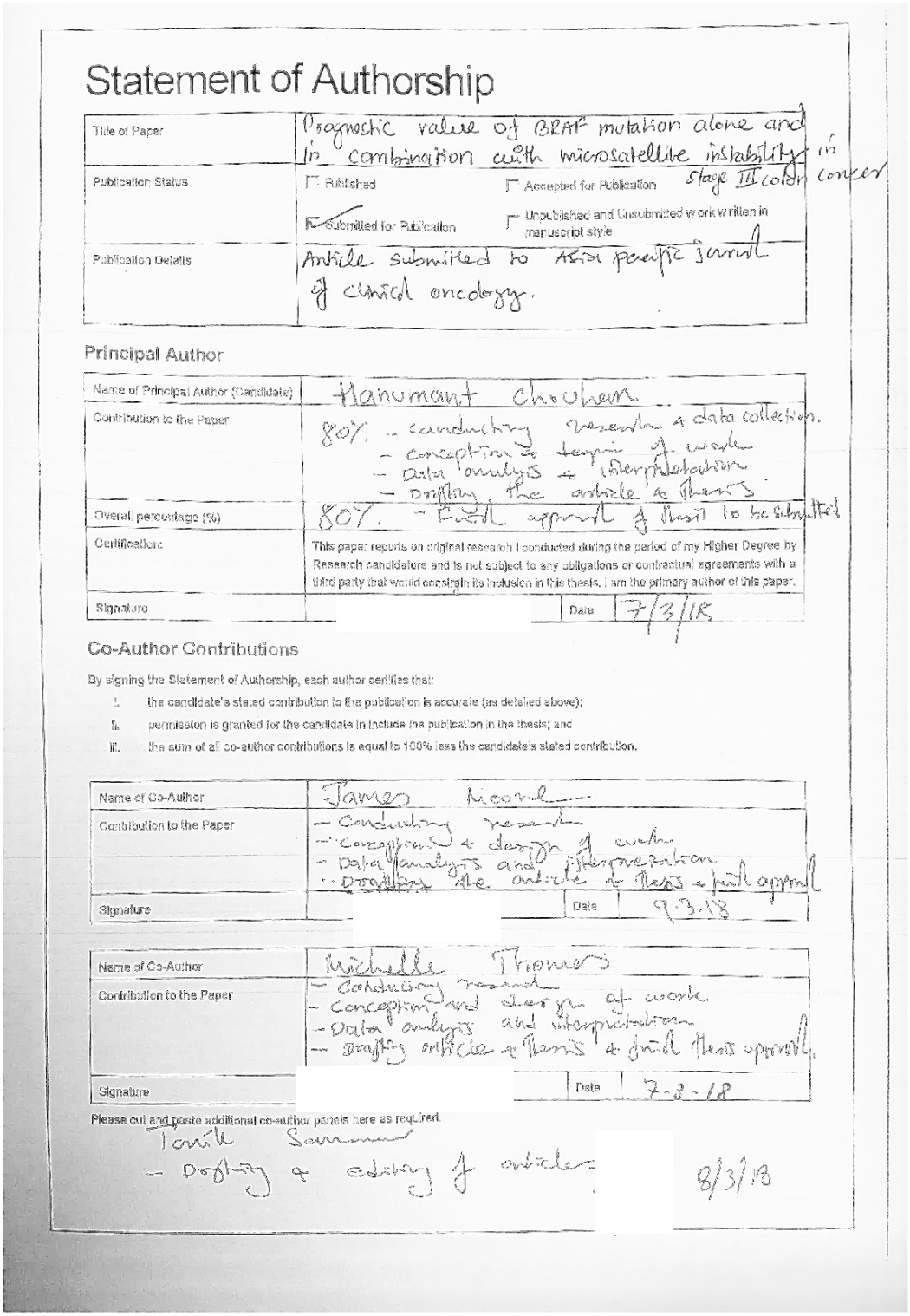
Statement of authorship



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Overall percentage (%)	80% - Final approval of thesis to be submitted
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 7/3/18

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 to 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.

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Abstract

Purpose: The prognostic value of biomarkers in colorectal cancer is still being defined. This study aimed to determine the prognostic value of BRAF mutation alone, and in combination with microsatellite instability (MSI), in stage III colon cancer.

Methods: Curatively resected stage III colon cancers were studied from a 33-year period. Clinicopathological data was collated (adjuvant chemotherapy, age, gender, obstruction, perforation, tumour location, grade, presence of mucin, nodal stage, extramural vascular and perineural invasion). MSI status was established and molecular testing for BRAF (V600E) was performed. Four mutation categories were examined: MSS and BRAF-ve (termed *"traditional*"), MSI and BRAF-ve (termed "*presumed Lynch*"), MSI and BRAF+ve (termed "*sporadic MSI*") and MSS and BRAF+ve (termed "*other BRAF*"). The endpoint was cancer specific survival.

Results: A total of 686 unselected cases met our inclusion criteria, of which 15.7% had a BRAF mutation and 13.8% showed MSI. On adjusted analysis, neither BRAF mutation nor MSI mutation were independently prognostic. On univariate analysis, survival in *presumed Lynch* cancers was similar to *traditional* cancers (5-year survival, 62 and 61%, respectively). While there was no difference in cancer specific survival between *sporadic MSI* and *other BRAF*, both these tumour groups had poorer outcome when compared to *traditional* or *presumed Lynch* cancers. Adjusted analysis of the four groups, however, showed that none of the subgroups were independently prognostic.

Conclusions: BRAF mutated cancers demonstrated a trend towards poorer outcomes, however, when adjusted for clinicopathological factors and chemotherapy, BRAF mutation was not found to be an independent prognostic biomarker in stage III colon cancer, even when combined with MSI.

Introduction

The future of personalised oncology relies on accurate prognostication of colorectal cancer (CRC). Understanding of colorectal tumorigenesis has evolved over the last two decades and CRC is now considered to be a multi-pathway disease. The chromosomal instability (CIN) pathway, adenoma to carcinoma sequence, leads to aneuploidy, oncogene activation and loss of tumour suppressor genes. While most CRCs exhibit CIN (60-70%), about 15-30% of cancers develop via an alternative serrated pathway of tumorigenesis. These cancers are formed via activation of the mitogen-activated protein kinase pathway (MAPK), and exhibit either BRAF or KRAS mutation (part of the RAS-RAF-MAP2K signalling pathway), a major driver of serrated cancer tumorigenesis. They may also demonstrate DNA CpG island hypermethylation (CIMP), with or without microsatellite instability (MSI) (3). It would seem that the CIN and serrated pathways are mutually exclusive, although the molecular biology of tumours involving the serrated pathway is very heterogeneous, and not yet completely characterised (3).

Most studies have shown that patients with early stage MSI CRC have a better 5-year survival compared to microsatellite stable (MSS) cases, with about half the risk of death (55, 56). Studies have also shown that patients with BRAF mutated cancer do poorly (59, 60). The presence of BRAF mutation in MSI colorectal cancer distinguishes sporadic MSI cases from Lynch syndrome, however, whether presence or absence of MSI has a prognostic impact on BRAF mutated cancers is yet to be established. The aim of this study was to determine prognostic value of both the BRAF mutation and the combination of BRAF/MSI in curatively resected stage III colon cancer.

Methods

This was a retrospective cohort study, comparing survival in patients with curatively resected stage III colon adenocarcinoma, looking for a compounding effect from clinical and pathological parameters and mutational status (BRAF, MSI or combination of BRAF plus MSI). All identifiable patients treated for colonic adenocarcinoma within the Central and Northern Adelaide Local Health Network over a 33-year period were screened for inclusion.

Patient data were derived and collated from regional and national cancer registries and pathology databases. Pathology reports were reviewed, and pathology reassessed from slides archived from the earlier cases to meet current reporting protocols. Patients were excluded if death occurred perioperatively, if surgical margin was positive for tumour (R1 resection), or if they had metachronous colorectal cancer within 5 years. Synchronous cancers were included. Appropriate ethics approval was obtained from the Royal Adelaide Hospital Human Research Ethics Committees (approval number 140108).

A collated clinicopathological database was created including data regarding patient age, gender, and whether they received adjuvant chemotherapy. Tumour characteristics, including obstruction (defined as proximal dilatation on the pathology report), perforation (defined as occurring through the tumour on the pathology report) and tumour location, were recorded. Proximal cancers were defined as cancers from the caecum to the splenic flexure inclusive and distal cancers defined as tumours distal to the splenic flexure. Rectal cancers were excluded. Histologic features including grade (well and moderately differentiated tumours were grouped together and poor differentiation recorded separately), mucinous component (positive if mucin present), T-stage (T1 and T2 grouped together, and similarly T3 and T4), nodal stage (N1 and N2), extramural vascular and perineural invasion were recorded. Death data were sourced from the South Australian State Cancer Registry, hospital cancer registry, patient case notes and by checking patients' final clinic visits. Hospital and state cancer registries are regularly updated with information from national databases to capture interstate deaths. Case notes were reviewed if required to capture missing data.

