ABL KINASE DOMAIN MUTATIONS IN NAÏVE AND MOLECULARLY TREATED CHRONIC MYELOID LEUKEMIA PATIENTS WITH *BCR-ABL* REARRANGEMENT

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ABSTRACT

ABL kinase domain (KD) mutation is the major mechanism associated with resistance or suboptimal response to tyrosine kinase inhibitors (TKI) in *BCR-ABL*-positive chronic myeloid leukemia (CML) patients. At present, no data currently exists with respect to the KD mutations in the Southeast Asian CML patients. The frequencies and types of mutations were determined in patients undergoing 1st generation (imatinib) and/or 2nd generation (nilotinib/dasatinib) TKI therapy. This study also asked if the KD mutations could be detected in the naïve CML cases without prior drug exposure. Denaturing high performance liquid chromatography (DHPLC) was selected as a mutation screening method in patient samples with wild-type and known KD mutated cell lines used as controls. Direct sequencing was performed when an abnormal DHPLC profile was observed. In addition, a single-tube allele specific-polymerase chain reaction (AS-PCR) was developed to specifically detect T315I resistant mutation which is strongly associated with therapeutic failure. Among 171 CML cases (80 naïve, 91 TKI-exposed), 21 types of mutations were discovered in 37 CML patients including 13 known mutations and 8 previously unidentified mutations. Thirty cases had a single mutation while 7 cases had multiple mutations. Twenty-four percent of patients receiving first-line imatinib, 63% of imatinib-resistant patients receiving 2nd generation TKI, and 75% of patients treated with front-line 2nd generation TKI had KD mutations. Interestingly, 9% of TKI-naïve CML patients were also found to carry the mutations (3.7% of newly diagnosed cases and 19.2 % of hydroxyurea-exposed cases). Five novel KD mutations were discovered in the naïve cases and three in the TKI-exposed cases. Mutations in the naïve cases were mainly localized in the C-helix domain and SH3 contact site whereas in the TKI-exposed cases predominantly in the drug contact site, P-loop, and catalytic domain. Overall, the most frequent mutation was T315I (24.3%), which was identified only in the TKI-exposed cases, followed by Y253H, M351T, and G250E. AS-PCR was able to specifically detect T315I mutant bands in the dilution mixtures containing as low as 0.5-1%. The detection sensitivity of DHPLC was 1.5-3% dilution whereas sequencing was unable to detect below 6.25% dilution.

In conclusion: 1) Thirteen known and 8 novel KD mutations were identified in the present study which represents the largest series ever reported from Asia; 2) Various types of sensitive and resistant mutations were detected in the naïve CML patients, suggesting that naturally occurring KD mutations were present in the leukemic cells prior to drug exposure; 3) Mutations in the naïve cases were predominantly localized in the different domains from the TKI-exposed cases and T315I potentially arises as a result of drug exposure as it was completely undetectable in the naïve cases; 4) A single-tube AS-PCR was developed and represents a rapid and sensitive screening method for T315I resistant mutation. Detection of the most resistant leukemic clone in CML patients undergoing TKI therapy should be feasible with this simple and inexpensive method.

KEY WORDS: ABL MUTATION / KD MUTATION / CHRONIC MYELOID LEUKEMIA/ IMATINIB REASITANCE / TKI-NAÏVE CML / SOUTHEAST ASIAN CML

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การกลายพันธุ์ของ *ABL* KINASE DOMAIN ในผู้ป่วยมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเรื้อรังที่มีการจัดเรียงใหม่ของยีน *BCR-ABL* ทั้งที่ได้รับและไม่ได้รับการรักษาในระดับโมเลกุล

ABL KINASE DOMAIN MUTATIONS IN NAÏVE AND MOLECULARLY TREATED CHRONIC MYELOID LEUKEMIA PATIENTS WITH *BCR-ABL* REARRANGEMENT

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บทคัดย่อ

การกลายพันธุ์ของยีนเอบีแอลที่บริเวณโดเมนของเอนซัยม์ไคเนส (KD) เป็นสาเหตุหลักของการดื้อต่อยามุ่งเป้ายับยั้งธัยโรซีนไคเนส (TKI) ในผู้ป่วยมะเร็งเม็ดเลือดขาวมัยอีลอยด์ชนิดเรื้อรัง (ซีเอ็มแอล) ที่มียีนลูกผสมบีซีอาร์-เอบีแอล ในปัจจุบันยังไม่มีรายงานการศึกษาถึงการกลายพันธุ์ดังกล่าวในชาวเอเชียตะวันออกเฉียงใต้ การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความชุกและชนิดของการกลายพันธุ์ของยีนเอบีแอลที่บริเวณ KD ทั้งในผู้ป่วยที่ได้รับยาอิมาทินิบ และ/หรือยาระดับสองที่มีระดับความแรงมากกว่า ได้แก่ นิโลทินิบและดาซาทินิบ รวมทั้งต้องการตอบคำถามว่า ผู้ป่วยที่ยังไม่เคยได้รับยาประเภทนี้มาก่อนจะมีการกลายพันธุ์ของยีนเอบีแอลอยู่แล้วหรือไม่ โดยเลือกใช้วิธี denaturing high performance liquid chromatography (DHPLC) ที่มีความไวสูงในการตรวจคัดกรองการกลายพันธุ์ และใช้เซลล์ปกติและเซลล์ที่ทราบว่ามีการกลายพันธุ์แน่นอนเป็นตัวควบคุม ผู้ป่วยที่ตรวจพบความผิดปกติโดยวิธี DHPLC จะได้รับการตรวจยืนยันด้วยวิธีตรวจลำดับเบส (direct sequencing) พร้อมกันนี้ได้ริเริ่มพัฒนาวิธีตรวจ allele specific-polymerase chain reaction (AS-PCR) ในหลอดเดียวกัน (single-tube) เพื่อตรวจหาการกลายพันธุ์ในตำแหน่ง T315I ซึ่งมีความเกี่ยวข้องกับการดื้อยาอย่างรุนแรงและนำไปสู่การรักษาที่ล้มเหลว การศึกษานี้ได้ทำการตรวจคัดกรองผู้ป่วยมะเร็งชนิดซีเอ็มแอลทั้งหมด 171 คน โดยเป็นผู้ป่วยที่ยังไม่เคยได้รับการรักษาด้วยยามุ่งเป้าจำนวน 80 คน และผู้ป่วยที่ได้รับการรักษาด้วยยาแล้วจำนวน 90 คน โดยสามารถพบการกลายพันธุ์ได้ถึง 21 ชนิดในผู้ป่วย 37 คน ประกอบด้วย 13 ชนิดที่เคยมีรายงานมาก่อน และ 8 ชนิดที่เป็นแบบใหม่ ผู้ป่วย 30 รายมีการกลายพันธุ์ในหนึ่งตำแหน่ง และผู้ป่วย 7 รายมีการกลายพันธุ์มากกว่าหนึ่งตำแหน่ง ทั้งนี้ตรวจพบการกลายพันธุ์ในร้อยละ 24 ของผู้ป่วยที่ได้รับยาอิมาทินิบ ร้อยละ 63 ของผู้ป่วยที่ดื้อยาอิมาทินิบ แล้วเปลี่ยนเป็นยาระดับที่สอง และร้อยละ 75 ของผู้ป่วยที่ได้รับยาระดับที่สองตั้งแต่แรก นอกจากนี้ ร้อยละ 9 ของผู้ป่วยที่ไม่เคยได้รับยาก็พบการกลายพันธุ์เช่นกัน (ร้อยละ 3.7 ของผู้ป่วยรายใหม่ก่อนการรักษาใดๆ และ ร้อยละ 19.2 ของผู้ป่วยที่เคยรักษาด้วยยาเคมีบำบัดกลุ่มฮัยดรอกซียูเรีย) การกลายพันธุ์ชนิดใหม่ 8 ชนิดแบ่งเป็น 5 ตำแหน่งใหม่ที่เกิดในผู้ป่วยที่ไม่เคยได้รับยามุ่งเป้าและ 3 ตำแหน่งใหม่ที่เกิดในผู้ป่วยที่เคยได้รับยามุ่งเป้า การกลายพันธุ์ในผู้ป่วยที่ไม่เคยได้รับยามุ่งเป้าส่วนใหญ่อยู่บนตำแหน่ง C-helix และ SH3 contact site ส่วนผู้ป่วยที่เคยได้รับยามุ่งเป้าส่วนใหญ่อยู่บริเวณ drug contact site บริเวณ P-loop และ catalytic domain ชนิดการกลายพันธุ์ที่พบบ่อยสุดได้แก่ T315I (ร้อยละ 24.3) ซึ่งพบเฉพาะในผู้ป่วยที่เคยได้รับยามุ่งเป้า ตามมาด้วยชนิด Y253H, M351T และ G250E วิธี AS-PCR ที่พัฒนาขึ้นสามารถตรวจหาการกลายพันธุ์ที่ตำแหน่ง T315I ได้ที่ระดับความไวร้อยละ 0.5-1 โดยวิธี DHPLC มีความไวประมาณร้อยละ 1.5-3 และวิธีตรวจลำดับเบสมีความไวร้อยละ 6.25

โดยสรุป 1) ได้ตรวจพบการกลายพันธุ์ที่เคยมีรายงานมาก่อน 13 ชนิด และพบชนิดใหม่อีกถึง 8 ชนิด โดยการศึกษานี้จัดเป็นการศึกษาขนาดใหญ่ที่สุดในทวีปเอเชีย; 2) พบการกลายพันธุ์ทั้งชนิดดื้อยาและไม่ดื้อยา ในผู้ป่วยที่ไม่เคยได้รับการรักษาด้วยยามุ่งเป้า บ่งชี้ว่าการกลายพันธุ์ของยีนเอบีแอลที่บริเวณโดเมนของเอนซัยม์ไคเนส สามารถเกิดขึ้นได้เองตามธรรมชาติตั้งแต่ก่อนได้รับการรักษาใดๆ; 3) ตำแหน่งของการกลายพันธุ์ในผู้ป่วยที่ยังไม่เคยได้รับยาส่วนใหญ่อยู่บนตำแหน่งที่ต่างจากที่พบในผู้ป่วยที่ได้รับยาแล้ว การเกิดการกลายพันธุ์ชนิดดื้อยารุนแรง T315I น่าจะเป็นผลของการได้รับยามุ่งเป้าโดยตรง เนื่องจากไม่สามารถตรวจพบการกลายพันธุ์ดังกล่าวในผู้ป่วยกลุ่มที่ไม่เคยได้รับยามุ่งเป้ามาก่อน; 4)การศึกษานี้ได้พัฒนาวิธีตรวจ AS-PCR ในหลอดเดียวกัน เพื่อตรวจหาการกลายพันธุ์ชนิดดื้อยารุนแรง T315I โดยพบว่าวิธีดังกล่าวมีความไวสูง รวดเร็ว จัดเป็นวิธีที่มีราคาถูก และทำได้ง่ายทั่วไป จึงเหมาะสมกับการใช้ตรวจวินิจฉัยการกลายพันธุ์ชนิดที่มีการดื้อยารุนแรงที่สุดในผู้ป่วยซีเอ็มแอลที่กำลังได้รับการรักษาด้วยยามุ่งเป้ายับยั้งธัยโรซีนไคเนส

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LIST OF ABBREVIATIONS

Abbreviations		Term
AP	=	accelerated phase
AS-PCR	=	allele specific-polymerase chain reaction
BC	=	blastic crisis
bp	=	base pair
BMT	=	bone marrow transplantation
°C	=	degree Celsius
CML	=	chronic myeloid leukemia
DT	=	dasatinib
cDNA	=	complementary DNA
CCA	=	clonal chromosome abnormalities
CP	=	chronic phase
CR	=	complete remission
dATP	=	deoxyadenosine-5’-triphosphate
dCTP	=	deoxycytosine-5’-triphosphate
dGTP	=	deoxyguanosine-5’-triphosphate
DHPLC	=	denaturing high performance liquid chromatography
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleotide triphosphate
dTTP	=	deoxythymidine-5’-triphosphate
EDTA	=	ethylenediamine tetraacetic acid
EtBr	=	ethidium bromide
F	=	phenylalanine
G	=	guanine
Hct	=	hematocrit
Hb	=	hemoglobin

LIST OF ABBREVIATIONS (cont.)

