

**MOLECULAR SCREENING FOR SOME
COMMON *BCR/ABL* GENE MUTATIONS IN
CHRONIC MYELOID LEUKEMIA PATIENTS
RESISTANT TO IMATINIB MESYLATE**

*A thesis submitted for Ph.D. degree in Science in
Biochemistry*

Presented by

Mohamed Ahmed Mohamed Ali

(M.Sc. in Biochemistry, 2006)

Under supervision of

Prof. Dr. Fahmy T. Ali

Prof. of Biochemistry
Faculty of Science
Ain Shams University

Prof. Dr. Wafaa H. El-Metenawy

Prof. of Hematology-Cancer Biology
Faculty of Medicine
Cairo University

Dr. Mohamed R. Mohamed

Ass. Prof. of Biochemistry
Faculty of Science
Ain Shams University

Dr. Yasser I.H. El-Nahass

Ass. Prof. of clinical Pathology
National Cancer Institute
Cairo University

Dr. Mahmoud M. Said

Lecturer of Biochemistry
Faculty of Science
Ain Shams University

**Faculty of Science
Ain Shams University**

2010

مسح جزيئي لبعض الطفرات الشائعة في جين
BCR/ABL في مرضى سرطان الدم النخاعي
المزمن المقاومين لعقار ميسيلات الإيماتينيب

رسالة للحصول على درجة دكتوراه الفلسفة في العلوم في الكيمياء الحيوية

مقدمة من

محمد أحمد محمد علي

(ماجستير في الكيمياء الحيوية ٢٠٠٦)

تحت إشراف

أ.د. وفاء حسن المتناوى

أستاذ أمراض الدم وبيولوجيا الأورام
كلية الطب- جامعة القاهرة

أ.د. فهمي توفيق علي

أستاذ الكيمياء الحيوية
كلية العلوم- جامعة عين شمس

د. ياسر إبراهيم حسن النحاس

أستاذ مساعد الباثولوجيا الإكلينيكية
معهد الأورام القومي- جامعة القاهرة

د. محمد رجا محمد

أستاذ مساعد الكيمياء الحيوية
كلية العلوم- جامعة عين شمس

د. محمود محمد سعيد

مدرس الكيمياء الحيوية
كلية العلوم- جامعة عين شمس

كلية العلوم
جامعة عين شمس

٢٠١٠

Abstract

The current study investigated the mechanism of resistance to imatinib in chronic myeloid leukemia (CML) patients through screening for point mutations in the *BCR-ABL* kinase domain.

Examination of serial measurements of Abelson-breakpoint cluster region (*BCR-ABL*) mRNA in 100 CML patients treated with imatinib using real time quantitative - polymerase chain reaction (RQ-PCR) revealed that 19 patients achieved a complete molecular response (CMR), 53 patients achieved a major molecular response (MMR) and 12 patients achieved a suboptimal response to imatinib, whereas 16 patients showed resistance to imatinib.

The frequency of mutations in patients with increasing *BCR-ABL* transcript levels (n=16), and those with stable or decreasing levels (n=32) was determined using allele specific oligonucleotide - polymerase chain reaction (ASO-PCR). Fourteen out of the sixteen patients (87.5%) with > 2-fold rise in the *BCR-ABL* transcript levels had detectable mutations, whereas none of the 32 patients with stable or decreasing *BCR-ABL* transcript levels had any detectable mutation ($P < 0.001$).

The presence of a mutation was significantly associated with a greater likelihood of subsequent progression to accelerated phase / blast crisis ($P < 0.001$) and shorter survival ($P < 0.001$). Patients harboring P-loop mutations showed poor overall survival ($P = 0.012$) and progression free survival ($P = 0.02$) compared with patients harboring non-P-loop mutations. Patients carrying T315I mutation seemed to have a particularly poor outcome in terms of

survival ($P = 0.014$), but not in terms of time to progression ($P = 0.450$) compared with patients harboring non-P-loop mutations.

These data suggest that a rise in *BCR-ABL* transcript levels of > 2 -fold can be used as a primary indicator to test patients for *BCR-ABL* kinase domain mutations and that ASO-PCR is a valuable tool allowing a timely detection of mutations. Moreover, early detection of *BCR-ABL* mutations may play a role in identifying patients who are likely to become resistant to imatinib therapy, for whom alternative therapeutic options should be considered.

ACKNOWLEDGEMENTS

First and foremost, my deep praises are due to almighty "Allah" who enabled me to finish this piece of work appropriately.

I express my deepest thanks to Prof. Dr. Fahmy T. Ali, for his valuable supervision, sincere guidance, constructive suggestions and wholehearted support throughout this work, and above all for his moral support and fatherly attitude.

I would like also to express my deep gratitude to prof. Dr. Wafaa H. El-metenawy, for giving me the privilege of working under her meticulous and kind supervision; for suggesting the thesis subject; for her constant support; valuable encouragement and guidance at every stage of this work.