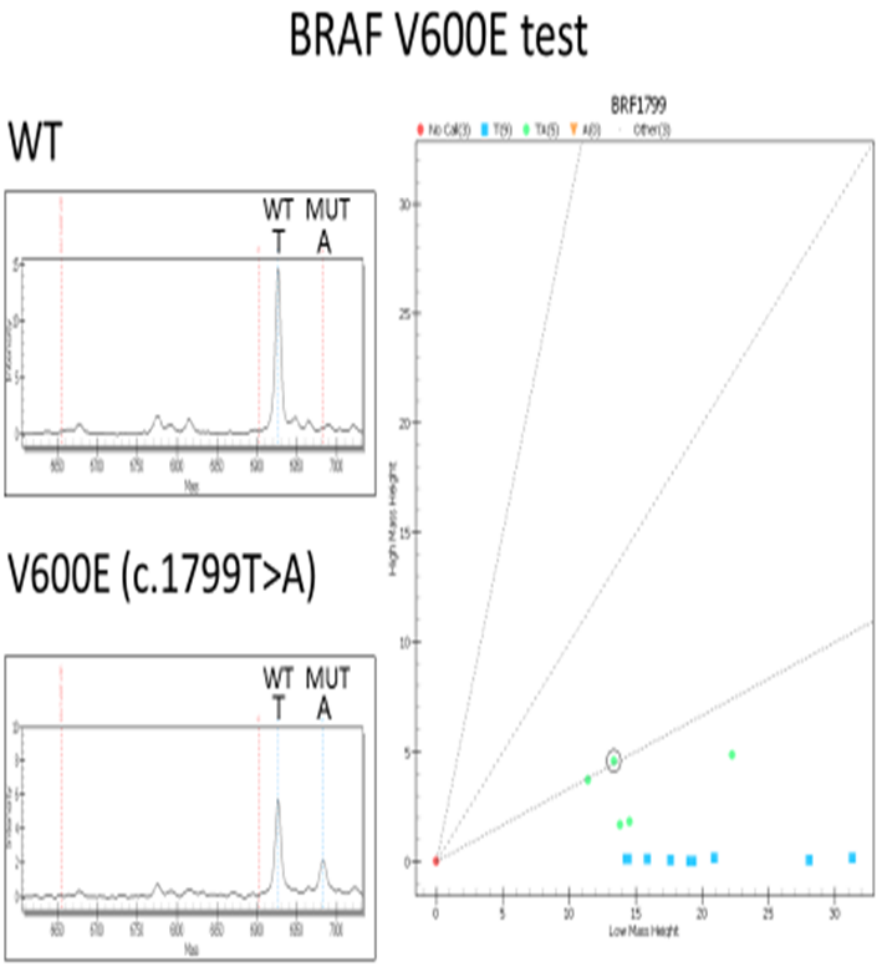
MSI status was established on formalin fixed paraffin embedded (FFPE) archived specimens from 1980 to 2002 by determining instability in mononucleotides sequences BAT40 and BAT26, the latter being highly specific for MSI high. The complete National Cancer Institute (NCI) panel was run on equivocal cases with MSI defined by instability in two or more markers. From 2002 onwards, MSI status has routinely been established on all through IHC expression of DNA mismatch repair (MMR) proteins and by molecular testing for the full NCI panel of markers (BAT25, BAT26, BAT40, D2S123, D10S197, D17S579, D18S34, D5S346

and D17S250). Tumours showing instability at two or more loci were defined as microsatellite unstable (MSI). Tumours showing no abnormal loci or instability at one locus were classified as microsatellite stable (MSS).

Mutation testing was performed on DNA isolated from paraffin-embedded tumour tissue. BRAF V600E (GRCh37/hg19, chr7:g.140,453,136A>T; NM_004333, c.1799T>A; p.Val600Glu) mutation was detected by the analysis of single base primer extension products as part of a larger screen on genomic DNA using chip-based MALDI-TOF mass spectrometry (Sequenom Inc., San Diego, CA, USA). The assay was designed to enable detection of both reference and mutant alleles, and quantification of mutant allelic load. In addition to colorectal cancer samples, samples positive and negative for BRAF (V600E) (using Sanger sequencing and/or a diagnostic Sequenom panel at 5% sensitivity performed under NATA accredited conditions) were included as controls.

Typer software (Version 4.0, Sequenom) was used to analyse the mass spectra (Figure 2). The software generated automated mutation calls using the default computational algorithms for SNP genotyping. Operator calling was performed blind (the operator being unaware of previous results) by optical inspection of the Typer Yield Call Cluster Plot, which compares the area under the curve of the extension product peak with that of the unextended primer peak (yield). Patient samples were called positive if they met the following criteria: negative control samples were clustered close to the origin, (2) plasmid standards were positioned away from the negative cluster, and (3) the positive control samples were confirmed to be positive (89).

Figure 2 – Typer software generated automated mutation calls



Statistical analyses were performed using the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The primary study endpoint was cancer specific survival. Non-cancer deaths were censored. Associations between BRAF, MSI and pathological factors were determined by logistic regression. Univariate analysis was performed using Kaplan-Meier survival curves and compared by a log rank test. Cox regression proportional hazard models were used for multivariate analysis with backward elimination. Cancer specific survival was compared among the groups MSI, BRAF and BRAF/MSI subgroups, and chemotherapy adjusted by various confounders. Four tumour categories were examined: MSS and BRAF-ve (termed "*traditional*"), MSI and BRAF-ve (termed "*presumed Lynch*"), MSI and BRAF+ve (termed "*sporadic MSI*") and MSS and BRAF+ve (termed "*other BRAF*"). The presence of a

BRAF (V600E) mutation confirms *sporadic MSI* rather than germline (52, 61). However, given we did not perform testing to identify the actual MMR mutation these germline MSI cases are labelled *presumed Lynch*. Backwards elimination was performed on each model to find a parsimonious model with all global $P < 0.1$ (except for the BRAF/MSI variate, which was kept in the model).

Results

Eight hundred and seventy-nine patients with stage III colon cancer were identified. Of these, 284 were excluded, 157 because insufficient tissue was available for molecular testing, 27 died in hospital perioperatively, five had metachronous cancers and four had a positive margin. This left 686 cases for analysis. Median follow-up was 52 months (0.4-329). BRAF mutation was identified in 15.7% (107/681) of tumours (some data for five patients were missing) and MSI observed in 13.8% (95/686).

Table 1 shows clinicopathological differences between BRAF+ve and BRAF-ve cancers. BRAF+ve cancers were more likely to be proximal tumours (84%) and poorly differentiated (45%), and to have a mucinous component (71%), extramural vascular invasion (66.4%) and higher nodal stage (71%, i.e., N2 disease).

Table 2 compares the BRAF/MSI subgroups. MSI cancers had a higher female preponderance regardless of BRAF status (63.8% *presumed lynch* and 73% *sporadic MSI*). *Presumed lynch*, *other BRAF* and *sporadic MSI* cancers had a higher rate of poor differentiation, were more likely to contain mucin and were more likely to be proximal compared to *traditional* cancers. The *other BRAF* group of cancers (MSS) had higher rate of extramural vascular invasion (70%) and perineural invasion (30.4%) compared to other groups.

Table 1 - Comparison of clinicopathologic characteristics of BRAF+ve and BRAF-ve patients

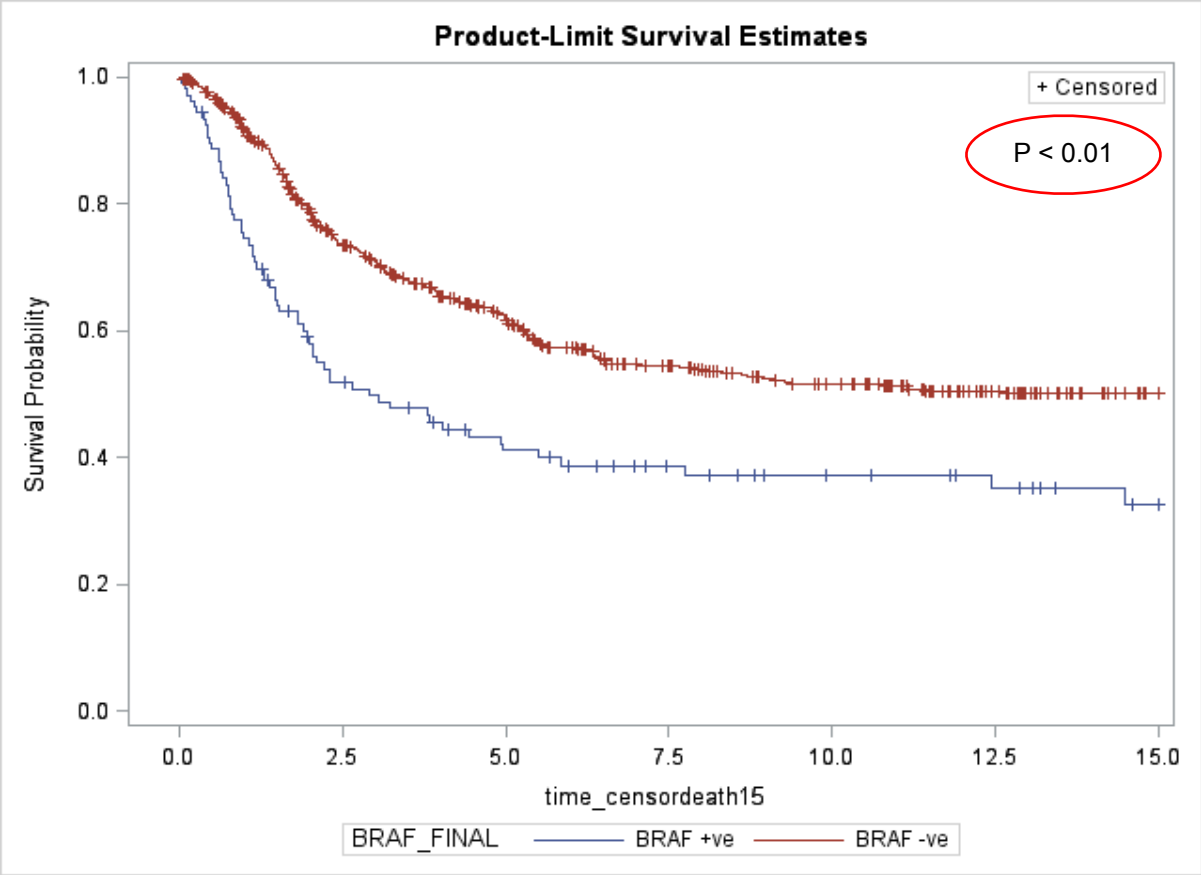
Subgroup	Subcategory	BRAF+ve	BRAF-ve	P
Median age		73.0	71.7	0.09
Number		107	574	
Death		65 (73%)	246 (59.3%)	0.02
Chemotherapy		38 (36%)	233 (40.6%)	0.32
Gender	Male	45 (42%)	286 (49.8%)	0.14
	Female	62 (57%)	288 (50.2%)	
Proximal location		89 (84%)	275 (48.4%)	<0.01
Poor differentiation	Poor	49 (46%)	93 (16.2%)	<0.01
Type	Mucinous component	76 (71%)	183 (31.9%)	<0.01
Nodal stage	N1	31 (29%)	391 (68.1%)	<0.01
	N2	76 (71%)	183 (31.9%)	
T-stage	T1 + T2	3 (3%)	31 (05.4%)	0.34
	T3 + T4	104 (97%)	543 (94.6%)	
Extramural		71 (66%)	308 (54.2%)	0.02
Perineural		27 (26%)	102 (18.0%)	0.06
Obstruction		24 (23%)	102 (17.9%)	0.23
Perforation		10 (10%)	25 (4.4%)	0.03