Abbreviations		Term
HU	=	hydroxyurea
IM	=	imatinib
kb	=	kilobase
KD	=	kinase domain
K562	=	human chronic myeloid leukemia cell Line
M	=	molar
MF	=	myelofibrosis
mg	=	milligram
min	=	minute
mL	=	millilitre
mM	=	millimolar
mo	=	months
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
N/A	=	not applicable, not available
nm	=	nanometre
NT	=	nilotinib
nt	=	nucleotide
OD	=	optical density
pmol	=	picomole
PB	=	peripheral blood
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
Ph^1	=	Philadelphia chromosome
PLT	=	platelets
PNA	=	peptide nucleic acid

LIST OF ABBREVIATIONS (cont.)

Abbreviations		Term
RNA	=	ribonucleic acid
RBC	=	red blood cells
RFLP	=	restriction fragment length Polymorphism
RQ-PCR	=	real-time quantitative PCR
RT-PCR	=	reverse-transcription PCR
SNP	=	single nucleotide polymorphism
T	=	thymine
Taq	=	*Thermus aquaticus*
TE	=	Tris-EDTA buffer
TBE	=	Tris-borate EDTA buffer
TKI	=	tyrosine kinase inhibitor
U	=	unit
V	=	valine
WBC	=	white blood cells
X	=	time

CHAPTER I
INTRODUCTION

Chronic myeloid leukemia (CML) is a chronic hematopoietic stem cell disorder characterized by extensive proliferation of clonally expanded immature and mature myeloid cells at varying stages of differentiation (1). CML is considered one of the most common leukemia in Thailand that significantly affects the young to middle-aged working populations of the country (2-3). The disease can be found incidentally during a routine medical check-up or it can manifest with significant symptoms and signs such as abdominal discomforts and early satiety due to splenomegaly or hyperviscosity syndrome due to markedly elevated granulocytes in the blood circulation (1, 4). Three different phases of CML have been well-recognized comprising 1) chronic phase, 2) accelerated phase, and 3) blastic crisis or transformation (4). In a chronic phase, patients are usually asymptomatic or have mild symptoms whereas in an accelerated phase or blastic crisis, patients are more ill with bone marrow failure symptoms similar to those of acute leukemia, and finally they all succumb to death (5-6). The majority of CML patients are predominantly diagnosed in a chronic phase although some patients may present in a very advanced phase (3).

The hallmark of CML is the Philadelphia (Ph^1) chromosome which occurs as a result of a reciprocal chromosomal translocation between chromosomes 9 and 22, creating a new fusion gene, *BCR-ABL*, with constitutive tyrosine kinase activity that is essential for the pathogenesis and disease progression (7). The *BCR* gene which is located on the long arm of chromosome 22 encodes a cytoplasmic protein, GTPase activating protein (GAP), with a complex regulatory function. The *BCR*'s chromosomal counterpart, the *ABL* proto-oncogene, is located on the long arm of chromosome 9 and encodes a nonreceptor tyrosine kinase (8-9). The BCR-ABL fusion protein has cellular effects through the activation of a number of intracellular oncogenic pathways via its constitutive tyrosine kinase activity. Such kinase activity leads to a clonal development of transformed cells with increased proliferation, decreased stromal

adherence, and decreased apoptosis associated with genomic instability (4, 9-11). The signaling pathways that can be activated by BCR-ABL include RAS, MYC, mitogen-activated protein kinases (MAPK), signal transducers and activators of transcription (STAT), and phosphatidylinositol 3-kinase (PI3K) (9-10). Moreover, dimerization of the BCR-ABL protein triggers autophosphorylation which results in the activation of downstream signaling cascades (10).

Targeting BCR-ABL-transfected cell lines and murine CML models with a variety of tyrosine kinase inhibitors (TKI) has led to a landmark discovery of a novel *BCR-ABL* targeting drug, imatinib mesylate (Glivec/Gleevec), which subsequently entered clinical trials, showed significant clinical benefits and has become a standard of care for CML patients worldwide (1,12-14). Structural data suggest that imatinib acts by binding to the BCR-ABL protein resulting in a prevention of phosphate transfer from ATP to the BCR-ABL's substrates and a block of the downstream signal transduction pathways (9, 15). With the invention of imatinib, the majority of CML patients who have a complete hematologic and cytogenetic response can now enjoy a near normal or normal quality of life with an overall survival over 85% and a transformation-free survival over 90% at the end of a 7-year follow-up period (16-17).

Unfortunately, a significant number of CML patients, particularly those who are in advanced phase, those who are intolerant to the drug, and about a third of chronic-phase cases, fail to respond to imatinib leading to their significant shortened survivals as contrasted to those who are able to continue the drug (18-22). Two major mechanisms of imatinib resistance are postulated, i.e. BCR-ABL independent resistant mechanism and BCR-ABL dependent resistant mechanism. Examples of the BCR-ABL independent resistant mechanism are clonal chromosomal evolution, inadequate plasma levels of imatinib, patients' noncompliance or incomplete adherence to the treatment, pharmacokinetic parameters, and activation of other signaling pathways (23-24). The main BCR-ABL dependent mechanisms are *BCR-ABL* amplification and *ABL* kinase domain (KD) mutation (25). *BCR-ABL* gene amplification produces a BCR-ABL protein overexpression which then leads to restoration of oncogenic signaling pathways in a given drug concentration (23, 26). Point mutations at the amino acid residues of the ABL KD, which are critical for direct imatinib binding or

stabilization of the inactive conformation of BCR-ABL, leads to impaired imatinib binding and reactivation of TK activity (9, 27).

ABL KD mutations are now recognized as the most common mechanism of imatinib resistance with their frequency varied from 30% to 50%, depending on the studied CML cohorts, type of resistance, and the sensitivity and specificity of the detection methods (21-22, 28-34). The majority of mutations in imatinib-resistant patients usually occur within the eight amino acid positions of the KD including M244V, G250E, Y253H/F, E255K/V, T315I, M351T, F359V, and H396 with varying sensitivities to imatinib (23-34). Some of these KD mutations not only resist to the first (1^{st})-generation TKI such as imatinib but also resist to the second (2^{nd})-generation TKI such as nilotinib and dasatinib (19, 35-37). Moreover, various resistant mutations could have been present before TKI therapy and may have an impact on the treatment outcome (19, 38-44).

Several methods have been utilized to detect *ABL* KD mutations such as a standard method direct sequencing (32, 38, 42, 45-48), subcloning and sequencing (27), polymerase chain reaction (PCR)-based pyrosequencing (49-50), denaturing high performance liquid chromatography (DHPLC) (19, 51-52), restriction fragment length polymorphism (RFLP) (26), double–gradient denaturing electrophoresis (53), allele-specific polymerase chain reaction (AS-PCR) (38, 42, 54-55), real-time quantitative PCR (RQ-PCR) (42), array-based assays (56) , and high-resolution melt curve analysis (HRM) (57-58), with varying sensitivities (0.01%- 25%) and specificities.

ABL KD mutations were mainly reported in the imatinib- resistant CML patients (19, 28-29, 33, 42-44, 54, 56, 59-62). Very few studies explored the frequency and types of KD mutations in the TKI-naïve cases who never received the drug. Moreover, the frequency and types of KD mutations had never been reported in the Southeast Asian populations. This study was thus designed to determine if and what types of KD mutations could be found in the Thai CML patients, particularly those who were treated with conventional chemotherapy and had no prior exposure to TKI since most of the published articles focused on the mutations that occurred in the TKI-exposed cases but very few concerned about the naïve CML population. Types of mutations were characterized with respect to the patients' history of drug exposure

and their association with the levels of *BCR-ABL* mRNA transcripts, hematologic parameters, and clinical outcome of CML patients.

In order to derive the results for all the above questions, I first set out to optimize the molecular methods for the detection of *BCR-ABL* fusion gene variants and *ABL* KD mutations. Reverse transcriptase (RT)-PCR and RQ-PCR were developed for the detection of *BCR-ABL* fusion gene variants whereas DHPLC and direct sequencing were chosen as my screening methods for *ABL* KD mutation detection. I then went on to develop a single-tube AS-PCR method to specifically detect T315I, which is the most resistant KD mutation, and compared its sensitivity and specificity to those of DHPLC and direct sequencing methods using dilution mixture with varying concentrations.

CHAPTER II
OBJECTIVES

1. To optimize the molecular methods for the detection of *BCR-ABL* fusion gene variants and *ABL* KD mutations

2. To explore if *ABL* KD mutation exists in naïve CML patients who had not been treated with imatinib

3. To determine the frequency and characteristics of *ABL* KD mutations that occurred during the course of imatinib therapy and their association with the levels of *BCR-ABL* mRNA transcripts, hematologic parameters, and clinical outcome of CML patients

CHAPTER III
LITERATURE REVIEW

3.1 Chronic myeloid leukemia (CML)

3.1.1 Definition of CML

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by an abnormal expansion of myeloid cells in the bone marrow (BM) and the peripheral blood (PB) (1).

3.1.2 Pathogenesis of CML

The cytogenetic hallmark of CML is the Philadelphia (Ph^1) chromosome, a shortened chromosome 22, which is observed in approximately 90-95% of CML patients (2, 3, 7). The Ph^1 chromosome arises as a result of a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11.2)] and leads to fusion of the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (8-9). The *BCR-ABL* fusion oncogene encodes the BCR-ABL oncoprotein with deregulated tyrosine kinase activity (4, 9-11), as shown Figure 3.1.

