

Role of matriptase (ST14) in Chronic Myeloid Leukaemia

Ka-chun Cheung

**MBBS (Hong Kong), Dip Pall Med (Cardiff), MRCP (UK), FRACP, FHKCP,
FHKAM**

**Leukaemia Biology Group, Department of Haematology, Centre for Cancer
Biology, SA Pathology, Adelaide**

&

School of Medicine, Faculty of Health Sciences, University of Adelaide

Supervisor:

**Professor Junia Vaz de Melo, MD, PhD, FRCPath
Department of Haematology, Centre for Cancer Biology, SA Pathology,
Faculty of Health Sciences, University of Adelaide**

**A thesis submitted to the University of Adelaide for the degree of Doctor of
Philosophy
July 2013**

THESIS DECLARATION	I
ACKNOWLEDGEMENTS	II
ABBREVIATIONS	IV
ABSTRACT	IX
1 INTRODUCTION:	1
1.1 HISTORICAL BACKGROUND ON CML	1
1.2 BIOLOGY OF CML	2
1.2.1 <i>Molecular biology of CML</i>	2
1.2.2 <i>Mechanisms underlying genomic instability</i>	5
1.2.3 <i>Molecular pathways to blastic transformation</i>	6
1.3 CLINICAL EVOLUTION OF CML	8
1.4 DISEASE MONITORING	8
1.5 THERAPY OF CML	10
1.5.1 <i>Chemotherapy</i>	10
1.5.2 <i>Interferon</i>	10
1.5.3 <i>Stem cell Transplantation</i>	10
1.5.4 <i>Tyrosine kinase inhibitors</i>	11
1.5.4.1 First generation TKI	11
1.5.4.2 Second generation TKIs	12
1.5.4.2.1 Nilotinib	12
1.5.4.2.2 Dasatinib	14
1.5.5 <i>Pan BCR-ABL inhibitors</i>	16
1.5.5.1 Ponatinib (Ariad 24534)	16
1.5.5.2 Danusertib (PHA-739358)	17
1.5.6 <i>Other agents</i>	17
1.5.6.1 Omacetaxine (HHT)	17
1.5.6.2 Arsenic compounds	19
1.5.6.3 Miscellaneous	19
1.6 BRIEF ACCOUNT OF IM RESISTANCE	20
1.6.1 <i>Pharmacological</i>	20
1.6.2 <i>Leukaemia cell related factors</i>	20
1.6.2.1 Amplification and overexpression of <i>BCR-ABL</i>	20
1.6.2.2 KD mutations	21
1.6.2.3 Drug transport	22
1.6.2.4 Quiescent Leukaemic stem cells (Q-LSC)	22
1.6.3 <i>Patient related</i>	22
1.7 RISK STRATIFICATION & PROGNOSTIC MARKERS	22
1.7.1 <i>Clinical prognostic markers</i>	22
1.7.1.1 Sokal (1984)	22
1.7.1.2 Hasford (1997)	23
1.7.1.3 Eutos (2011)	23
1.7.2 <i>Drug trans-membrane transporters</i>	24
1.7.3 <i>Patient compliance and early response to IM</i>	24
1.7.4 <i>Gene expression profiling</i>	26
1.8 ST14 AS A BCR-ABL EFFECTOR	27
1.9 AIMS OF THIS PROJECT	29
2 MATERIALS AND METHODS	31
2.1 CELL LINES	31
2.2 PREPARATION OF RNA	31
2.3 PREPARATION OF CDNA	32
2.4 PCR	32
2.5 TAQMAN REAL-TIME PCR (RQ-PCR)	33
2.6 TAQMAN® CUSTOM ARRAY (TLDA)	34
2.7 SYNTHESIS OF PROTEIN LYSATE	35
2.8 PROTEIN QUANTIFICATION	35
2.9 WESTERN BLOTTING	35