Table 2 - Comparison of clinicopathologic characteristics of the four MSI/BRAF subgroups

Subgroup	Subcategory	*Traditional*	*Presumed Lynch*	*Sporadic MSI*	*Other BRAF*	P
Median age		71.7	72.0	75.4	71.6	0.01
Number		516 (75.8%)	58 (8.4%)	37 (5.4%)	70 (10.3%)	
Chemotherapy		210 (40.4%)	23 (39.7%)	9 (24.3%)	29 (44.4%)	0.26
Gender	Male	251 (48.6%)	37 (63.8%)	27 (73.0%)	35 (50.0%)	0.007
	Female	265 (51.4%)	21 (36.2%)	10 (27.1%)	35 (50.0%)	
Proximal location		225 (44.1%)	50 (86.2%)	34 (91.9%)	55 (79.7%)	<0.01
Poor differentiation	Poor	63 (12.2%)	30 (51.7%)	23 (62.2%)	26 (37.1%)	<0.01
Type	Mucinous component	145 (28.1%)	38 (65.5%)	28 (75.7%)	48 (68.6%)	<0.01
Nodal stage	N1	381 (73.8%)	41 (70.7%)	22 (59.9%)	39 (55.7%)	<0.01
	N2	135 (26.2%)	17 (29.3%)	15 (40.5%)	31 (44.3%)	
T-stage	T1 + T2	30 (5.8%)	1 (1.7%)	0 (0%)	3 (4.3%)	0.36
	T3 + T4	486 (94.2%)	57 (98.0%)	37 (100%)	67 (95.7%)	
Extramural		280 (54.9%)	28 (48.3%)	22 (59.5%)	49 (70.0%)	0.06
Perineural		96 (18.9%)	6 (10.3%)	6 (16.7%)	21 (30.4%)	0.03
Obstruction		94 (18.4%)	8 (13.8%)	5 (13.9%)	19 (27.5%)	0.16
Perforation		25 (4.9%)	0 (0%)	4 (11.4%)	6 (8.6%)	0.05

Univariate analysis indicated a statistically significant difference in cancer specific survival between BRAF+ve vs BRAF-ve cancers, with BRAF+ve cancers having shorter survival than BRAF-ve cancers (Figure 3).

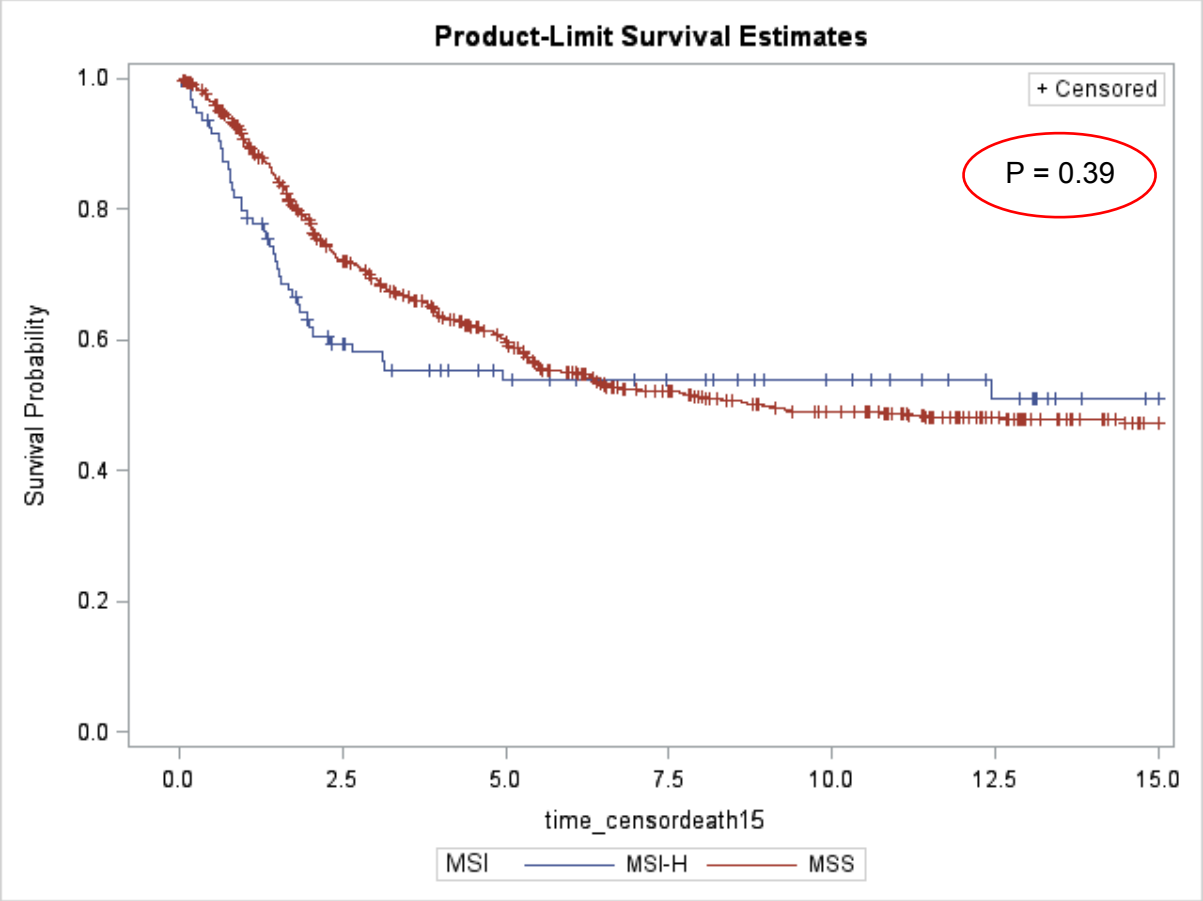
Figure 3 – Kaplan-Meier curve comparing cancer specific survival between BRAF+ve and BRAF-ve cancers



At 5 years	Survival	Failure	Number fail	Number left
BRAF+ve	0.41	0.58	60	36
BRAF-ve	0.61	0.38	199	276

There was no difference in cancer specific survival between MSI and MSS cases in this cohort of node positive patients (Figure 4).

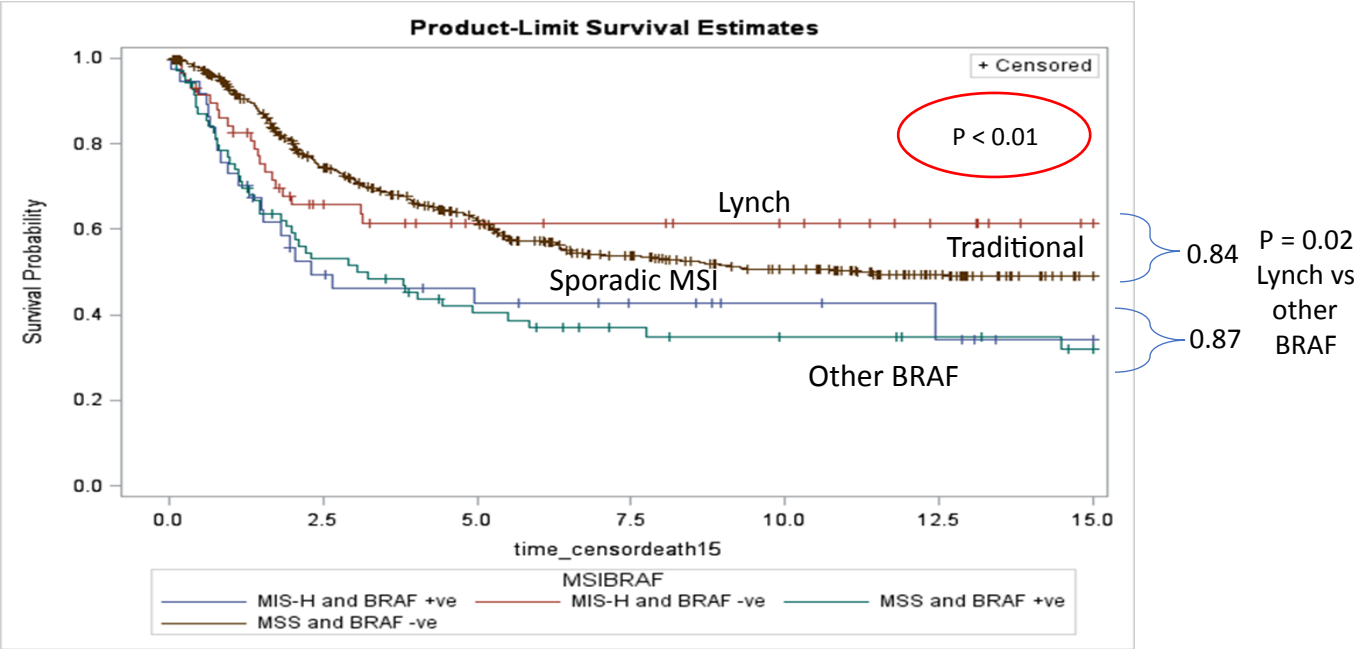
Figure 4 - Kaplan-Meier curve comparing cancer specific survival between MSI and MSS cancers



At 5 years	Survival	Failure	Number fail	Number left
MSI	0.53	0.46	41	35
MSS	0.59	0.40	218	280

When the four subgroups are examined (Figure 5) there was a statistically significant difference in cancer specific survival across the groups. *Presumed Lynch* and *traditional* cancers had a similarly good survival compared to the BRAF cancers, both *sporadic MSI* and *other BRAF*, which were similar.