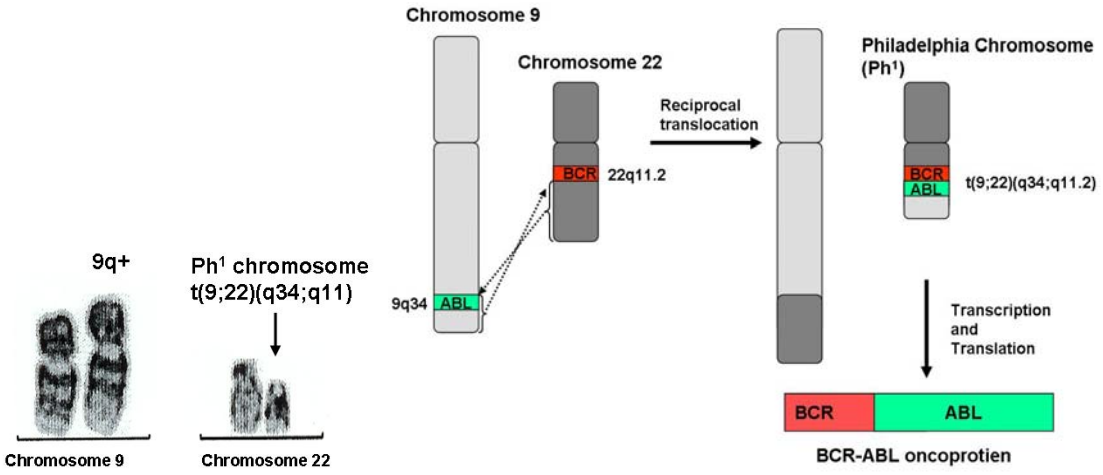


Figure 3.1 Philadelphia (Ph^1) chromosome and *BCR-ABL* fusion gene. Adapted from Frazer R *et al* (4, 63).

3.1.3 Epidemiology of CML

In the western reports, CML constitutes about 15% of all leukemias and occurs with an annual incidence of 1.5 per 100,000 (1,4). CML is considered a rare disease in the young children and the incidence increases with age with a male preponderance and a median age at onset of 60 years old (64). Previous studies in Thailand, however, indicated that Thai patients with CML developed the disease at a much younger age than the Caucasian populations with the median age at diagnosis of 30-50 years old (2-3).

3.1.4 Clinical features of CML

Clinical symptoms of CML may be absent or may include fatigue, anorexia, early satiety, splenomegaly, hepatomegaly, hyperviscosity syndrome, or abnormal bleeding (1-6). In a chronic phase, patients are usually asymptomatic or have mild symptoms whereas in an accelerated and blastic phase, patients frequently present with bone marrow failure symptoms similar to those of acute leukemia. The majority of CML patients are initially diagnosed in the chronic phase although some patients may present in very advanced phase. Disease acceleration and blastic transformation usually arise in chronic-phase CML patients after a period of 3-5 years (1-6, 65).

3.1.5 Cytogenetic evolution in CML

The acquisition of additional chromosomal abnormalities, i.e. clonal evolution is commonly reported in CML patients entering the accelerated phase or blastic crisis, and is also well-recognized as a criterion for such conditions (5-6). About 80% of CML patients had additional clonal cytogenetic abnormalities (CCA) in the Ph^1-positive leukemic cells (CCA/Ph^1+) during the terminal phase which reflects their leukemic cells' genomic instability (66). The most frequent secondary cytogenetic abnormalities are trisomy 8, isochromosome 17, and a duplicate Ph^1 chromosome (resulting in *BCR-ABL* overexpression) (15). Other less common abnormalities are trisomy 19, trisomy 21, trisomy 17, and deletion 7. The secondary cytogenetic abnormalities are more frequently associated with myeloid than lymphoid blast crisis CML (9). Approximately 10-15% of CML patients carry the deletion in

the derivative chromosome 9, which may indicate the inherent genetic instability that is an indicator for more rapid disease progression (67).

3.1.6 Treatment of CML

Treatments of CML patients have significantly evolved in the last 150 years. Initially, most of the treatments were aimed to relieve symptoms associated with marked leukocytosis (5). The most commonly used oral chemotherapy has been hydroxyurea, a ribonucleotide reductase inhibitor which can induce myelosuppression leading to decreased blood cell production and decreased splenomegaly (5). Hematologic remission can thus occur in hydroxyurea-treated cases but cytogenetic remission, i.e. loss or partial reduction of the Ph^1 chromosome could rarely be demonstrated (2,5). The only curative treatment before the TKI era was high-dose myeloablative chemotherapy followed by stem cell transplantation (SCT) from related or unrelated donors which was generally offered to all CML patients within the first 12 months of diagnosis (1,2). Although SCT could cure 50%-70% of CML patients, particularly those in a chronic phase, the procedure itself is associated with a high morbidity and mortality rate as a result of graft versus host disease, organ dysfunctions and severe opportunistic infections (68). Nowadays, the molecularly targeted therapy with various TKIs has replaced SCT as a standard treatment for *BCR-ABL* positive CML patients (9,14).

3.2 *BCR-ABL* fusion gene

3.2.1 Structure of *BCR* and *ABL* gene

The *BCR* gene, located on the long arm of chromosome 22, encodes a cytoplasmic protein, GTPase activating protein (GAP), with a complex regulatory function. The *ABL* gene, located on the long arm of chromosome 9, is a proto-oncogene that encodes a nonreceptor tyrosine kinase. ABL function depends on its subcellular localization. In the cytoplasm, ABL plays a role in cell proliferation and survival (8, 9).

3.2.2 Structure of *BCR-ABL* fusion gene

The breakpoints in the *BCR* gene can occur in various different sites while the location of the *ABL* breakpoint is usually constant (8, 9, 69). The three main *BCR* breakpoints are m-bcr, M-bcr, and µ-bcr as shown in Figure 3.2.

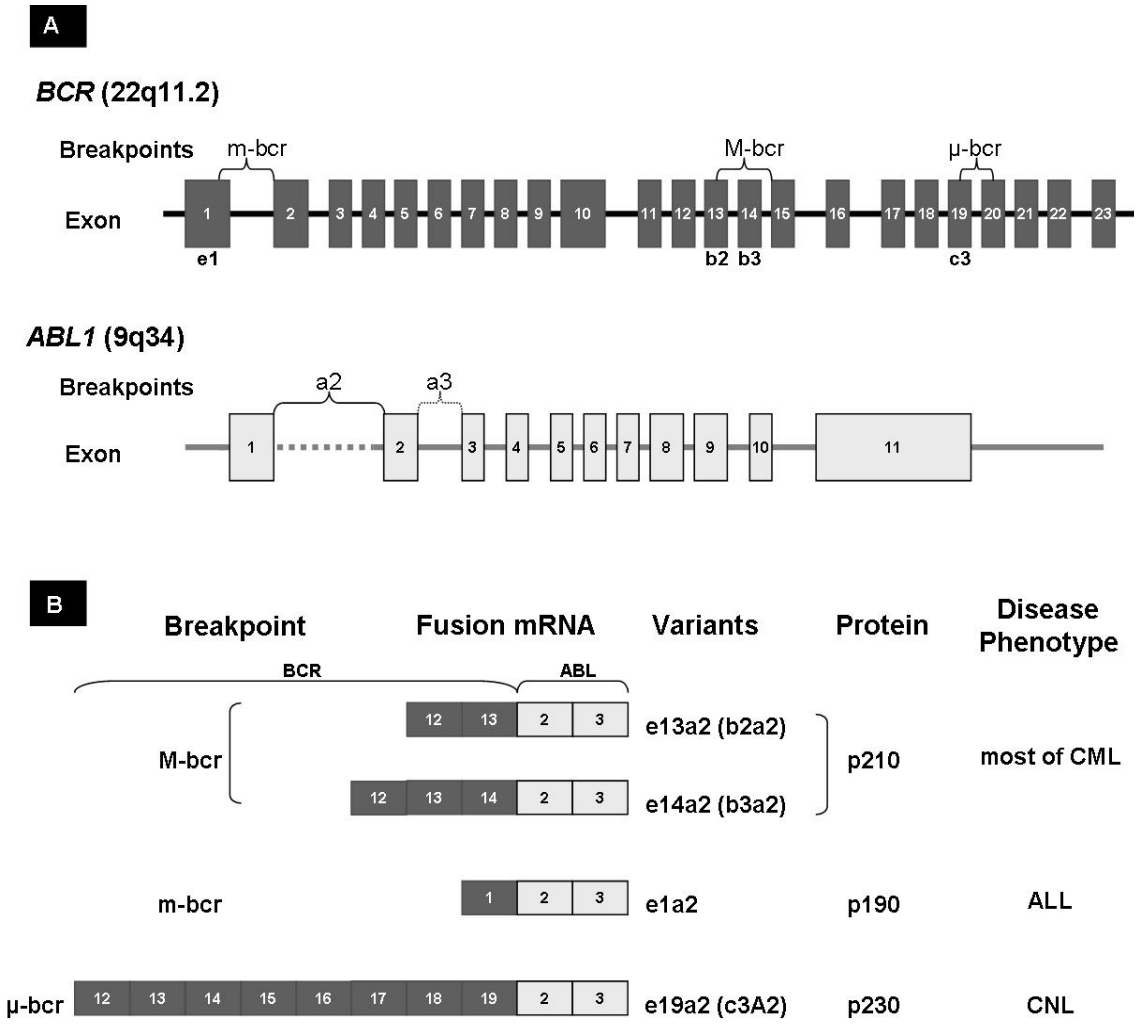


Figure 3.2 The cDNA structure of the *BCR-ABL* fusion gene. *BCR-ABL* breakpoints (A), BCR-ABL transcripts (B). Adapted from Ou J *et al* (69).

3.2.3 *BCR-ABL* gene variants in CML

Three different types of fusion mRNA are produced and subsequently transcribed into three distinct products, i.e. 190 kd, 210 kd, and 230 kd, respectively (1, 8). The most common protein in CML patients is a 210-kd protein (p210 BCR-

ABL), which is encoded from either b2a2 (e13a2) or b3a2 (e14a2) fusion mRNA (1, 70). On the other hand, p190 is found more frequently in acute lymphoblastic leukemia (ALL) whereas p230 is associated with chronic neutrophilic leukemia (CNL) (1, 69).

3.2.4 Levels of *BCR-ABL* transcripts

Quantitative detection of *BCR-ABL* transcripts by real-time quantitative polymerase chain reaction methodology (RQ-PCR) has become essential in the TKI era (71-72). CML patients who have a molecular remission, i.e. *BCR-ABL* transcripts are not detectable or minimally detectable, have a longer overall survival than patients who continue to have a significantly high level of *BCR-ABL* transcripts (73, 74). Increased levels can signify a molecular relapse followed by a cytogenetic relapse and eventually a hematologic relapse and clinical relapse. Wang L *et al* identified increasing *BCR-ABL* transcript levels in patients who developed *BCR-ABL* mutations while no transcripts were detectable in patients who had a good response to TKI (75).