2.10	CONSTRUCTION OF VECTORS	36
2.10.1	<i>Retroviral and lentiviral vectors overexpressing ST14</i>	36
2.10.2	<i>Construction of miR-27b retroviral vector</i>	37
2.11	TRANSFORMATION INTO COMPETENT BACTERIA.....	38
2.12	DNA MINIPREP AND MIDIPREP.....	38
2.13	DNA SEQUENCING.....	39
2.14	CULTURE OF 293T CELLS.....	39
2.15	TRANSFECTION OF 293T	40
2.16	INFECTION OF HAEMOPOIETIC CELL LINES	40
2.17	TRANSFECTION OF HAEMOPOIETIC CELL LINES	41
2.18	PROLIFERATION ASSAY.....	42
2.19	VIABILITY ASSAY	42
2.20	CELL CYCLE ANALYSIS	42
2.21	MIGRATION ASSAY	44
2.21.1	<i>Transwell system and PI staining</i>	44
2.21.2	<i>Tilted dish migration method</i>	45
2.22	MATRIGEL INVASION ASSAY	46
2.23	FACS ANALYSIS FOR ST14 EXPRESSION	46
2.24	CD34 SELECTION.....	47
2.25	INFECTION OF PRIMARY CELLS BY RETRONECTIN BOUND VIRUS METHOD (RBV)	48
2.25.1	<i>Preparation of Retronectin Plate</i>	48
2.25.2	<i>RBV method</i>	48
2.26	SELECTION OF CLONES BY FACS SORTING	49
2.27	COLONY FORMING ASSAY (CFU ASSAY).....	49
2.28	IM SENSITIVITY ASSAY	50
2.28.1	<i>IM Intracellular Uptake and Retention Assay (IUR-IM) and OCT-1 activity</i>	50
2.28.2	<i>IC50 Assay for IM</i>	51
2.29	FLUIDIGM ANALYSES OF ST14 AND HAI-1	52
2.30	STATISTICAL ANALYSIS	53
3.	RESULTS	54
3.1	BCR-ABL AND MATRIPTASE/ST14.....	54
3.1.1	<i>Gene expression quantification</i>	54
3.1.2	<i>The effect of BCR-ABL inhibition on ST14 expression</i>	60
3.2	GENERATION OF BCR-ABL CELL LINES OVEREXPRESSING ST14	62
3.2.1	<i>Construction of retroviral and lentiviral vectors overexpressing ST14 and GFP</i>	62
3.2.2	<i>Infection of Ph-pos cell lines with retroviral vector (PBPG/ST14) and lentiviral vector (pLV411G/ST14)</i>	63
3.2.3	<i>ST14 expression of Ph-pos infected cell lines by Taqman RQ-PCR and western blotting</i>	65
3.2.4	<i>FACS analysis of surface ST14 expression</i>	67
3.3	PHENOTYPES OF BCR-ABL CELL LINES OVEREXPRESSING ST14	68
3.3.1	<i>Cell proliferation</i>	69
3.3.2	<i>Cell viability</i>	70
3.3.3	<i>Cell migration assay</i>	71
3.3.4	<i>Matrigel assay</i>	72
3.3.5	<i>CFU assay</i>	73
3.3.6	<i>Effect of BCR-ABL downstream signalling pathway inhibitors on ST14 expression</i>	76
3.3.7	<i>Relationship between BCR-ABL, miR-27b and ST14 in Ph-neg cell lines with or without p210 by Taqman RQ-PCR</i>	81
3.3.8	<i>Effect of miR-27b on ST14 expression in haematopoietic cell lines</i>	82
3.4	KCL22 AND IM RESISTANCE	84
3.4.1	<i>Viability of KCL22/ST14 cell line with IM</i>	84
3.4.2	<i>ST14 expression in IM-sensitive and IM-resistant cells by Taqman RQ-PCR</i>	85
3.4.3	<i>ST14 and miR-27b expression in KCL22-s and KCL22-r</i>	86
3.4.4	<i>Viability of KCL22-s with downregulation of ST14 expression treated with 1 μM IM</i> ..	87
3.4.5	<i>Possible mechanisms of the reversal of IM resistance in KCL22 cell line through ST14</i>	90
3.4.5.1	<i>Feedback mechanism of BCR-ABL expression</i>	90
3.4.5.2	<i>LYN expression in Ph-pos cell lines overexpressing ST14</i>	91