Moreover, I would like to thank heartily Dr. Mohamed R. Mohamed, for the experience he

gave me, for his kind supervision, and great help throughout this work.

I also owe my sincere thanks and gratitude to Dr. Yasser J.H. El-nahass, for his great support, helpful advice, valuable technical assistance, and fruitful comments without which this work would have never been accomplished.

I am also deeply indebted to Dr. Mahmoud M. Said, for his moral help, patience, meticulous observation, continuous encouragement and generous advice during this work.

Finally, I would like to convey my thanks to my family for their unfailing support and encouragement.

Mahamed Ahmed Mahamed Ali

*This thesis has not been submitted
to this or any other university*

Mahamed Ahmed Mahamed Ali

Aim of the work

Point mutations within the Abelson-breakpoint cluster region (*BCR-ABL*) kinase domain of the *BCR-ABL* gene in imatinib-treated chronic myeloid leukemia (CML) patients are the most commonly identified mechanism of resistance to imatinib. As alternative therapies are available for imatinib-resistant CML patients, early detection of mutations may provide clinical benefit by allowing early intervention. Because the emergence of a detectable mutant clone leads to a rise in the *BCR-ABL* transcript levels measured by real time quantitative - polymerase chain reaction (RQ-PCR), this rise can therefore be used as a sensitive trigger to screen for mutations.

The present study was carried out to shed further light on the frequency, distribution, and prognostic significance of *BCR-ABL* mutations in imatinib-resistant CML patients.

Contents

	Page
★ Abbreviations.	i
★ List of figures.	vii
★ List of tables.	xi
★ Abstract.	
★ Introduction.	1
★ Aim of the work.	6
★ Review of literature.	7
➤ Chronic myeloid leukemia.	7
✓ Epidemiology.	7
✓ Etiology.	8
✓ Clinical characteristics.	8
✓ Diagnosis.	9
✓ Natural history.	10
✓ Molecular genetics of the Philadelphia chromosome.	11
✓ The <i>ABL</i> gene and <i>ABL</i> protein.	13
✓ The <i>BCR</i> gene and <i>BCR</i> protein.	14
✓ The <i>BCR-ABL</i> gene and <i>BCR-ABL</i> protein.	15
✓ Frequency of the Philadelphia chromosome in leukemia.	20
✓ Mechanisms of <i>BCR-ABL</i> –mediated malignant transformation.	20
I. Altered adhesion properties.	20
II. Activation of mitogenic signaling.	21
1) RAS and MAP kinase pathways.	21
2) JAK-STAT pathway.	22
3) PI 3-Kinase pathway.	22
4) MYC pathway.	23
III. Inhibition of apoptosis.	24

➤ Treatment of chronic myeloid leukemia.	26
I. Radiotherapy.	26
II. Chemotherapy.	26
1) Busulfan.	27
2) Hydroxyurea.	27
III. Allogeneic hematopoietic stem cell transplantation.	28
IV. Interferon alpha (IFN- α).	28
V. Tyrosine kinase inhibitors (TKIs).	29
1) First generation TKI (Imatinib mesylate; IM).	30
✓ Mechanism of action of imatinib.	31
2) Second generation tyrosine kinase inhibitors (SG-TKIs).	36
a) Nilotinib.	36
b) Dasatinib.	38
➤ Monitoring chronic myeloid leukemia patients on imatinib.	40
✓ Definitions of imatinib response criteria.	41
1) Hematologic response.	41
2) Cytogenetic response.	42
3) Molecular response.	42
✓ Guidelines for monitoring CML patients receiving imatinib.	43
✓ Evaluation of response to imatinib.	44
1. Optimal response to imatinib.	44
2. Suboptimal response to imatinib.	44
3. Imatinib failure.	45
✓ Imatinib intolerance.	46
➤ Imatinib resistance.	48
✓ Primary resistance (refractoriness).	48

✓ Secondary (acquired) resistance.	49
✓ Mechanisms of resistance to imatinib.	49
I. <i>BCR-ABL</i> –dependent mechanisms.	50
1) <i>BCR-ABL</i> amplification.	50
2) <i>BCR-ABL</i> overexpression.	50
3) <i>BCR-ABL</i> kinase domain mutations.	50
II. <i>BCR-ABL</i> –independent mechanisms.	51
1) Drug influx and efflux.	51
2) Drug Binding.	53
3) Drug Concentration.	54
4) Activation of alternative signaling pathways.	54
✓ <i>BCR-ABL</i> kinase domain mutations.	55
I. Mutations that directly impair imatinib binding.	56
1) T315I mutation.	56
2) F317L mutation.	58
3) F359V mutation.	58
II. ATP binding loop (P-loop) mutations.	58
III. Catalytic domain (C-loop) mutations.	59
IV. Activation loop (A-loop) mutations.	60
V. Mutations in other regions.	62
✓ Frequency of <i>BCR-ABL</i> mutations.	64
✓ Clinical significance of <i>BCR-ABL</i> mutations.	66
✓ Indications for mutation analysis.	68
✓ Methods for detecting <i>BCR-ABL</i> mutations.	68
✓ Novel therapeutic strategies to overcome imatinib resistance.	70
I. Imatinib dose escalation.	70
II. Combination with conventional cytotoxic drugs.	71
III. Second generation tyrosine kinase	71