3.3 BCR-ABL oncoprotein

3.3.1 Structure of the BCR-ABL oncoprotein

The structure of BCR, ABL protein and BCR-ABL oncoprotein are shown in Figure 3.3 (8, 9). BCR-ABL has several domains and also a number of interacting substrates. The important functional domains of the ABL protein include the SRC-homology 3 and 2 regulatory domains (SH3 and SH2, respectively), the SH1 domain with its ATP-binding site (aka, PTKc_Abl, catalytic domain of the protein tyrosine kinase, abelson kinase, or kinase domain), the DNA-binding domain, and F-actin DNA-binding domains (conserved domains, NCBI) (8). The TK activity of the normal ABL protein ($p145^{ABL}$) encoded by its SRC-homology 1 (SH1) domain is kept under a tight control by the intramolecular binding of an N-terminal cap region encompassed by the first exon (1b or 1a) and the first part of exon 2a. In the BCR-ABL fusion protein with a lack of the ABL cap region and a dimerization domain encoded by the first exon of BCR are responsible for constitutive activation of the ABL SH1 domain, resulting in uncontrolled signal transduction (8, 9).

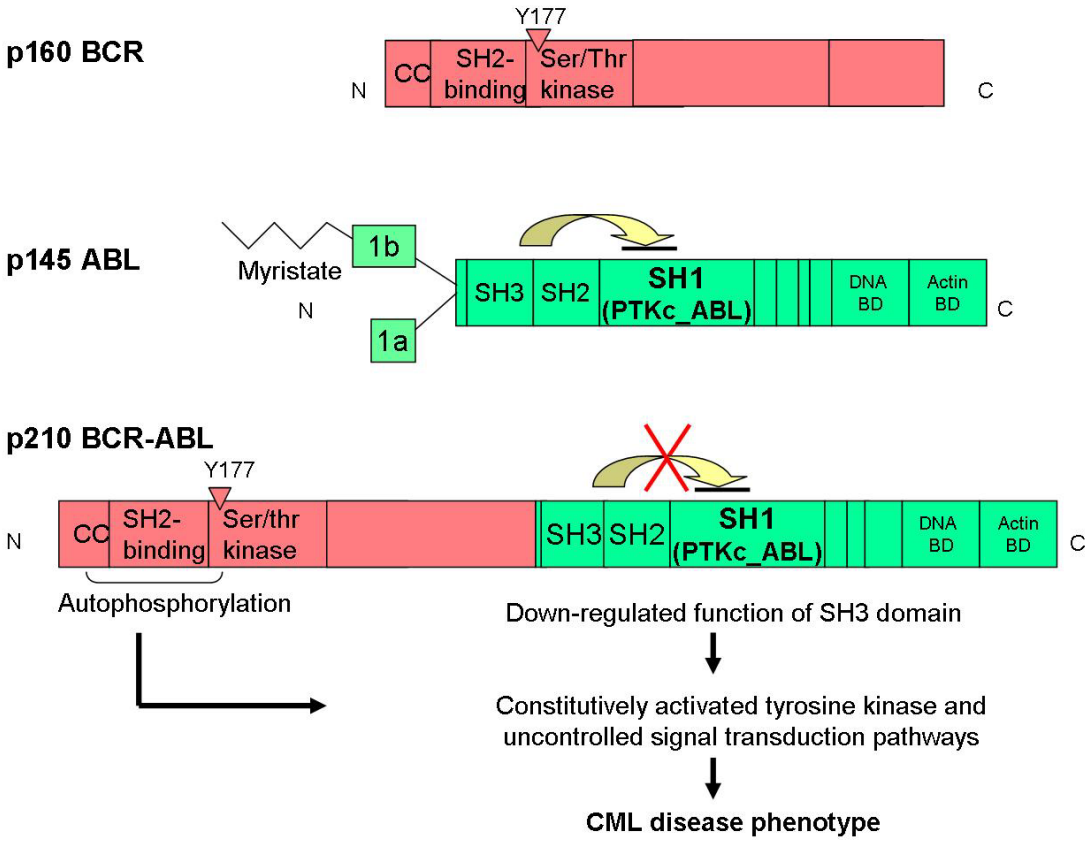


Figure 3.3 Structure of the BCR, ABL protein and BCR-ABL oncoprotein (8,9).

3.3.2 BCR-ABL signaling pathways

The BCR-ABL protein has cellular effects through the activation of a number of intracellular signaling pathways that transduce the oncogenic signals responsible for the activation or suppression of gene transcription (4,10). Such kinase activity results in transformation of cells that have increased proliferation, decreased apoptosis, decreased adherence, and genomic instability. The signaling pathways known to be activated by BCR-ABL include RAS, mitogen-activated protein kinases (MAPK), signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K), and MYC. Dimerization of BCR-ABL also triggers autophosphorylation (Figure 3.4) (4,9-11,69,76,77).

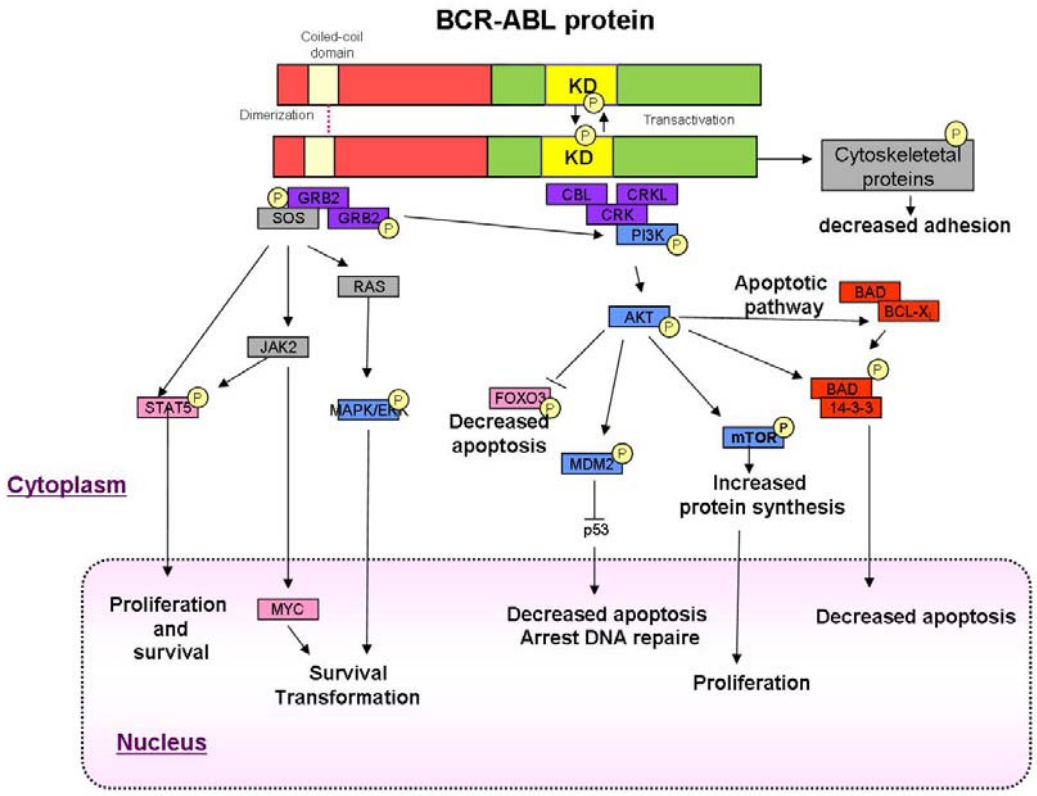


Figure 3.4 Signal transduction pathways affected by BCR-ABL protein (1, 10).

3.4 Tyrosine kinase inhibitors (TKI)

Various TKI drugs have been synthesized and tested in the past 30 years. Some of these drugs have been found to have a significant clinical benefit in many types of malignancies. In CML, imatinib myselate (STI571, Glivec or Gleevec) was the first drug shown to be beneficial and has become the first-line treatment for CML patients since 2002 (33).

3.4.1 Imatinib as the first-generation TKI

Imatinib is an effective inhibitor of the oncogenic tyrosine kinase which is deregulated in 95% of CML patients and in 20% of adult Ph^1-positive ALL patients (33). Structural data of the ABL KD in complex with imatinib showed that imatinib acts by binding exclusively to the catalytic inactive conformation ABL kinase protein which results in prevention of phosphate transfer from ATP to substrates and block of

the downstream signal transduction pathway (9,15). The structure of imatinib binding site on BCR-ABL KD is illustrated in Figure 3.5.

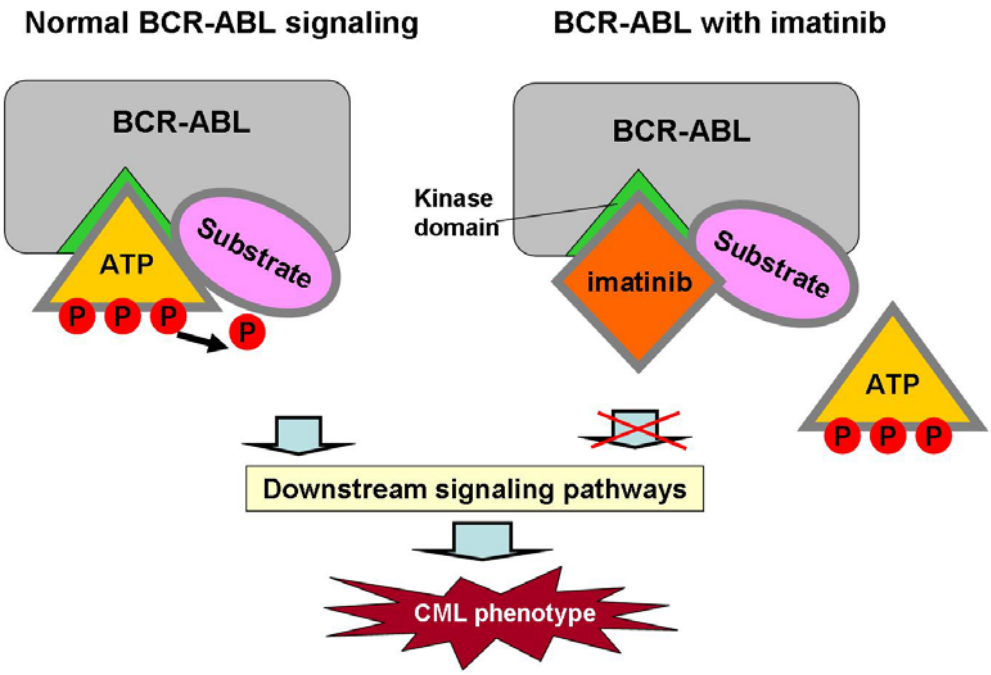


Figure 3.5 Illustration of the imatinib mechanism in competitive binding of ATP to BCR-ABL oncoprotein (8).

3.4.2 Second-generation TKI

There are two newer TKI that have been approved for CML therapy, i.e. nilotinib and dasatinib (11, 21, 35, 48). Nilotinib is an imatinib derivative with enhanced selectivity and potency for BCR-ABL as compared to imatinib (35). Nilotinib also binds an inactive conformation of ABL like imatinib. However, in vitro study showed that nilotinib is more potent with a 30-fold more BCR-ABL inhibition than imatinib (35,78). Other kinase targets for nilotinib include platelet-derived growth factor-receptor (PDGFR) and c-Kit receptors (11, 78). Dasatinib, a SRC/ABL inhibitor, is about 325 fold more potenct for BCR-ABL inhibition than imatinib (78). Dasatinib binds to both the active and inactive conformations. In addition to blocking the BCR-ABL kinase activity, dasatinib also inhibits the alternative kinase targets and signaling pathways including SFKs, c-Kit, PDGFR, and ephrin-A receptor (11, 79).