3.4.5.3 Intracellular IM uptake (IUR) and OCT-1 activity of KCL22 infected cell lines	92
3.4.5.4 ST14 enhancing IM induced apoptosis	94
3.4.5.5 Viability of KCL22-r treated with PD98059 alone and in combination with IM	96
3.4.5.6 Western blot of pro-survival and pro-apoptotic protein expression in KCL22-s and KCL22-r treated with IM and PD98059	98
3.5 ST14 EXPRESSION IN PRIMARY CELLS AND ITS PROGNOSTIC IMPLICATION	99
3.5.1 Predictors for CCyR at 12 months	104
3.5.2 Clinical utility of ST14 percentage to predict CCyR at 12 months	105
3.6 RESULTS FROM FLUIDIGM ANALYSES	106
4. DISCUSSION	109
4.1 NOVELTY OF THE PRESENT STUDY	109
4.1.1 ST14 and CML	109
4.1.2. Regulatory pathway of ST14/matriptase	111
4.2 ST14 AND IM RESISTANCE	114
4.2.1. IM resistance in KCL22 overexpressing ST14	114
4.2.2. Possible role of ST14 percentage as a prognostic marker	118
4.3 RESULT OF FLUIDIGM ANALYSIS	120
4.4 SUGGESTIONS FOR FUTURE STUDY	121
4.4.1. Which is the culprit for IM resistance in KCL22? ST14 or miR-27b?	121
4.4.2. Validation of prognostic value of ST14 percentage by clinical trial and potential indication for novel therapy	123
4.5 CONCLUSIONS	124
5. APPENDIX	126
APPENDIX A: REAGENTS AND PROTOCOLS	126
A.1 RF-10 Complete Medium	126
A.2 Dulbecco's Modified Eagle's Medium (DMEM) for adherent cells	126
A.3 Culture for primary cells (pre-stimulation for retroviral infection)	126
A.4 cDNA mix	127
A.5 PCR mix	127
A.6 PCR Program for G6PD	128
A.7 100 bp marker mix (working stock of loading sample)	128
A.8 Sample specific PCR mix for TLDA	128
A.9 Preparation of protein lysates protocol	128
A.9.1. Protease inhibitor (PI) Cocktail (10x)	128
A.9.2. Phenylmethylsulphonyl Fluoride (PMSF)	129
A.9.3. Final lysis buffer	129
A.10 Sample buffer	129
A.11 SDS-PAGE gel	130
A.12 Running buffer (5X)	130
A.13 Transfer buffer (with SDS)	131
A.14 TBS-T	131
A.14.1 TBS (5X)	131
A.14.2 TBS-T	131
A.15 PCR Program for Amplification of miR-27b from genomic DNA	131
A.16 LB Agar	132
A.17 LB broth	132
A.18 Sequencing reactions for dsDNA PCR products	132
A.19 Preparation of WEHI conditioned media (CM) as an IL3-rich supplement for cultures of growth-factor dependent murine haemopoietic cell lines RF-10 Complete Medium	132
A.20 MACS buffer	133
A.21 FACS fix	133
A.22 Laemmli Sample Buffer – 5X	134
A.23 Preparation of BCR-ABL signalling pathway inhibitors	134
A23.1 Imatinib (IM)	134
A23.2 other downstream inhibitors	134
APPENDIX B: ANTIBODIES IN THIS PROJECT	135
APPENDIX C: CELL LINES IN THIS PROJECT	136
APPENDIX D: PCR PRIMERS AND PROBES	137
Appendix D.1 Assay ID for Taqman primer and probe sets/ TLDA assay	137

<i>Appendix D.2 Standard PCR primers</i>	138
<i>Appendix D.3 Standard RQ-PCR primer and probe set</i>	138
<i>Appendix D.4 PCR primers for amplifying miR-27b</i>	138
APPENDIX E: ST14, miR-27B CDNA SEQUENCE.....	139
<i>Appendix E1: ST14 cDNA sequence</i>	139
<i>Appendix E2: miR-27b sequence (with 200 bp upstream and downstream of sequence)</i>	139
APPENDIX F. PERMISSIONS.....	141
6. REFERENCES	148

Thesis Declaration

I declare that this thesis is the result of my own work carried out in the Department of Haematology, Centre for Cancer Biology, SA Pathology, between 2008 and 2013.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Ka-chun Cheung 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.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed

Ka-chun Cheung

Acknowledgements

First of all, I would foremost like to thank my supervisors Professor Junia V. Melo and Professor L-Bik To for taking me on as a PhD student, for your kind help in organising my PhD study, your guidance and patience to ensure I have learnt and developed my intellectual rigor and completed my thesis.

Secondly, I would like to thank my colleagues in the Leukaemia Biology group, both past and present: Ljiljana Semendric, Vicki Wilczek, Duncan Hewett, Brett Johnson, Jun Ishiko, Debora Casolari, Bradley Chereda, Gink, Fong Choy and everybody in the group for your invaluable support in assistance my work in the laboratory and sharing many laughs and fun in the last few years.

I am also very grateful for the hospitality provided by the members in various laboratories in IMVS. I thank Verity Saunders and Amity Frede from Melissa White Laboratory for their supervision and guidance in some of lymphoprep/CD34 selection and imatinib uptake experiments. A big thank-you to Samdy, Katherine Pilkington and Alan Bishop from Deltmold Family Image Facility (Flow cytometry and sorting) at IMVS, for their helping of the FACS sorting.