Figure 5 – Kaplan-Meier curve comparing cancer specific survival between four MSI/BRAF subgroups



At 5 years	Survival	Failure	Number fail	Number left
Lynch	0.61	0.38	21	28
Traditional	0.62	0.37	178	253
Sporadic MSI	0.42	0.57	20	12
Other BRAF	0.40	0.59	40	24

On the multivariate model (Table 3, adjusting for chemotherapy, age, T-stage, nodal stage, obstruction, perforation, extramural and perineural invasion), neither BRAF nor MSI status was prognostically significant. There was a trend towards poorer outcomes in BRAF+ve (HR 1.27, 95% CI 0.89-1.80, P = 0.18) and MSI (HR 1.17, 95% CI 0.75-1.83, P = 0.48) cancers.

Table 3 - Cox proportional hazard model, adjusting for clinicopathological factors, BRAF and MSI

Variate	HR	95% CI	P
BRAF+ve	1.27	0.89 - 1.80	0.18
MSI	1.17	0.75 - 1.83	0.48
Chemotherapy	0.66	0.49 - 0.88	<0.01
N1	0.50	0.39 - 0.66	<0.01
T1 + T2	0.39	0.14 - 1.07	0.06
Extramural	1.67	1.26 - 2.21	<0.01
Perineural	1.67	1.22 - 2.29	<0.01
Obstruction	1.43	1.06 - 1.91	0.01
Perforation	2.20	1.45 - 3.34	<0.01

Backward stepwise elimination, excluding non-significant variates >0.1, i.e., no gender, grade, location and type.

Adjusted analysis on the four subgroups showed that none were independently prognostically significant despite the univariate findings (Table 4). Notably the BRAF+ve MSS (*other BRAF*) cancers did not have a poorer outcome after adjustment for other factors. In an adjusted model comparing *other BRAF* to all other tumours as a single group, there was no significant difference in cancer specific survival (HR 0.78, 95% 0.55-1.10, p = 0.15).

Table 4 - Cox proportional hazard model, adjusting for clinicopathological factors and four subgroups

Multivariate analysis of four subgroups			
Variate	**HR**	**95% CI**	**P**
Non-Lynch vs Lynch	1.02	0.65 - 1.61	0.90
Non-sporadic MSI vs sporadic MSI	0.94	0.58 - 1.53	0.81
Non-other BRAF vs other BRAF	0.78	0.55 - 1.10	0.15
Non-traditional vs traditional	1.14	0.86 - 1.51	0.34

Excluding non-significant variates >0.1

Discussion

This was a retrospective cohort study investigating the impact of BRAF mutation (with and without MSI) on survival. BRAF mutation was associated with poorer survival when compared to BRAF wild type on univariate analysis. However, on multivariate analysis, there was no statistically significant relationship between BRAF and survival regardless of MSI status in this group of node positive colon cancer patients.

The BRAF V600E mutation occurs at a high frequency in serrated cancer and can help distinguish inherited from sporadic cancers (52, 61). Similar to previous reports (59), we found that BRAF mutation was associated with some adverse features, including poor differentiation, presence of mucin, extramural invasion and high nodal stage, suggesting BRAF mutated cancer will do poorly. However, when multivariate analysis was conducted on stage III colon cancer patients only, we found that BRAF mutation was not an independent prognostic biomarker. Published data have been conflicting (50, 60, 64). A meta-analysis of 26 colorectal cancer studies (published in 2013) suggested that BRAF mutation is independently associated with poor survival (59). However, most of the studies analysed were heterogenous, with not all studies adjusting for clinicopathological variables and chemotherapy, and many including cancers at all stages as well as rectal cancer cases. In our study, MSI status was not found to be prognostically significant in stage III colon cancer, on adjusted analysis. It may be that the inclusion of stage III cases only influenced the results, suggesting that the favourable impact on survival of MSI reported in previous studies might be in early-stage disease only (55, 56).

We also investigated whether the combination of BRAF mutation and MSI status, might provide useful prognostic information beyond the evaluation of either factor alone. Consistent with previous studies (47, 83, 90), adjusted analysis of the *other BRAF* group (BRAF+ve/MSS) with poor histological features revealed non-significant trend towards a poor outcome (Table 4). The inability in our study to reach statistical significance, despite such a big difference in survival rates, may reflect the low number of patients with this

tumour phenotype (BRAF+ve/MSS). We did not observe the better outcome that some others have shown in Lynch patents (55, 56).

The current study has some limitations. The incidence of BRAF mutation in our study was similar to that reported in literature (91), but we found a slightly higher percentage of MSI and *presumed Lynch* cases (8.4%), which likely reflects more comprehensive testing. Data from a large RCT have shown that addition of oxaliplatin to adjuvant 5-FU may further improve prognosis of MSI CRC (92). However, we only included patients who received 5-FU-based regimens to allow for appropriate comparisons. Retrospective data collected from 33 years may contain biases. To overcome this, we included a large number of patients, adjusted for pathological variables and chemotherapy and limited the study to stage III colon cancers. In addition, to ensure accuracy of data in our study, pathology was reassessed from slides the earlier cases to meet current reporting protocols. Clinical information was also extensively cross checked from different sources.

Conclusions

BRAF mutation was associated with poorer survival when compared to BRAF wild type on univariate analysis of stage III colorectal cancer. However, on multivariate adjusted analysis there was no statistically significant relationship between BRAF and survival in stage III colon cancer regardless of MSI status.

Chapter 3

Article 3

The interaction between BRAF mutation and microsatellite instability status in determining survival outcomes after adjuvant 5-FU based chemotherapy in stage III colon cancer

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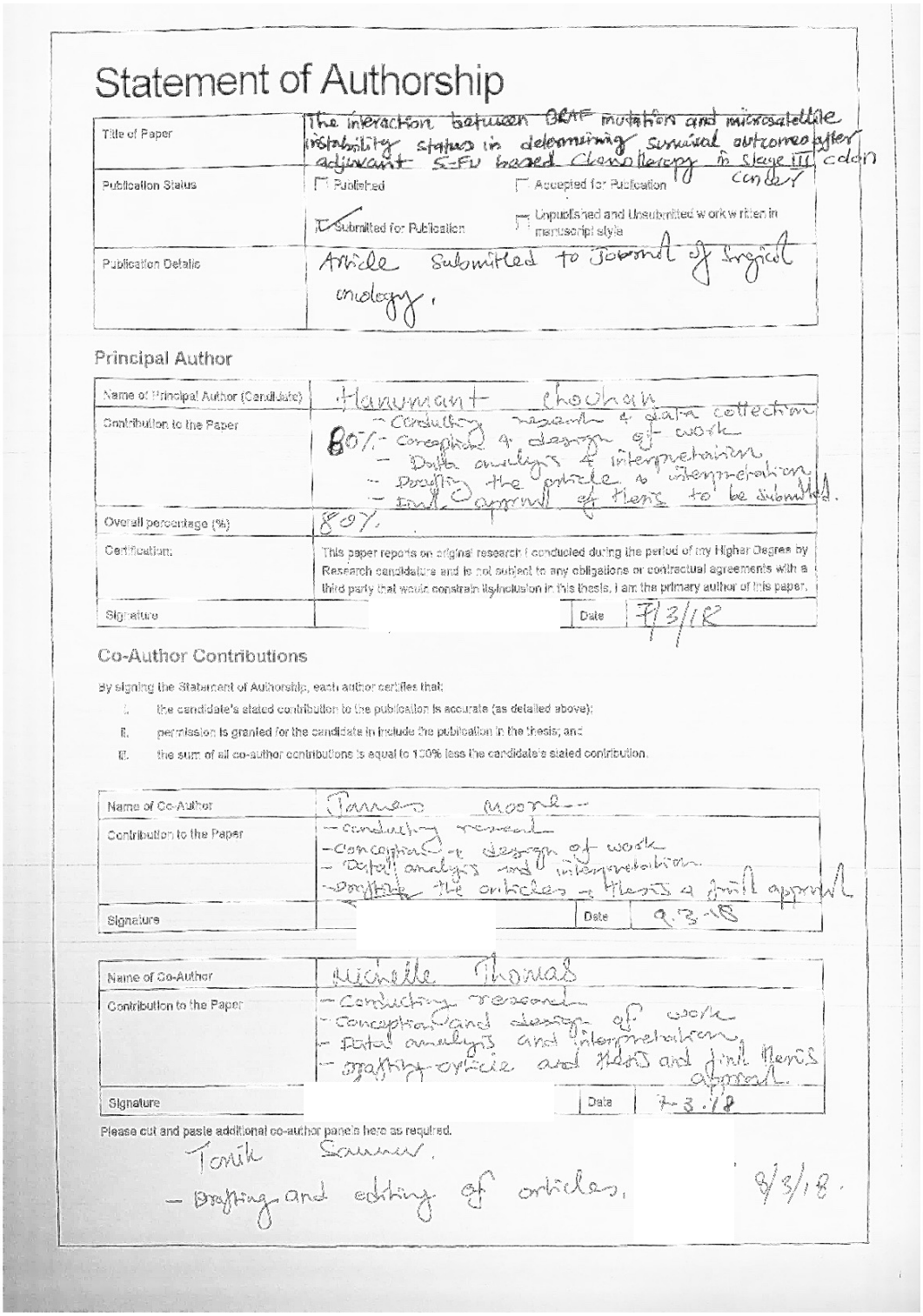

Statement of Authorship

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Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Article Submitted to Journal of Surgical oncology.