The fact that dasatinib works through other signaling pathways such as SFKs, it may be of use to overcome imatinib resistance caused by BCR-ABL overexpression as well as BCR-ABL-independent mechanism of imatinib resistance (80).

3.5 Response to imatinib

3.5.1 Definition of imatinib response

Imatinib is an oral drug which makes it easy to administer and is not associated with serious adverse side effects that usually occur in patients receiving conventional chemotherapy. At present, molecularly targeting therapy with imatnib is accepted as the standard treatment for patients with *BCR-ABL*-positive CML worldwide. The aim of treatment with imatinib in CML patients at present is to achieve complete response. The three levels of TKIs responses that be classified according to the recently update of concepts and management recommendations of European LeukemiaNet 2009 (81) are demonstrated in Table 3.1 and Table 3.2. Hematologic response (HR) is defined as the normalization of blood counts and resolution of organomegaly. Cytogenetic response (CyR) is determined by chromosome analyses which show the absence of the Ph^1 chromosome. A molecular response (MR) is defined by absence of the *BCR-ABL* transcripts (complete MR, CMR) or at least 3 log reduction of the *BCR-ABL* transcript level (0.1% *BCR-ABL* to control gene ratio) from the a standardized baseline using RQ-PCR method (major MR, MMR) (82,83).

Table 3.1 Definitions of TKI response (81)

Response by Type	Definition
Hematologic response	
Complete (CHR)	WBC< 10 x10^9/L Basophils <5% No myelocytes, promyelocytes, myeloblasts in the differential Platelet count <450x 10^9/L Spleen nonpalpable
Cytogenetic response	
Complete (CCyR)	No Ph^1+ metaphases
Partial (PCyR)	1% - 35% Ph^1+ metaphases
Minor (mCyR)	36% - 65% Ph^1+ metaphases
Minimal (minCyR)	66% - 95% Ph^1+ metaphases
None (noCyR)	> 95% Ph^1+ metaphases
Molecular response	
Complete (CMR)	Undetectable *BCR-ABL* mRNA transcripts by RQ-PCR and/or nested PCR in two consecutive blood samples of adequate quality (sensitivity >10^4)
Major (MMR)	*BCR-ABL/ABL* (%ratio) ≤0.1% IS*

*Ratio of *BCR-ABL* to *ABL* (or other housekeeping genes) according to the international scale (IS)

Table 3.2 2009 European LeukemiaNet monitoring recommendation of imatinib response (81)

Response to Imatinib		
Hematologic **At diagnosis** **Every 15 days** until CHR has been achieved and confirmed **at least every 3 months** or as required	**Cytogenetic** **At diagnosis** at 3 months, and 6 months **Every 6 months** until a CCyR has been achieved and confirmed **Every 12 months** if regular molecular monitoring cannot be assured **Always** for occurrences of treatment failure (primary or secondary resistance), and for occurrences of unexplained anemia, leukopenia, or thrombocytopenia	**Molecular** **RQ-PCR:** **Every 3 months** until MMR has been achieved and confirmed at least every 6 months **Mutational analysis:** In occurrences of suboptimal response or failure; always required before changing to other TKIs or other therapies

3.5.2 Frequency of imatinib response

The overall survivals of CML patients with chronic phase who have a CCR are now reaching over 85% with the transformation-free survivals over 90% at the end of a 7-year follow-up period (16, 17, 84). Moreover, a high incidence of freedom from progression to accelerated phase/blast crisis was 93% (16). Patients with accelerated phase had a CHR rate of 37%, a CCyR rate of 19%, and a transformation-free survival of 40% at a 3-year follow-up period (85). Patients with blast crisis had a CHR rate of 25% and CCyR could be obtained in some cases, but a transformation-free survival was short (85).

3.5.3 Monitoring molecular methods to determine imatinib response

RQ-PCR is the current recommended method for monitoring *BCR-ABL* transcript levels based on the proportion of the *BCR-ABL* transcript copy numbers over the control gene copy numbers (83). It is recommended for disease monitoring every

3 months until MMR has been achieved and then confirmed at least every 6 months. The *BCR-ABL* transcript levels have been correlated with the numbers of residual leukemic cells in the CML samples as demonstrated in Figure 3.6 (85). Ph^1-positive CML patients who achieve a MMR at 12-month usually have a longer time to disease progression (49,86,87). The newest recommendation for imatinib treatment response assessment from European LeukemiaNet is demonstrated in Table 3.3.

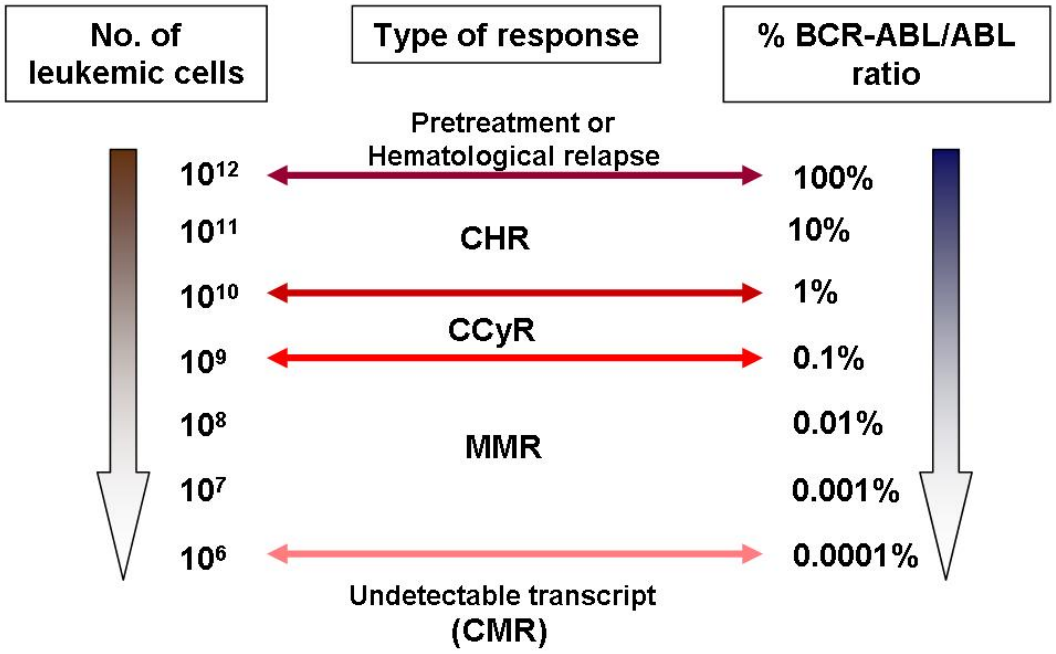


Figure 3.6 A schematic representation of relationship between responses, the number of leukemic cells, and the BCR-ABL transcript levels. (adapted from Baccarani M et al (85))

Table 3.3 Definitions of imatinib response in CML patients (81)

Time	Response			Warnings
	Optimal response	**Suboptimal response**	**Failure**	
Diagnosis	N/A	N/A	N/A	High Risk CCA/Ph+[3]
3 mo.	CHR, at least Minor CgR	No CgR	Less than CHR	N/A
6 mo.	At least PCgR	Less than PCgR	No CgR	N/A
12 mo.	CCyR	PCgR	Less than PCgR	Less than MMR[4]
18 mo.	MMR[4]	Less than MMR[4]	Less than CCyR	N/A
Any time (during treatment)	Stable or improving MMR[4]	Loss of MMR[4] Mutations[1]	Loss of CHR, Loss of CCyR, Mutations[2] CCA/Ph+[3]	Increase in transcript levels CCA/Ph-

[1] *ABL* KD mutation type still sensitive to imatinib

[2] *ABL* KD mutation type poorly sensitive to imatinib

[3] CCA/Ph[1]+ is a "warning" factor at diagnosis, although its occurrence during treatment (i.e., clonal progression) is a marker of treatment failure

[4] MMR indicates a ratio of *BCR-ABL1* to *ABL1* or other housekeeping genes of ≤0.1% on the IS.

3.6 Resistance to imatinib

3.6.1 Mechanism of imatinib resistance

Although imatinib is effective in inducing hematologic, cytogenetic and molecular response in most CML patients and is now considered the best drug ever developed for leukemia, resistant to imatinib has been reported to occur in some patients, especially those patients in an advanced phase (18-22, 32). The mechanisms that trigger imatinib resistance can be categorized in to *BCR-ABL* dependent resistant mechanism and *BCR-ABL* independent resistant mechanism (23-25).

3.6.1.1 *BCR-ABL* dependent resistant mechanism

The most common mechanism of *BCR-ABL* dependent resistant mechanism in CML patients is a point mutation at the amino acid residues critical for direct imatinib binding to BCR-ABL and at residues critical for stabilization of inactive conformation of BCR-ABL resulting in impaired imatinib binding and reactivation of TK activity (25). Another mechanism is *BCR-ABL* gene amplification which leads to increased BCR-ABL mRNA levels and subsequent BCR-ABL oncoprotein overexpression resulting in restoration of oncogenic signaling pathway in a given drug concentration (26). BCR-ABL expression has been reported in 18% of CML patients undergoing imatinib treatment (23, 32).

3.6.1.2 *BCR-ABL* independent resistant mechanism

BCR-ABL independent resistant mechanism are mostly related to the drug itself such as inadequate plasma levels of TKI, patients' incomplete adherence to the TKI regimen, drug metabolisms, intracellular uptake of imatinib (ATP-binding cassette (ABC) transporter, organic cation transporter 1 (OCT1)), and activation of other signaling pathways (26). For example, the Lyn, a Src family kinase, was found to be overexpressed and activated in imatinib resistant cell lines in the presence of imatinib during incubation and in samples from imatinib-resiatant CML (23,24).

3.6.2 Type of imatinib resistance

Two types of imatinib resistance are defined, i.e. primary resistance and secondary resistance. Primary or intrinsic resistance is defined as the inability to achieve an initial response to imatinib which was reported in 5% of patients in a

chronic phase, 24% of patients in an accelerated phase, and 66% of patients with a blastic crisis, respectively (88,89). The secondary or acquired resistance is defined as a treatment failure after an initial period of response with disease progression into an accelerated phase or blast crisis, loss of a sustained CHR without transformation, the development of new cytogenetic abnormalities in the Ph^1–positive clone, an increase of 30% or more of Ph^1–positive cells examined at an interval of 3 months or longer, loss of a major CyR, or loss of a CCR (45). The secondary resistance has been reported in 4% of patients in a chronic phase, 51% of patients in an accelerated phase, and 88% of patients with a blastic crisis, respectively (88,89).