My thanks are also due to Dr Mhairi Copland and Dr David Irvine from Paul O Gorman Leukaemia Research Centre, Glasgow, United Kingdom for collaborating with us and providing the data on *ST14* expression from their experiments and Dr Chen-Yong Lin from Georgetown University Medical Centre, Washington, USA, for kindly donating the pcDNA3.1 constructs containing the *ST14* cDNA sequence.

I would also like to thank the Department of Haematology in IMVS, Professor Timothy Hughes and his colleagues, and all the patients and healthy donors who provided blood/bone marrow samples for part of this research.

I could not have been able to finish this PhD study without the financial support. My particular gratitude to the Croucher Foundation Scholarship from Hong Kong for my PhD funding.

I would also like to thank Adeline Lyn and Dr Robert Yiu, Professional Editors, for editing the English of my thesis. My great gratitude to Dr Cally Guerin, co-ordinator of higher degree program, whom was helping and guiding me on my academic writing during the first year of PhD introductory programme organised by the University.

My friends and family have also given me endless support and encouragement, even though they are often neglected during my PhD study. The biggest thank you of all goes to my wife Melva for all of the encouragement and love that you have given me for these challenging years. Thanks also to my daughters, Annecy, Joycey, Gracie, and my friends, David Yeung, Agnes Yong, Chung-Kok, Ashanka Beligaswaste, Dan Thomas, Devendra Hiwase, Pratyush Giri, Oi-lin Lee from IMVS and everybody that I might have left out for their kind support and wonderful time over last few years.

Thank you very much.

Abbreviations

Abl	Abelson proto-oncogene
ALL	acute lymphoblastic leukaemia
Allo-SCT	allogeneic haematopoietic stem cell transplantation
AML	acute myeloid leukaemia
Ab	antibody
AP	accelerated phase
APOD	apolipoprotein D
ATO	arsenic trioxide
ATP	adenosine triphosphate
BC	blast crisis
BCL2	B-cell CLL/lymphoma 2
BCR	breakpoint cluster region
BM	bone marrow
BMI1	BMI1 polycomb ring finger oncogene
bp	base pair
BSA	bovine serum albumin
BUS	busulfan
cDNA	copy DNA
cGMP	cyclic guanosine monophosphate
CCyR	complete cytogenetic response (0% Ph chromosome)
CHR	complete haematological response
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CMR	complete molecular response
CP	chronic phase
Ct	cycle threshold value
CUB	complement protein C1r/C1s, urchin embryonic growth factor and bone morphogenic protein 1 domain
DAB	3,3'-Diaminobenzidine

DAS	dasatinib
DLI	donor lymphocyte infusion
ds	double stranded
DUB1	deubiquitinating enzyme 1
DUB2	deubiquitinating enzyme 2
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epithelial growth factor
EGR1	early growth response 1
ELA	elastase
ERK	extracellular signal-regulated kinase
FACS	flow cytometry and fluorescence activated cell sorting
FSC	forward scatter
FTI	farnesyl transferase inhibitors
GADD45	growth arrest and DNA-damage-inducible
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GLI	GLI family zinc finger
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	granulocyte-macrophage progenitor
GUSB	glucuronidase, beta
GVHD	graft versus host disease
HAI-1	hepatocyte growth factor activator inhibitor-1
HDI	histone deacetylase inhibitors
HGF/SF	hepatocyte growth factor/scattering factor
HHT	homoharringtonine
HLA	human leukocyte antigen
HMOX1	heme oxygenase (decycling) 1
HRP	horseradish peroxidase
HSC	haemopoietic stem cells
HSP70	heat shock protein 70 (HSPA1B)
HSP90	heat shock protein 90
HU	hydroxyurea/hydroxycarbamide
IFN	interferon

IgG	immunoglobulin type G
IL-3	interleukin-3
IL-5	interleukin-5
IM	imatinib
IRES	internal ribosome entry site
IVT	in vitro transcription
JAK2	Janus kinase 2
Kb	kilo base
KDa	kilo Dalton
LPS	lipo-polysaccharides
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1
LTR	long terminal repeat
LYN	v-src-1 Yamaguchi sarcoma viral related oncogene homolog
MACS	magnetic antibody coated bead separation
MAPK	mitogen activated protein kinase
MATK	megakaryocyte associated tyrosine kinase
mCyR	minimal cytogenetic response (35%-95% Ph chromosome)
MEP	megakaryocyte-erythroid progenitor
Met	proto-oncogene (hepatocyte growth factor receptor)
MFI	mean fluorescence intensity
MGG	May-Grunwald-Giemsa stain
miR	micro-RNA
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MMR	major molecular response
MNC	mononuclear cells
MPP	multipuripotent progenitor
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MT-SP1	membrane type serine protease 1
MYD116	myeloid differentiation primary response gene 116
NANOS1	nanos homolog 1 (Drosophila)
NDR1	N-myc downstream regulated gene 1 (NDRG1)
NF-κB	nuclear factor kappa B