Principal Author

Name of Principal Author (Candidate)	Hanumant Chouhan
Contribution to the Paper	80% - Conducting research & data collection - Conception & design of work - Data analysis & interpretation - Drafting the article & interpretation - Final approval of thesis to be submitted.
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 7/3/18

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 to 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.

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- Drafting and editing of articles. 8/3/18.

Abstract

Purpose: The predictive role of biomarkers in colon cancer is still being defined. The aim of this study is to determine the interaction between BRAF mutation and microsatellite instability (MSI) status in determining survival benefit after adjuvant 5-FU based chemotherapy in stage III colon cancer.

Methods: We performed a retrospective cohort study including all curatively resected stage III colon cancer cases over a 33-year period. A clinicopathologic database was collated (adjuvant chemotherapy, age, gender, obstruction, perforation, tumour location, grade, mucin, nodal stage, extramural vascular and perineural invasion). BRAF (V600E) mutation testing was performed and MSI status established by immunohistochemistry for mismatch repair proteins and molecular testing for National Cancer Institute panel markers. Patients were categorised into four groups for comparison: MSS and BRAF-ve (termed "*traditional*"), MSI and BRAF-ve (termed "*presumed Lynch*"), MSI and BRAF+ve (termed "*sporadic MSI*") and MSS and BRAF+ve (termed "*other BRAF*"). The primary endpoint was cancer specific survival. Interaction testing was conducted to determine whether there were different responses to chemotherapy between groups.

Results: A total of 686 unselected cases met inclusion criteria and had tissue available, of which 15.7% had BRAF mutation (BRAF+ve) and 13.8% had MSI. Thirty-nine percent received chemotherapy. Overall, adjuvant chemotherapy produced a cancer specific survival benefit (HR 0.66, 95% CI 0.49-0.88, P < 0.01). On adjusted analysis, neither BRAF nor MSI status were individually predictive of survival benefit. On adjusted analysis specifically of the chemotherapy effect in each subgroup, only patients in the *presumed Lynch* (HR 0.260, 95% CI 0.09-0.80, P < 0.01) and *other BRAF* groups (HR 0.45, 95% CI 0.23-0.87, P < 0.01) had a significant survival benefit from chemotherapy. On interaction testing of subgroups, adjusting for all the clinicopathological parameters, only patients in the *presumed Lynch* group (HR 0.277, 95% CI 0.10-0.75, P < 0.01) gained a differentially greater benefit from chemotherapy than other groups.

Conclusions: In this historical cohort, MSI testing is predictive of response to adjuvant chemotherapy in stage III colon cancer, but only when results are interpreted in combination with BRAF. This supports the role of routine testing for these biomarkers.

Introduction

5-Fluorouracil (5-FU) based chemotherapy regimens are commonly used in the adjuvant treatment of stage III colon cancer and are known to moderately reduce the risk of recurrence and death in this subset of patients (8-10%% improvement in overall survival) (68, 93). Given the potential toxicity, cost and inconvenience associated with chemotherapy treatment, the use of predictive biomarkers to help individualise adjuvant treatment is appealing.

Studies looking at microsatellite instability (MSI) as a predictive biomarker for chemotherapy response are inconsistent. While many studies have suggested that MSI tumours do not respond to 5-FU based chemotherapy (42, 80), and that chemotherapy may even have a detrimental effect on survival, others have found that MSI tumours gain similar survival benefit from adjuvant chemotherapy as non-MSI cancers (57, 72, 81). This has led to variation in the recommended management due to difference in interpretation of the published data.

The role of BRAF V600E mutations is reasonably well established in metastatic colorectal disease. This mutation is responsible for conferring anti-EGFR (epithelial growth factor receptor) antibody resistance and is considered to be partly responsible for the 12-15% of patients who do not respond to anti-EGFR treatment in the palliative setting (94). The National Comprehensive Cancer Network (95) guidelines now recommend BRAF mutation testing in all patients with metastatic colorectal disease (95). In the adjuvant setting, BRAF status has not generally been considered to be a predictive biomarker, however, research in this area has been relatively limited (72, 73, 83).

The aim of this study was to determine the interaction between BRAF mutation and MSI status in determining survival outcomes after adjuvant 5-FU based chemotherapy in stage III colon cancer.

Methods

This was a retrospective cohort study. All identifiable patients treated for colonic adenocarcinoma over a 33-year period within the Central and Northern Adelaide Local Health Network in Australia were screened for inclusion. Adult patients who underwent surgery with curative intent were included. Patients were excluded if death occurred perioperatively, if the surgical margin was positive for tumour (R1 or R2 resection), or if they had metachronous colorectal cancer within 5 years. Synchronous cancers were included. Patient data were derived, linked and collated from regional and national cancer registries and pathology databases. Individual pathology reports were reviewed, and pathology reassessed from slides on the earlier cases to meet current standardised reporting protocols.

A clinicopathologic database was created including data on patient age, gender and whether they received adjuvant 5-FU chemotherapy. Patients receiving other chemotherapy agents were excluded. Tumour characteristics, including obstruction (defined as proximal dilatation on the pathology report), perforation (defined as occurring through the tumour on the pathology report) and tumour location, were recorded. Proximal cancers were defined as cancers from the caecum to the splenic flexure inclusively and distal cancers defined as tumours distal to the splenic flexure. Histologic features, including grade (well and moderately differentiated tumours were grouped together, and poorly differentiated tumours recorded separately), mucinous component (positive if mucin present on histology), T-stage (T1 and T2 grouped together, and similarly T3 and T4), nodal stage (N1 vs N2), extramural vascular and perineural invasion, were recorded. Death data were sourced from the South Australian State Cancer Registry, hospital cancer registry, patient case notes and by checking patients' final clinic visit. Hospital and state cancer registries are regularly updated with information from national databases to capture interstate deaths. Patient case notes were reviewed if required to replace missing data.

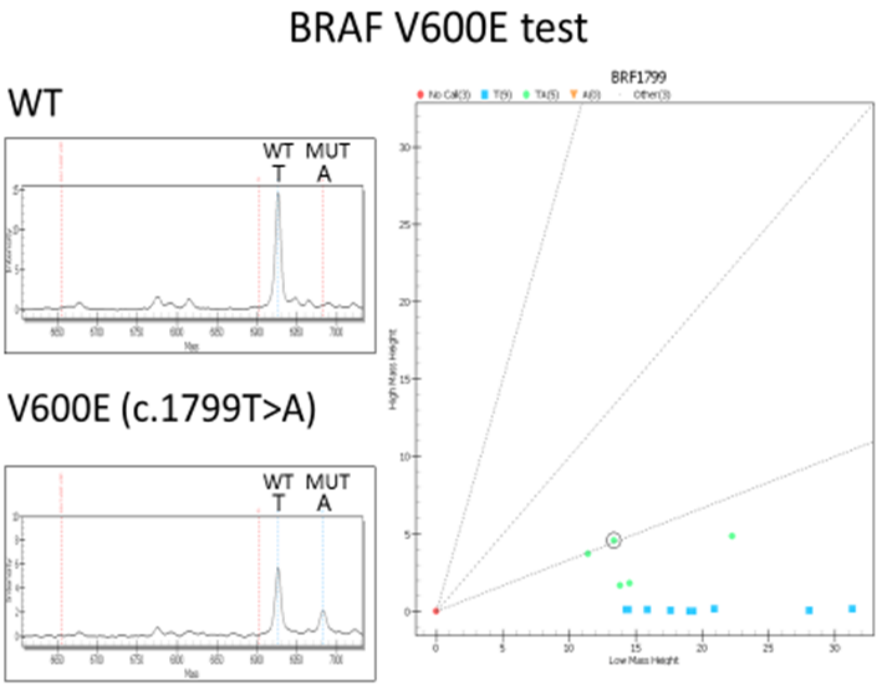
MSI status was established on formalin fixed paraffin embedded (FFPE) archived specimens from 1980 to 2002 by determining instability in mononucleotide sequences BAT40 and

BAT26, the latter being highly specific for MSI high (96). The complete National Cancer Institute (NCI) panel was run on equivocal cases with MSI defined by instability in two or more markers. From 2002 onwards, MSI status has routinely been established on patients through IHC expression of DNA mismatch repair (MMR) proteins and by molecular testing for the full NCI panel of markers (BAT25, BAT26, BAT40, D2S123, D10S197, D17S579, D18S34, D5S346 and D17S250). Tumours showing instability at two or more loci were defined as microsatellite unstable (MSI). Tumours showing no abnormal loci or instability at one locus were classified as microsatellite stable (MSS).