3.7 *ABL* kinase domain (KD) mutations

ABL KD is the main target of genetic mutations in CML patients with imatinib resistance (27). The frequency of *ABL* KD mutations varies from 30% to 50% depending on the studied CML cohorts, type of resistance, and the sensitivity and specificity of the detection methods (21,22,28-34). *ABL* KD mutations confer resistance either directly by blocking imatinib binding or indirectly by altering the conformation of BCR-ABL (90).

3.7.1 Incidence of *ABL* KD mutations

Typically, mutations are seen in about 10%, 30%, 60%, and 90% of patients in early chronic phase, late chronic phase, accelerated phase, and blast crisis, respectively (69, 91-93). To date, more than 100 types of *ABL* point mutations have been identified and at least 40 different amino acid substitutions have been found with the overall mutation prevalence of 30-60% in patients who are imatinib-resistant (18, 27-29, 33, 59, 78, 82,94). Ten common mutant residues within the eight amino acid positions of KD (M244V, G250E, Y253H/F, E255K/V, T315I, M351T, F359V, and H396) have been described in about 85% of mutated patients (23, 33-34). Imatinib-resistant patients who have a baseline mutation prior to 2^{nd} generation TKI (nilotinib/dasatinib) exposure have a higher likelihood to develop further mutations and 83% of relapsed patients on 2^{nd} generation TKI contained additionally new mutations after baseline mutations (62).

3.7.2 Type of *ABL* KD mutations

Mutations can be clustered in four main regions; 1) ATP-binding loop (P-loop) that surrounds a phosphate group of the binding pocket, 2) a drug contact site, 3) catalytic domain (C-domain) which may participate to stabilize the A-loop in a certain conformation, and 4) the activation loop (A-loop), which is crucial for maintaining the inactive conformation of the pocket required for imatinib binding (Figure 3.7). Imatinib inserts its pyridinyl group underneath the helix C, displacing ATP and trapping the kinase in its inactive conformation (9, 34).

Although the first mutation detected in imatinib-resistant patients was the replacement of threonine by isoleucine at ABL amino acid position 315 (T315I), the most prevalent types of mutations in imatinib-resistant patients involve the P-loop (amino acid 248-256), which is the site where imatinib binds, and the drug contact site (amino acid 315 and 317), which provides a crucial H-bond interaction between imatinib and ABL (10, 27, 34). Leukemic cells with *ABL* mutations at these amino acid positions have been shown to have increased transforming potential as compared with the wild type (90).

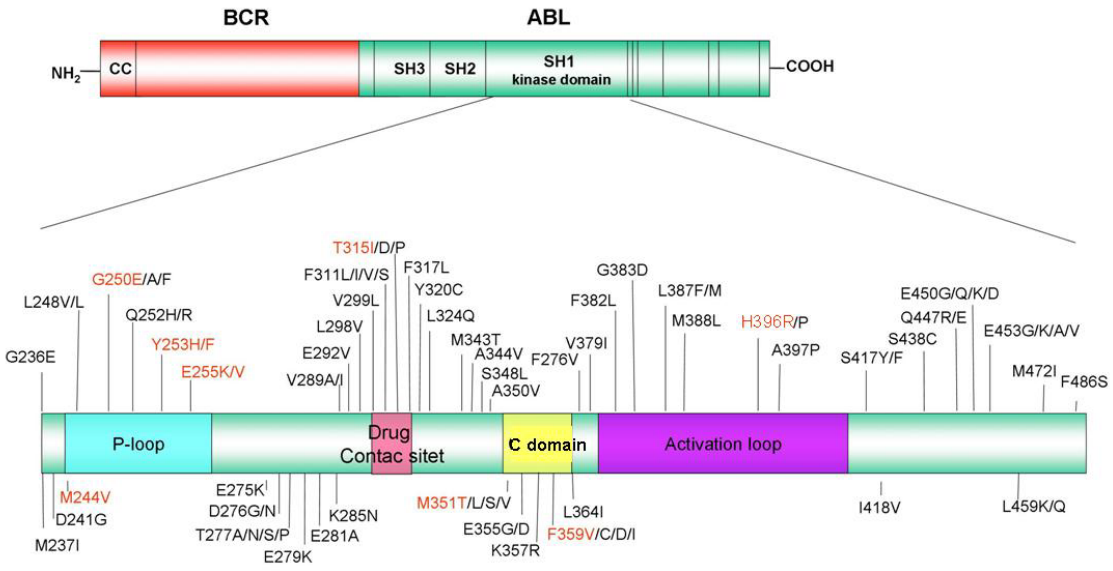


Figure 3.7 A schematic representation of previously reported *ABL* KD mutations within *BCR-ABL* gene of imatinib-resistant CML patients. Adapted from previous reports (23, 61,95).

Most common *ABL* KD mutation in imatinib resistant patient can be overcome by the 2nd generation TKI drugs although some mutations are less sensitive to nilotinib (Y253H, E255K/V, and F359V/C) whereas other mutations are less sensitive to dasatinib (V299L, F317L/V/C, and T315A). The only type of KD mutation that is truly resistant to imatnib as well as the 2nd generation TKI (nilotinib and dasatinib) (49,96,97) is T315I which changes the three-dimensional structure of the BCR-ABL binding site conformations and prevents binding of imatinib into the ATP-binding pocket (11,19,35-37). Recent CML treatment programs recommend discontinuation of imatinib treatment if T315I- or P-loop mutant clones are detected. Detection of baseline mutation before starting TKI treatment or in imatinib-resistant patients could be of prognostic significance to predict the response to TKI treatment (35).

3.7.3 Imatinib sensitivity level of various *ABL* KD mutations

In vitro sensitivity of unmutated *BCR-ABL* and *ABL* KD mutations to imatinib and 2nd generation TKI (nolotinib and dasatinib) can be described based on the IC50 level (Table 3.4) (4, 18, 34, 81, 117). The data from 10 different studies of the in vitro sensitivity levels of the common *ABL* KD mutations are summarized in Table 3.4 (81). IC50 is the drug concentration that inhibited 50% of the growth in culture of mouse lymphoblastoid Ba/F3 cells transfected with wild-type *BCR-ABL* gene or the *ABL* KD mutants.

3.7.4 Types of single nucleotide polymorphisms (SNPs)

The SNP must be distinguished from the mutations. Previously reported types of single nucleotide polymorphism (SNP) of *ABL* KD (amino acid 235-497) are listed in Table 3.5. None of them has ever been proposed to have any effect on TKI binding (98, 99). Therefore, in cases with a suspected novel point mutation, detection the normal ABL allele should be performed to exclude a SNP (98).

Table 3.4 The in vitro sensitivity level of the more common *ABL* KD mutations (81).

BCR-ABL Gene Mutation	IC50 Range by Agent (ng/mL)		
	Imatinib	Nilotinib	Dasatinib
Unmutated	153-400	< 5-13	0.4-0.9
M244V	944-1,829	20.1-20.6	0.7
L248V	1,101-5,900	26-486	4.7
G250E	796 to > 11,800	25-116	0.9-4.1
Q252H	433-1,841	8-37	1.7-2.8
Y253F	1,114-5,274	30-66	0.7-1.5
Y253H	> 3,800-10,442	238-688	1.3-10
E255K	1,873 to > 5,900	63-299	2.8-6.6
E255V	3,605-5,282	96-384	3.2-5.6
D276G	677	19	1.3
E279K	1,104	19-40	1.5
V299L	319-480	13	8-9.1
F311L	283-767	12	0.7
T315I	> 3,800 to > 11,800	369 to > 5,300	69.3 to > 500
T315A	448	NA	63
F317L	620-4,425	21-48	3.7-9.1
F317V	207-295	185	27
F317C	708	NA	NA
M351T	519-2,891	4.1-20.1	0.6-0.8
E355G	1,404	NA	NA
F359V	826-1,077	48-93	1.1-1.4
F359C	708	NA	NA
V379I	590-962	27	0.4
L384M	398-1,652	21-22	2
L387M	590-649	26	1
H396R	1,032-3,186	22-29	0.7-1.5
H396P	850-2,537	22-23	0.3-1.0
F486S	1,609-5,369	17-46	2.8

Table 3.5 Single nucleotide polymorphism on *ABL* KD (SNP database, NCBI, http://www.ncbi.nlm.nih.gov/projects/SNP/)

SNP ID	**Nucleotide** (NM_005157.4)	**Class**	**Type**	**Amino Acid** (NP_005148.2)
Nucleotide substitution				
rs79052735	T/C	SNP	Non-synonymous coding	S206P
rs56271006	G/T	SNP	Non-synonymous coding	R220L
rs111371946	C/A	SNP	Synonymous coding	P230P
rs2229069	G/A	SNP	Synonymous coding	T240T
rs34549764	A/G	SNP	Synonymous coding	K247R
rs1141212	G/A	SNP	Synonymous coding	L298L
rs1141213	G/A	SNP	Synonymous coding	L354L
rs1064156	G/A	SNP	Non-synonymous coding	E459K
rs62638716	G/A	SNP	Non-synonymous coding	R473Q
rs2227985	A/G	SNP	Synonymous	E499E
Insertion				
rs34470251	c.1009_1010insC		Frameshift coding	337
rs67495456	c.1222_1223insC		Frameshift coding	408
rs67981317	c.1317_1318insC		Frameshift coding	439
rs66887049	c.1399_1400insA		Frameshift coding	467

3.8 *ABL* KD mutation detection

Recent recommendation suggests that the *ABL* KD mutation analysis should be performed in all cases with imatinib failure, suboptimal response, and rising *BCR-ABL* level (85, 100). Furthermore, the 2008 National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology recommends *ABL* KD mutation screening for patients in a chronic phase because it may provide useful information in cases where there is no initial response or any sign of loss of response (83, 101). Mutation testing is also performed in those who have a non-optimal response to imatinib and are likely to become resistant to imatinib, in patients who are receiving dose escalation, combination therapy, or administration of novel 2nd generation TKI (57, 81, 83). Continuing imatinib treatment at the current dose the patients fail to respond is no longer appropriate (19, 35-37). In addition, because of the rapidly disease progression in cases with resistance to 2nd generation TKI, assessment for the high risk types of mutations should be considered (35,81). Disease monitoring after treatment is the essential management strategy of CML to assess the response and to detect early relapse.

The available methods which have been used to detect the existence of *ABL* KD mutation in CML patients are summarized in Table 3.6. The sensitivity and specificity of each method is also shown. The following section describes only the 3 methods that are relevant to the present study.

3.8.1 Direct sequencing (34)

Direct sequencing is widely used to investigate the *ABL* KD mutations with a low sensitivity of about 15-20% (27,32,38,42,45-47). It also has a limited sensitivity, costs more and takes more time to finish.

3.8.2 Denaturing high performance liquid chromatography (DHPLC) (34)

Denaturing high performance liquid chromatography (DHPLC) is a newer, faster, and more sensitive method (0.5-5%) than sequencing. It is increasing used for *ABL* KD mutation detection (19, 38 51,52,102,103). This method detects a mutation based on a heteroduplex formation by PCR products amplified from the wild-type and

mutant alleles. Under optimized denaturing conditions, these heteroduplexes can be separated from homoduplexes (19,51,102). DHPLC can be used to identify known and new mutations.