inhibitors.	
IV. Allogeneic hematopoietic stem cell transplantation.	80
* Patients and Methods.	81
✓ Study group.	81
✓ Patient eligibility.	81
✓ Treatment.	82
✓ Study design.	83
✓ Quantitation of <i>BCR-ABL</i> mRNA transcript levels by RQ-PCR.	84
✓ RNA extraction.	84
✓ cDNA synthesis.	92
✓ Real time quantitative - polymerase chain reaction (RQ-PCR).	96
✓ Categorization of patients according to molecular response criteria.	112
✓ Mutation analysis.	114
✓ DNA extraction.	115
✓ ASO-PCR assay.	120
✓ DNA agarose gel electrophoresis.	126
✓ Statistical analysis.	133
* Results.	135
✓ Real time quantitative-polymerase chain reaction (RQ-PCR) for <i>BCR-ABL</i> transcript levels.	135
✓ Mutation analysis.	138
✓ Frequency of <i>BCR-ABL</i> kinase domain mutations in imatinib-resistant CML patients.	147
✓ Correlations between mutation analysis and RQ-PCR results.	150
✓ Correlations between mutations frequency,	168

disease phase and location in the <i>BCR-ABL</i> kinase domain among imatinib-resistant patients.	
✓ Correlations between mutations and patient outcome.	170
I. Overall survival (OS) and progression free survival (PFS) analyses of mutation screened patients according to presence or absence of mutations.	170
II. Overall survival (OS) and progression free survival (PFS) analyses of imatinib-resistant patients with mutations according to the type of mutation.	173
III. Overall survival (OS) analysis of imatinib-resistant patients with mutations according to disease phase.	177
* Discussion.	179
* Summary.	211
* References.	215
* الملخص العربي	
* المستخلص	

Abbreviations

A	: Alanine (Ala).
AB	: Actin binding domain.
ABC	: ATP binding cassette.
ABC	: Acute blast crisis.
ABL	: Abelson.
AGP	: α 1- acid glycoprotein.
AKT	: Murine thymoma serine/threonine kinase oncogene homolog.
ALL	: Acute lymphoblastic leukemia.
Allo-HSCT	: Allogeneic - hematopoietic stem cell transplantation.
A-loop	: Activation loop.
ALT	: Alanine aminotransferase.
AML	: Acute myeloid leukemia.
AP	: Accelerated phase.
Ara-C	: Arabinoside cytosine (cytarabine).
ASO-PCR	: Allele specific oligonucleotide -polymerase chain reaction.
AST	: Aspartate aminotransferase.
ATP	: Adenosine 5'-triphosphate.
BAD	: BCL2-associated antagonist of cell death.
BC	: Blast crisis.
BCL2	: B-Cell leukemia/Lymphoma 2.
BCL-X	: A member of the BCL2 family of proteins.
BCR	: Breakpoint cluster region serine/threonine kinase.
BCR-ABL	: Breakpoint cluster region-Abelson.
Bp	: Base pair.
BT	: Blastic transformation.
C	: Cysteine (Cys).
CBL	: Casitas B-lineage lymphoma.
CcyR	: Complete cytogenetic response.

CD34	: Cluster of differentiation 34.
CDKs	: Cyclin-dependent kinases.
CG	: Control gene.
CHR	: Complete hematologic response.
C-loop	: Catalytic loop.
CML	: Chronic myeloid leukemia.
CMR	: Complete molecular response.
CP	: Chronic phase.
CP-CML	: Chronic phase - chronic myeloid leukemia.
CR	: Common reverse.
CRK	: CT 10 sarcoma oncogene homolog.
CRKL	: CT 10 sarcoma oncogene homolog-like protein.
Ct	: Cycle threshold.
D	: Aspartic acid (Asp).
DB	: DNA binding domain.
DEAE	: Diethylaminoethyl.
DEPC	: Diethyl pyrocarbonate.
D-HPLC	: Denaturing - high performance liquid chromatography.
dNTP	: Deoxynucleotide triphosphate.
DSBs	: Double strand breaks.
DTT	: Dithiothreitol.
E	: Glutamic acid (Glu).
E2F	: E2F transcription factor.
EAC	: Europe against cancer.
EDTA	: Ethylenediaminetetraacetic acid.
ERK	: Extracellular signal-regulated serine/threonine kinase.
F	: Phenylalanine (Phe).
FAM	: 6-carboxyfluorescein.
FDA	: Food and drug administration.
FG	: Fusion gene.
FISH	: Fluorescence in situ hybridization.