Mutation testing was performed on DNA isolated from paraffin-embedded tumour tissue. BRAF V600E (GRCh37/hg19, chr7:g.140,453,136A>T; NM_004333, c.1799T>A; p.Val600Glu) mutation was detected by the analysis of single base primer extension products as part of a larger screen on genomic DNA using chip-based MALDI-TOF mass spectrometry (Sequenom Inc., San Diego, CA, USA). The assay was designed to enable detection of both reference and mutant alleles, and quantification of mutant allelic load. In addition to colorectal cancer samples, samples positive and negative for BRAF (V600E) (using Sanger sequencing and/or a diagnostic Sequenom panel at 5% sensitivity performed under NATA accredited conditions) were included as controls.

Typer software (Version 4.0, Sequenom) was used to analyse the mass spectra (Figure 6). The software generated automated mutation calls using the default computational algorithms for SNP genotyping. Operator calling was also performed blind (the operator being unaware of previous results) by optical inspection of the Typer Yield Call Cluster Plot, which compares the area under the curve of the extension product peak with that of the unextended primer peak (yield). Patient samples were called positive if they met the following criteria: negative control samples were clustered close to the origin, (2) plasmid standards were positioned away from the negative cluster, and (3) the positive control samples were confirmed to be positive (89).

Figure 6 – Typer software generated automated mutation calls



Statistical analyses were performed using the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Four patient categories were examined: MSS and BRAF-ve (termed "*traditional*"), MSI and BRAF-ve (termed "*presumed Lynch*"), MSI and BRAF+ve (termed "*sporadic MSI*") and MSS and BRAF+ve (termed "*other BRAF*"). The primary study endpoint was cancer specific survival (non-cancer deaths were censored). This was compared between the groups, and associations between BRAF, MSI and pathological factors were determined by logistic regression. Univariate survival analysis was performed using Kaplan-Meier survival curves and compared using a log rank test.

Adjusted analysis was performed using Multivariate Cox Proportional Hazard Models with backward elimination adjusting for the identified confounders. Subgroup analysis was performed on each of the four MSI/BRAF combination groups determining the hazard ratio of chemotherapy effect in that subgroup. From the adjusted model of the whole group, interaction testing was performed to determine if one of the four MSI/BRAF subgroups had a greater effect from chemotherapy than the others by creating an eight-variate model with

each subgroup with or without chemotherapy, with $P < 0.10$ (except for the BRAF/MSI variate, which was retained in the model).

Appropriate ethics approval was obtained from the Royal Adelaide Hospital Human Research Ethics Committees (approval number 140108).

Results

Eight hundred and seventy-nine patients with stage III colon cancer were identified. Of these, 284 were excluded, 157 because insufficient tissue was available for molecular testing, 27 died in hospital perioperatively, five had metachronous cancers and four had a positive margin. This left 686 cases for analysis. Median follow-up was 52 months (0.4-329). BRAF mutation was identified in 15.7% (107/681) of tumours (five patients had missing data) and MSI observed in 13.8% (95/686). Of the 681 patients that had complete molecular profile, 75.8% (516/681) were in the *traditional* group, 8.5% (58/681) were *presumed Lynch*, 5.4% (37/681) were *sporadic MSI* and 10.3% (70/681) were *other BRAF*. Two hundred and seventy-one patients received postoperative adjuvant chemotherapy and 415 patients did not, partly due to inclusion of patients before the routine use of adjuvant chemotherapy (n = 140) circa 1991.

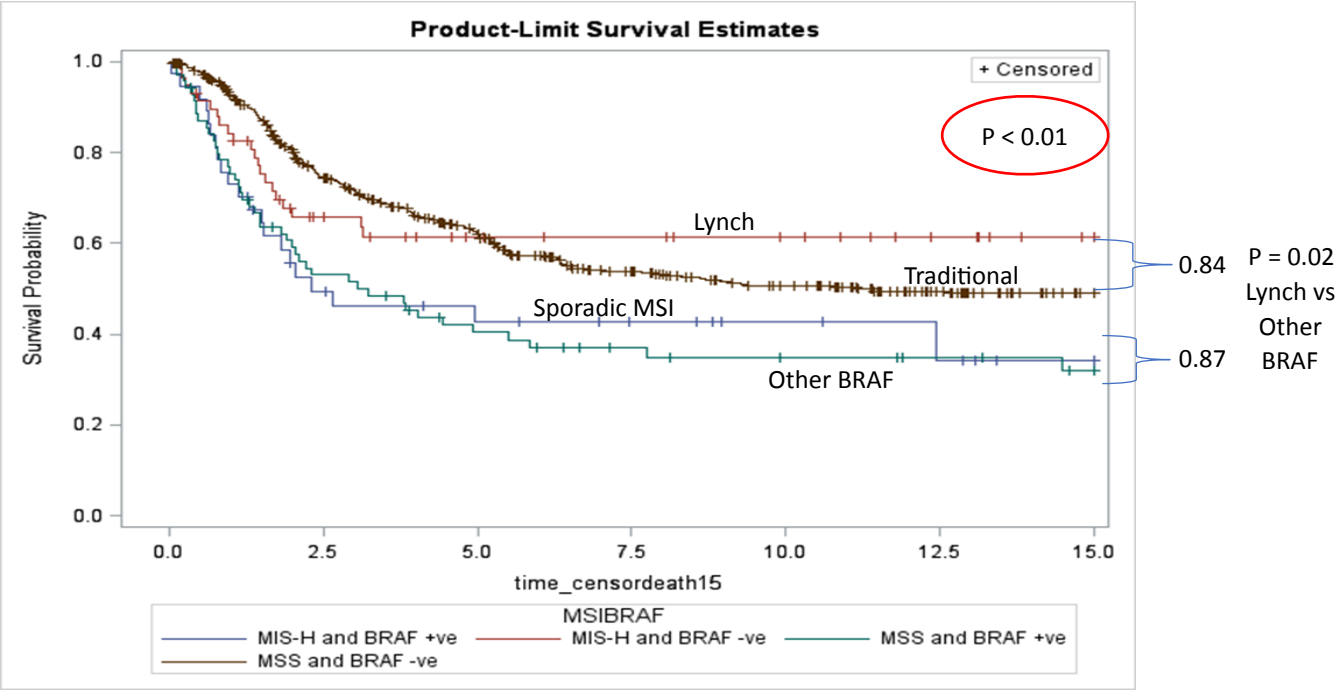
Table 5 shows clinicopathological differences between tumours in patients who received adjuvant chemotherapy versus those who did not. Patients who received chemotherapy were younger, more likely to be male, had a higher rate of extramural and perineural invasion, but had improved cancer specific survival. The two groups were otherwise well matched, particularly with regards to BRAF and MSI status. There was no significant difference in the rates of adjuvant chemotherapy use in any of the four subgroups (Table 5).

Table 5 - Comparison of clinicopathologic differences between patients who received chemotherapy and patients who did not

	Chemo	No chemo	P
Age at surgery (mean±SD)	65.3±11.0	73.8±11.9	<0.01
Male	154 (57%)	179 (43%)	<0.01
Female	117 (43%)	236 (57%)	
Cancer-related death	164 (61%)	343 (83%)	<0.01
Differentiation - well/moderate	212 (78%)	331 (80%)	
Differentiation - poor	59 (22%)	84 (20%)	0.63
Mucinous component	99 (37%)	160 (39%)	0.59
Obstruction	43 (16%)	83 (20%)	0.18
Perforation	15 (6%)	20 (5%)	0.68
Extramural	163 (61%)	216 (52%)	0.03
Perineural	61 (23%)	68 (17%)	0.04
MSI	32 (12%)	63 (15%)	0.21
BRAF	38 (14%)	69 (17%)	0.32
Traditional	210 (77%)	306 (75%)	
Sporadic MSI	9 (3.3%)	28 (6.8%)	0.26
Lynch	23 (8.4%)	35 (8.5%)	
Other BRAF	29 (11%)	41 (10%)	

Univariate analysis of cancer specific survival indicated statistically significant overall differences between the four patient groups (Figure 7).