3.8.3 Allele specific-polymerase chain reaction (AS-PCR) (34, 54)

In most of the mutated cases, the proportion of the mutant allele to wild-type was very small, and may require more sensitive techniques, such as polymerase chain reaction with allele specific oligonucleotides (ASO-PCR) (28,54,55). The AS-PCR was the earliest assay that was shown to be more sensitive than conventional DNA sequencing (4). Detection of mutation by AS-PCR is based on amplification of allele of interest using allell- specific primers. The additional mismatch at the third base from the 3' end was introduced to increase the specificity of primer-template binding. The internal control primer was also added to determine the efficiency of amplifications.

Table 3.6 Detection methods for *ABL* KD mutations

Method	Sensitivity %	Type of mutations	References
Direct sequencing	15-25	unknown	(27,32,38,42,45-47)
Subcloning and sequencing	10	unknown	(27)
DHPLC	0.5-5	unknown	(19,38,51,52,102,103)
RFLP	5	known	(26)
AS-PCR	0.01-0.5	known	(28, 34, 38,42,51,54,55,105,106)
AS real-time PCR	0.01	known	(42)
Pyrosequencing	5	unknown	(104)
Double-gradient denaturing electrophoresis	5	unknown	(53)
Fluorescent PCR and PNA clampling	0.2	known	(40)
Taqman-based RQ PCR	0.1	known	(107)

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Patient samples

Peripheral blood (PB) and/or bone marrow (BM) were collected from 171 CML patients with Ph^1 chromosome and/or *BCR-ABL* fusion gene who received medical care at the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. Eighty newly diagnosed or naïve CML cases without prior TKI exposure and 91 CML cases with a history of TKI exposure were recruited into this study. Among the latter, 72 cases were being treated with imatinib, 15 cases had failed imatinib and were subsequently switched to 2^{nd} generation TKI, and the rest four cases were being treated with 2^{nd} generation TKI (nilotinib or dasatinib) as a front-line therapy. Approximately 0.5-2 mL of BM and/or 3-5 mL PB were collected from each patient. The diagnosis of CML was made by clinical characteristics and standard morphological, cytogenetic, and molecular methods. The present study was approved by the Ethical Committee for Human Research, Faculty of Medicine Siriraj Hospital, Mahidol University (certificate no. 563/2551(EC2)).

4.1.2 Control samples

Normal samples (*BCR-ABL* negative control) were obtained from consenting healthy people. K562 cell lines (*BCR-ABL* wild-type positive control) were purchased from American Type Culture Collection (ATCC, USA). BA/F3 cell lines (BA/F3 mouse pro-B cells) expressing the wild-type full-length *BCR-ABL* fusion gene and ABL KD mutants (M244V, G250E, Y253F, Y253H, E255K, E255V, T315I, M351T, and F359V) cell lines were kindly provided by Professor Brian J Druker and Professor Michael W Deininger (Oregon Health & Science University, USA) (14).

4.1.3 Oligonucleotide primers

RT-PCR of *BCR-ABL* fusion gene was performed using 2 pairs of primers. The list of nucleotide sequences, PCR product sizes, and nucleotide position is demonstrated in Table 4.1.

Table 4.1 Nucleotide sequences of the primers for the amplification of *BCR-ABL* fusion gene by RT-PCR

Primer name	Nucleotide sequence 5'-3' sequence	No. of nucleotides	Product size (bp)
bcr_f	CTCCAGACTGTCCACAGCATTCCG	24	168 (e14a2)
abl_r	CAGACCCTGAGGCTCAAAGTCAGA	24	93 (e13a2)
β-actin_f	GTGGGGCGCCCCAGGCACCA	20	540
β-actin_r	GTCCTTAATGTCACGCACGATTTC	24	

Table 4.2 shows the list of nucleotide sequences, nucleotide position, and PCR product sizes after amplifications with all primers in the nested RT-PCR for *BCR-ABL* KD while Table 4.3 shows the list of nucleotide sequences, nucleotide position, and PCR product sizes after amplifications with all primers in the real-time quantitative PCR (RQ-PCR) for BCR-ABL transcript levels. In a preliminary study of T315I mutation, a single tube AS-PCR was developed using three-paired primers consisting of T315I mutant primers, wild-type primers, and internal control gene (*β*-actin) as shown in Table 4.4.

The schematic representations of the primers used in this study which correspond to those listed in Table 4.1 to 4.4 are demonstrated in Figure 4.1 to Figure 4.4, respectively. Specifically, the location of the two primer pairs designed to amplify the KD of BCR-ABL in the 2nd round nested RT-PCR is shown in Figure 4.2.

4.1.4 Other materials

Chemicals, instruments, enzymes, reagents and others are listed in the appendix.

Table 4.2 Sequences of PCR primers used in the nested RT-PCR

Primer name	5'-3' sequence	Position	PCR product size (bp)
1st round PCR			
B2A _f	acagcattccgctgaccatcaataag	*BCR* exon12	1643 (e13a2) or 1719 (e14a2)*
BA_r	atggtccagaggatcgctctct	*ABL* exon 11	
2nd round PCR			
abl_1f	tggttcatcatcattcaacggtgg	*ABL* exon 4	447
abl_1r	tctgagtggccatgtacagcagc	*ABL* exon 6	
abl_2f	tcatgacctacgggaacctc	*ABL* exon 5	333
abl_2r	atactccaaatgcccagacg	*ABL* exon 7	

The sequence of *BCR* and *ABL*1 gene according to GenBank Accession no. Y00661 and NM_005157.4, respectively. *Size of the 1st round PCR product depends on the respective *BCR-ABL* transcript on the patients.

Table 4.3 Sequences of primers and hybridization probes used in RQ-PCR assay

Primer name	5'-3' sequence	Position
B2A	TTCAGAAGCTTCTCCCTGACAT	BCR exon 13
PE1+	CAGATCTGGCCCAACGATGG	BCR exon 1
A2N	CCCAACCTTTTCGTTGCACTGT	ABL exon 2
NA4-	CGGCTCTCGGAGGAGACGTAGA	ABL exon 4
Hybridization probes		
5'Probes	LC Red 640-AATGGGGAATGGTGTGAAGCCCAAA-	ABL exon 3
3'Probe	P TGAAAAGCTCCGGGTCTTAGGCTATAATCA-F	ABL exon 3

HP, Hybridization probe; LC Red 640, LightCycler fluorescence dye 640; F, fluorescein; P, phosphate.

Table 4.4 Oligonucleotide primers used in the AS-PCR of the T315I mutation

Primer name	Nucleotide sequence 5'-3' sequence	No. of nucleotide	Product size (bp)
T315I Mutant			
MT_F	GCCCCCGTTCTATATCATaAT	21	158
MT_R	GGATGAAGTTTTTCTTCTCCAG	22	
T315 Wild type			
WT_F	TGGTTCATCATCATTCAACGGTGG	24	374
WT_R	GTTCCCGTAGGTCATGAACTCAG	23	
Internal control			
β-actin_R	GTGGGGCGCCCCAGGCACCA	20	540
β-actin_R	GTCCTTAATGTCACGCACGATTTC	24	

Underlined text indicates a nucleotide complementary to the mutant allele. The lower case shows a mismatch at the 3rd nucleotide from the 3' end which is introduced to increase the specificity of the T315I mutant allele.

(A.) B2A2 (e13a2)

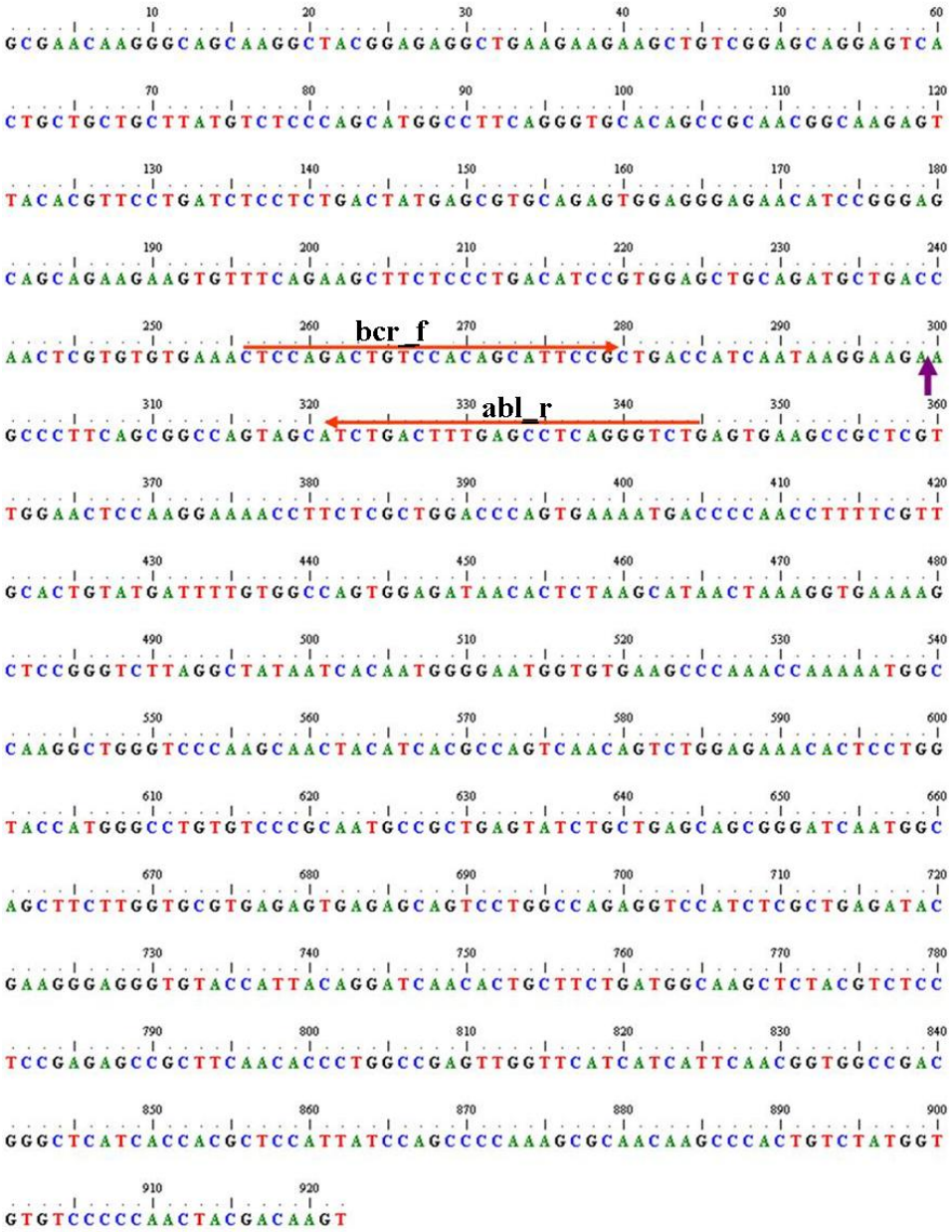


Figure 4.1 Reference nucleotide sequences of *BCR-ABL* fusion cDNA. The break point is indicated by a vertical purple arrow. Arrows indicate the 5' to 3' direction of the primers (bcr_f and abl_r). The PCR amplification of exon 13 of *BCR* and exon 2 of *ABL* gene (Figure 4.1A) and of exon 14 of *BCR* and exon 2 of *ABL* gene (Figure 4.1B) correspond to B2A2 and B3A2 variants, respectively.