NIL	nilotinib
NPM	nucleophosmin
Oligos	oligonucleotides
OS	overall survival
PAR2	protease-activated receptor 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCyR	partial cytogenetic response (1%-35% Ph chromosome)
PDGF	platelet-derived growth factor
PFS	progression free survival
Ph	Philadelphia chromosome
Ph-pos	Philadelphia positive
Ph-neg	Philadelphia negative
PI	propidium iodide
PI-3K	phosphatidylinositol-3 kinase
PMSF	phenylmethylsulfonyl fluoride
POLK	polymerase (DNA directed) kappa
PPV	positive predictive value
PR3	protease 3
PTCH1	patched 1 (Drosophila)
RHOJ	Ras homolog gene family, membrane J (ARHJ)
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rotations per minute
RQ-PCR	quantitative real time reverse transcriptase polymerase chain reaction
RT	room temperature
SCT	stem cell transplantation
sc-uPA	single chain urokinase-type plasminogen activator
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEA	sea urchin sperm protein, enterokinase and agrin domain
shRNA	short hairpin RNA
siRNA	short interfering RNA

SLC2A1	solute family carrier 2 (facilitated glucose transporter), member 1 (GLUT1)
Smo	smoothened, frizzled family receptor
SOCS2	suppressor of cytokine signalling 2
ss	single stranded
ST14	suppression of tumorigenicity 14 (colon carcinoma)
TAF1	TAF1 RNA polymerase II, TATA box binding protein –associated factor
TBP	TATA box binding protein
TE	Tris-EDTA buffer
TGF	transforming growth factor
TKTL1	transketolase-like 1
TLDA	Taqman Low Density Array
TLE6	transducin-like enhancer of split 6 homolog (Drosophila)
TNF	tumour necrosis factor
TNFRSF6	tumour necrosis factor superfamily member 6 (FAS)
WBC	white blood cells count
UV	ultraviolet

Abstract

Chronic myeloid leukaemia (CML) is characterised by a reciprocal chromosomal translocation that gives rise to a 22q-, or Philadelphia (Ph) chromosome and a derivative 9+. The translocation results in a chimaeric *BCR-ABL* gene which is expressed as a 210 kDa protein. The tyrosine kinase inhibitor imatinib mesylate (IM) specifically blocks the enzymatic activity of the BCR-ABL fusion protein and has been successfully employed for the treatment of CML. However, some patients develop resistance to IM and progress to blastic crisis (BC) which is usually fatal. Previous microarray screening of murine cell clones ectopically expressing graded amounts of BCR-ABL showed that *ST14* mRNA levels correlated with those of BCR-ABL. *ST14*, encoding matriptase, acts as a tumour suppressor gene in solid tumours, but has also been linked to metastasis and invasion. The aim of my project is to understand the function of *ST14* in CML. Taqman real time quantitative PCR showed that there was downregulation of *ST14* expression in CD34 cells from CML chronic phase (CP) as compared to those from healthy individuals. These findings were further confirmed by experiments demonstrating upregulation of *ST14* expression in Ph-pos cell lines upon IM treatment, a phenomenon not observed in Ph-neg lines. In order to study the effect of *ST14* overexpression in CML cell lines, a retroviral vector encoding *ST14* was used to infect Ph-pos cell lines (i.e. K562, KCL22 and LAMA84). Overexpression of *ST14* in CML cell lines and primary cells significantly enhanced their migratory and invasion capacities. Intriguingly, it restored IM sensitivity in the KCL22 cell line which is intrinsically resistant to IM. The rescue of IM sensitivity in KCL22 overexpressing *ST14* was independent of the level of *BCR-ABL* expression and unrelated to intracellular IM uptake, *LYN* and *MET* expression. The increased sensitivity to IM of KCL22 overexpressing *ST14* was found to be associated with downregulation of *BCL2* expression, a pro-survival protein. Furthermore, the analysis of *ST14* expression in a group of IM treated CML CP patients from a clinical trial showed that a lower *ST14* expression was associated with poorer molecular responses. The *ST14* percentage at baseline was found to be a better predictor of achieving complete cytogenetic response (CCyR) at 12 months than the Sokal score. Overall, our findings were consistent with a

tumour suppressor role for *ST14*. Future studies should focus on delineating the role of *ST14* as a prognostic indicator in CML patients and its role in the mechanism of disease progression as well as IM resistance.