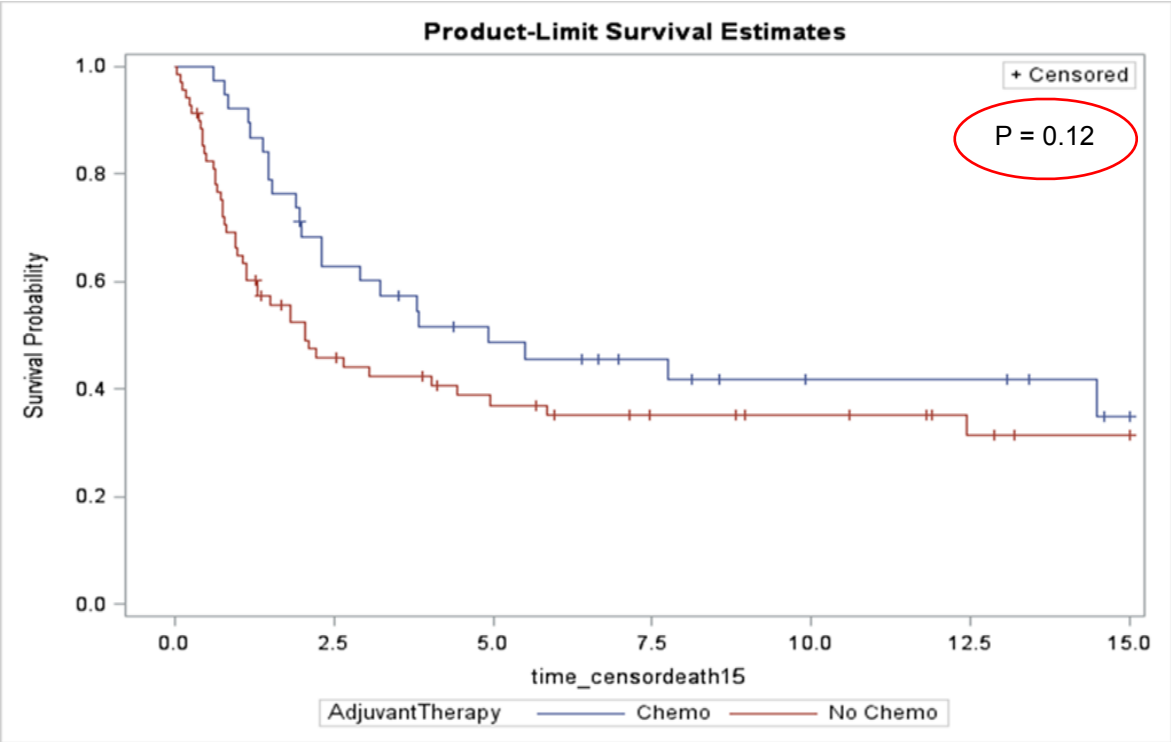
Figure 7 – Kaplan-Meier curve comparing cancer specific survival between four MSI/ BRAF subgroups



At 5 years	Survival	Failure	Number fail	Number left
Lynch	0.61	0.38	21	28
Traditional	0.62	0.37	178	253
Sporadic MSI	0.42	0.57	20	12
Other BRAF	0.40	0.59	40	24

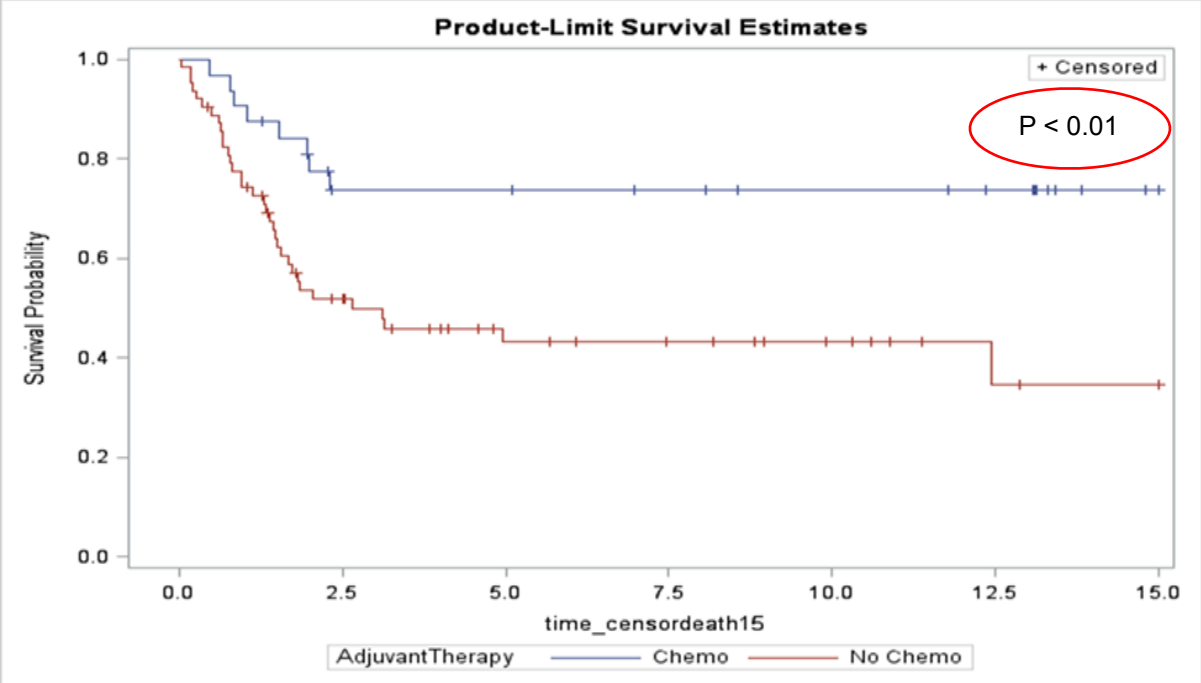
In patients who were BRAF+ve (combining *sporadic MSI* and *other BRAF* groups) there was no apparent survival benefit from adjuvant chemotherapy (Figure 8), whereas, in patients who exhibited MSI (including both *presumed Lynch* and *sporadic MSI* groups), there was a clear survival benefit (Figure 9).

Figure 8 – Kaplan-Meier curve comparing cancer specific survival in BRAF+ve group with or without chemotherapy



At 5 years	Survival	Failure	Number fail	Number left
BRAF+ve Chemo	0.48	0.51	19	16
BRAF+ve No chemo	0.37	0.63	41	20

Figure 9 - Kaplan-Meier curve comparing cancer specific survival in MSI group with or without chemotherapy

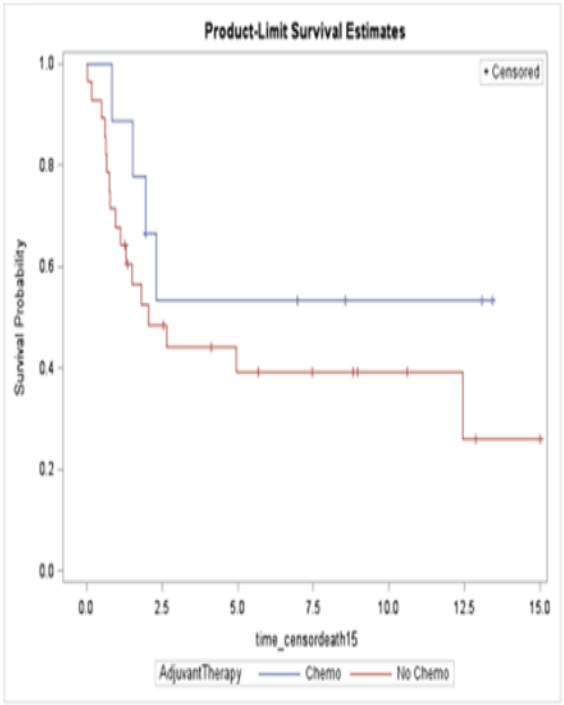


At 5 years	Survival	Failure	Number fail	Number left
MSI Chemo	0.73	0.26	8	20
MSI No chemo	0.43	0.56	33	16

When the four distinct patient groups were examined on univariate analysis, a survival benefit for chemotherapy could only be demonstrated in the *presumed Lynch* group (MSI/ BRAF-ve) (Figure 10). Interestingly, the benefit in *traditional* cancer, when examined in isolation without adjustment, failed to reach significance (p = 0.09, Figure 11).

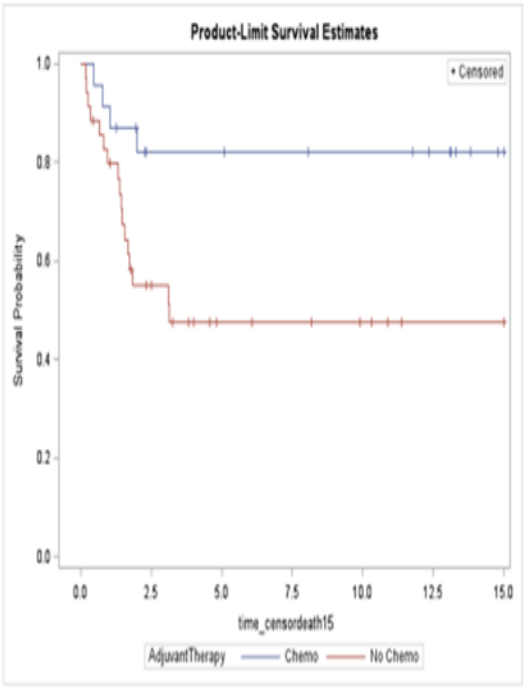
Figure 10 – Kaplan-Meier curve comparing cancer specific survival benefit from chemotherapy in MSI subgroups (*sporadic MSI* and *presumed Lynch*)

Sporadic MSI chemo vs no chemo (MSI and BRAF+ve)



P = 0.26

Presumed Lynch chemo vs no chemo (MSI and BRAF-ve)



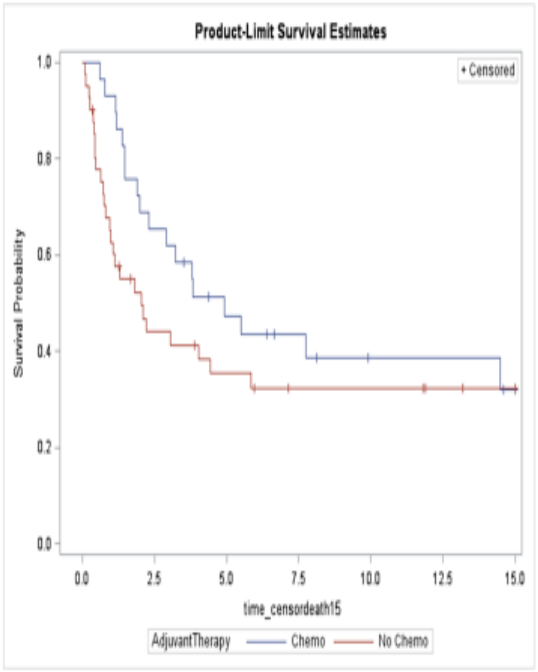
P =0.01

At 5 years (*Sporadic MSI*)	**Survival**	**Failure**	**Number fail**	**Number left**
Chemo	0.53	0.46	4	4
No chemo	0.39	0.60	16	18

At 5 years (*Presumed Lynch*)	**Survival**	**Failure**	**Number fail**	**Number left**
Chemo	0.82	0.17	4	17
No Chemo	0.47	0.52	17	13

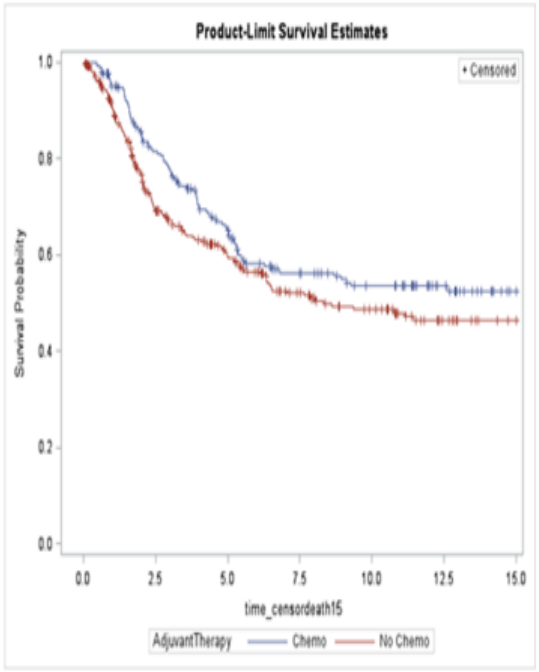
Figure 11 - Kaplan-Meier curve comparing cancer specific survival benefit from chemotherapy in *other BRAF* and *traditional* subgroups

Other BRAF Chemo vs no chemo (MSS and BRAF+ve)



P = 0.22

Traditional Chemo vs no chemo (MSS and BRAF-ve)



P = 0.09

At 5 years (*Other BRAF*)	**Survival**	**Failure**	**Number fail**	**Number left**
Chemo	0.47	0.52	15	12
No chemo	0.35	0.64	25	12

At 5 years (*Traditional*)	**Survival**	**Failure**	**Number fail**	**Number left**
Chemo	0.65	0.34	68	117
No chemo	0.59	0.40	110	136

On analysis adjusting for other prognostic factors in each of the four subgroups determining the chemotherapy effect on cancer specific survival, the *presumed Lynch* and other BRAF (MSS/BRAF+ve) groups reached significance (Table 6). However, interestingly, interaction testing reached significance for the *presumed Lynch* (MSI/BRAF-ve) group suggesting the chemotherapy benefit was highest in this group (Table 7).

Table 6 - Adjusted analysis of the chemotherapy effect on cancer specific survival in each subgroup

Multivariate Hazard Model: Chemotherapy effect in each subgroup				
Group	**N**	**HR**	**95% CI**	**P**
Traditional	516 (75.8%)	0.76	0.57 – 1.03	0.08
Sporadic MSI	37 (5.4%)	0.64	0.23 – 1.99	0.44
Other BRAF	70 (10.3%)	0.45	0.23 – 0.87	0.01
Presumed Lynch	58 (8.5%)	0.26	0.08 – 0.79	0.01

Table 7 - Interaction testing of the chemotherapy effect in each subgroup

Multivariate Hazard Model: Interaction testing of the chemotherapy effect in each subgroup					
Group	**N**	**HR**	**95% CI**	**P**	**Overall P**
Traditional	516 (75.8%)	0.80	0.62 - 1.05	0.11	<0.01
Sporadic MSI	37 (5.4%)	0.54	0.18 – 1.61	0.27	
Other BRAF	70 (10.3%)	0.68	0.37 – 1.24	0.21	
Presumed Lynch	58 (8.5%)	0.27	0.10 – 0.75	0.01	

Discussion

Our findings suggest that MSI/BRAF subtyping has a predictive role in the clinical setting of curatively resected stage III colorectal cancer. While there was an overall benefit from adjuvant chemotherapy, the *presumed Lynch* group gained differentially greater benefit from chemotherapy than the other groups when taking into account clinicopathologic parameters. Our study differs from published studies as we determined the predictive role of MSI and BRAF in combination, whereas previous research focused on MSI and BRAF separately. This may explain some of the conflicting results in the literature.

The role of BRAF V600E mutations as a predictive biomarker in the adjuvant setting is unclear, with very few studies looking at this specifically (72, 73, 83). Hutchins et al., analysing data from the QUASAR trial, studied BRAF mutation in predicting recurrence and benefit from chemotherapy in stage II colon cancer and found the BRAF mutation not to be predictive of chemotherapy benefit (72). However, this study had limited statistical power to demonstrate any interactions between the molecular markers and the efficacy of chemotherapy due to few recurrences in early stage disease (72). Similarly, the MRC FOCUS trial found no evidence of impact on overall survival, however, the relative infrequency of BRAF mutation in the studied patient cohort made this study underpowered to detect or exclude any BRAF-specific effect (73). Interestingly, Ogino et al. found a non-significant trend towards better survival in stage III colorectal cancer patients with BRAF V600E mutation on adjusted analysis (83).

There is considerable literature on the predictive role of MSI, but the findings are conflicting. Evidence from earlier in vitro studies has indicated that inactivation of the MMR system can result in resistance to 5-FU treatment (74-76). Several early retrospective studies suggested greater chemotherapy benefit in MSI-H cases (40, 41, 77), but may have only served to highlight the overall better prognosis seen in MSI-H cases (74, 75, 89). In contrast, to prior studies, in 2003, Ribic et al. retrospectively analysed data from five RCTs and found a trend towards MSI showing less benefit from 5-FU based chemotherapy (42). Subsequently in 2010, Sargent et al. pooled data from the Ribic et al. study with the addition of 457 cases

and reported similar results in stage III cancer (80). However, more recent evidence, in contrast to Ribic and Sargent et.al study, found MSI status does not affect chemotherapy responsiveness (57, 72, 81). Interestingly, when Sinicrope et al. re-analysed data from previous RCT's with patients from Ribic and Sargent studies, they found MSI status to be predictive of chemotherapy benefit, however, the effect was limited to germline cases (*presumed Lynch*) (82), a finding that is consistent with our current results. Unlike our study, Sinicrope et al. did not use BRAF V600E (a more reliable way to differentiate sporadic from germline MSI cancers) in all patients to subclassify MSI cancers, instead in some patients assuming Lynch syndrome if there was MLH1 loss in patients under 55 years at diagnosis.

The incidence of BRAF mutation in our study was similar to that reported in the literature, but we found a slightly higher percentage of MSI and *presumed Lynch* cases (91). The higher rate of *presumed Lynch* cases (8.4%) likely reflects our stringent testing protocols, and the recent adoption of routine testing. Nevertheless, our study has some limitations. Non-randomised data have potential bias, but to overcome this we included cases from an era prior to routine use of chemotherapy in stage III colon cancer (circa 1990). Patients in the chemotherapy group were 8 years younger than those in the non-chemotherapy group, indicating that age may have influenced who was selected for adjuvant treatment, however, to overcome this we comprehensively adjusted for all clinicopathological factors and age. The strengths of the study include the large number of patients, the inclusion of a homogeneous group of stage III colon cancers (i.e. no rectal cancers were included), and adjustment for pathological variables and chemotherapy. To ensure accuracy of data, we re-examined all pathology reports and pathology was reassessed from slides on the earlier cases to meet current reporting protocols. Also, clinical information, in particular mortality, was extensively cross checked from multiple sources.

While we did not have mutation testing to confirm Lynch syndrome, we tested for BRAF V600E mutation in all cases (unlike previous studies) and used this as a marker to identify *presumed Lynch* patients.

Conclusions

In conclusion, our study emphasises the importance of testing for BRAF mutation and MSI in stage III colon cancer. Doing so may help individualise adjuvant treatment, particularly for MSI cancers, who may not have been considered for 5-FU chemotherapy based on older published data but may derive benefit if BRAF-ve.

Chapter 4

Thesis Summary

Summary

In this study, on initial analysis, it appeared that patients with cancer with BRAF mutation had a poorer survival. However, after adjusting for clinicopathologic parameters, BRAF mutation was not found to be associated with a poorer prognosis, even when considered in combination with MSI. Interestingly, MSI cases were found to benefit from adjuvant chemotherapy, however, it was the germline MSI cases that had a significantly greater benefit from chemotherapy than the other groups.

By using this historical cohort, we have attempted to overcome some of the shortcomings of earlier studies. Those studies included rectal cancers, which are known to be distinct from colon cancer, particularly at stage III, and patients with these cancers often receive neoadjuvant radiation/chemotherapy treatment. Also, the patterns of recurrence and survival are different for colon and rectal cancers. Therefore, the exclusion of rectal cancer from our study improves the reliability of the conclusions. Unlike some earlier studies, we only included stage III colon cancers, which allowed us to form conclusions specific to that stage

The greatest strength of this study is its large sample size, carefully-validated dataset, and the inclusions of patients over a 33-year period. We comprehensively adjusted for prognostic clinicopathological parameters and looked for compounding effect of chemotherapy for individual and combined biomarkers.

Future Research

The trends identified in our study need further evaluation. CRC cancer tumorigenesis is particularly heterogeneous, and has proven difficult to understand and fully delineate, particularly all the molecular alterations. However, as knowledge of the molecular landscape of colorectal tumorigenesis advances, new molecular biomarkers with potential prognostic and predictive value will be discovered. These biomarkers may lead to improved tumour molecular classification, and potentially help individualise treatment of colon cancer patients.

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