B3A2 (e14a2)

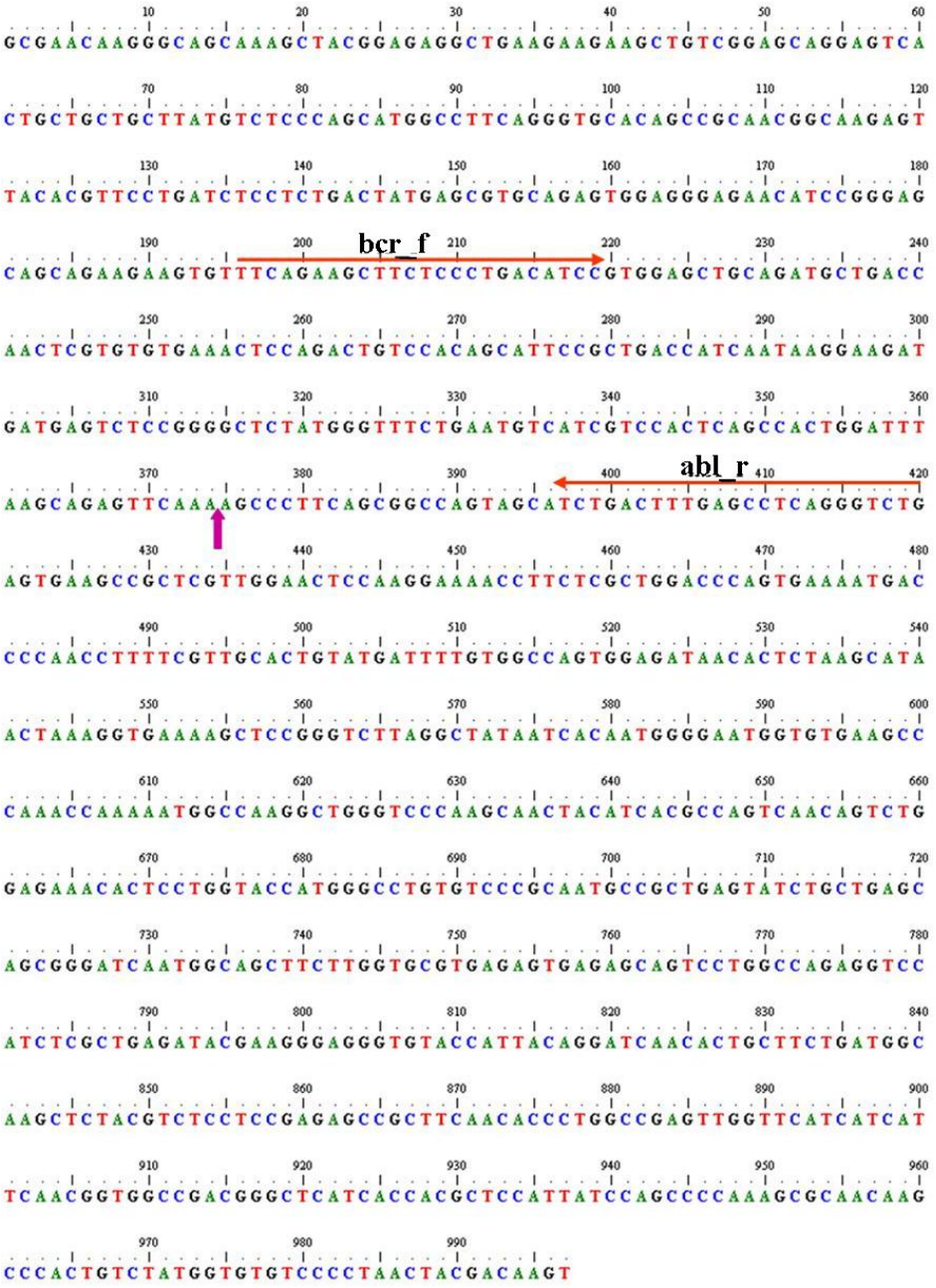


Figure 4.1 Reference nucleotide sequences of *BCR-ABL* fusion cDNA (cont.). The break point is indicated by a vertical purple arrow. Arrows indicate the 5' to 3' direction of the primers (bcr_f and abl_r). The PCR amplification of exon 13 of *BCR* and exon 2 of *ABL* gene (Figure 4.1A) and of exon 14 of *BCR* and exon 2 of *ABL* gene (Figure 4.1B) correspond to B2A2 and B3A2 variants, respectively.

Figure 4.2 Locations of the two primer pairs designed to amplify the *BCR-ABL* KD used in the study. Arrows indicates the 5' to 3' direction of the primers and the location where the primers for the 2nd round nested RT PCR bound to the template. The yellow highlight indicates the sequences of the KD. (*ABL1* sequence was retrieved from GenBank accession number NM_005157.4).

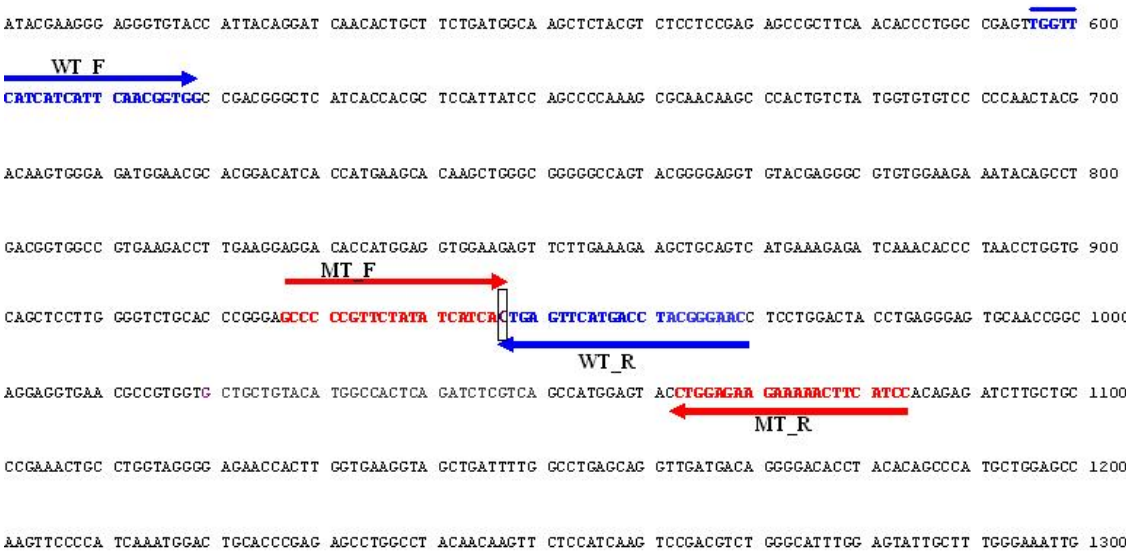


Figure 4.3 A schematic representation of *ABL* KD gene with the AS-PCR primers. Red and blue alphabets indicate the nucleotide sequences and the location where the primers for T315I mutant and T315wild type, respectively, bound to the cDNA template. Arrows indicates 5'→3' direction of the primers. KD cDNA sequence of the *ABL1* gene (accession number NM_005157.4) was retrieved from GenBank database. The C in the box is the position of T315I (c.944C>T).

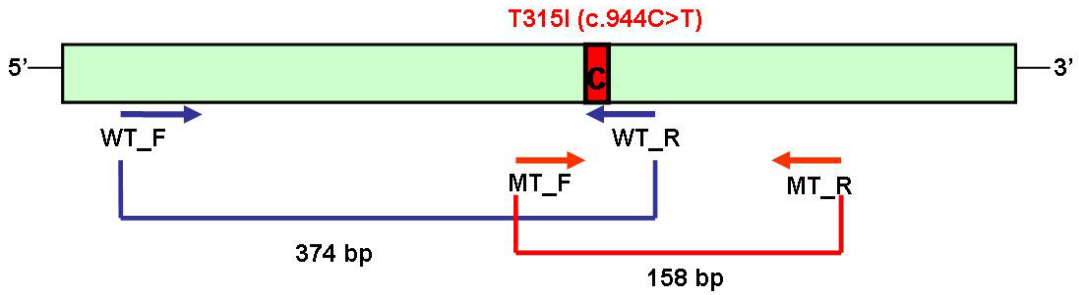


Figure 4.4 A schematic representation of the product of AS-PCR for T315I mutation detection. The C in the box is the position of T315I (c.944C>T). Red and blue alphabets arrow indicate the nucleotide location and the direction of transcription where the primers for T315I mutant and wild type, respectively, bound to the cDNA template.

4.2 Methods

4.2.1 Experimental strategy

Two groups of CML patients were identified. The first group was the naïve CML patients (chronic phase and blastic phase) who had never been treated with imatinib and the second group was CML patients who were currently receiving imatinib or 2nd generation TKI. Leukocytes were isolated from BM or PB samples and total RNA was isolated from leukocytes by standard phenol/chloroform method using the TRIzol ® reagent (Invitrogen, CA, USA), following the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by SuperScript III cDNA synthesis kit (Invitrogen, Canada). The presence of *BCR-ABL* fusion gene was determined by the RT-PCR in all CML cases.

The molecular methods for *ABL* KD mutation were developed using DHPLC and direct sequencing. K562 leukemic cell lines were used as a wild-type *BCR-ABL* control while BA/F3 cell lines with *ABL* KD mutation represented known mutant positive controls and normal blood samples represented *BCR-ABL* negative controls. The PCR products from the first nested RT-PCR assay was used for the 2nd round PCR whereby the two overlapping fragments corresponding to *ABL* KD were amplified. PCR products were screened for *BCR-ABL* mutation by DHPLC. Abnormal DHPLC peaks were confirmed by direct sequencing. The sequencing analysis was performed to identify the nucleotide alterations and the mutation type of *ABL* KD as compared with *ABL1* (accession no. NM_005157.4). The amounts of *BCR-ABL* fusion gene transcripts were quantified by RQ-PCR to determine the BCR-ABL/ABL ratio in patients who were receiving TKI therapy and carried the *ABL* KD mutation. I also developed a single-tube AS-PCR assay specifically for T315I resistant mutation detection. The sensitivity of sequencing, DHPLC, and AS-PCR for T315I were evaluated and compared. For AS-PCR, RNA from T315I mutant cell lines was serially diluted by wild-type BA/F3 cells to prepare 10 dilutions with indicated percentages of T315I mutants, and the PCR product serial dilutions were used in sequencing and DHPLC sensitivity tests. Thirty RNA samples from non-leukemic patients were used as negative control samples to optimize the AS-PCR condition. Correlation between KD mutations and the BCR-ABL/ABL ratio,

hematologic parameters, karyotype, and clinical data were determined. The experimental strategy is delineated in Figure 4.5.

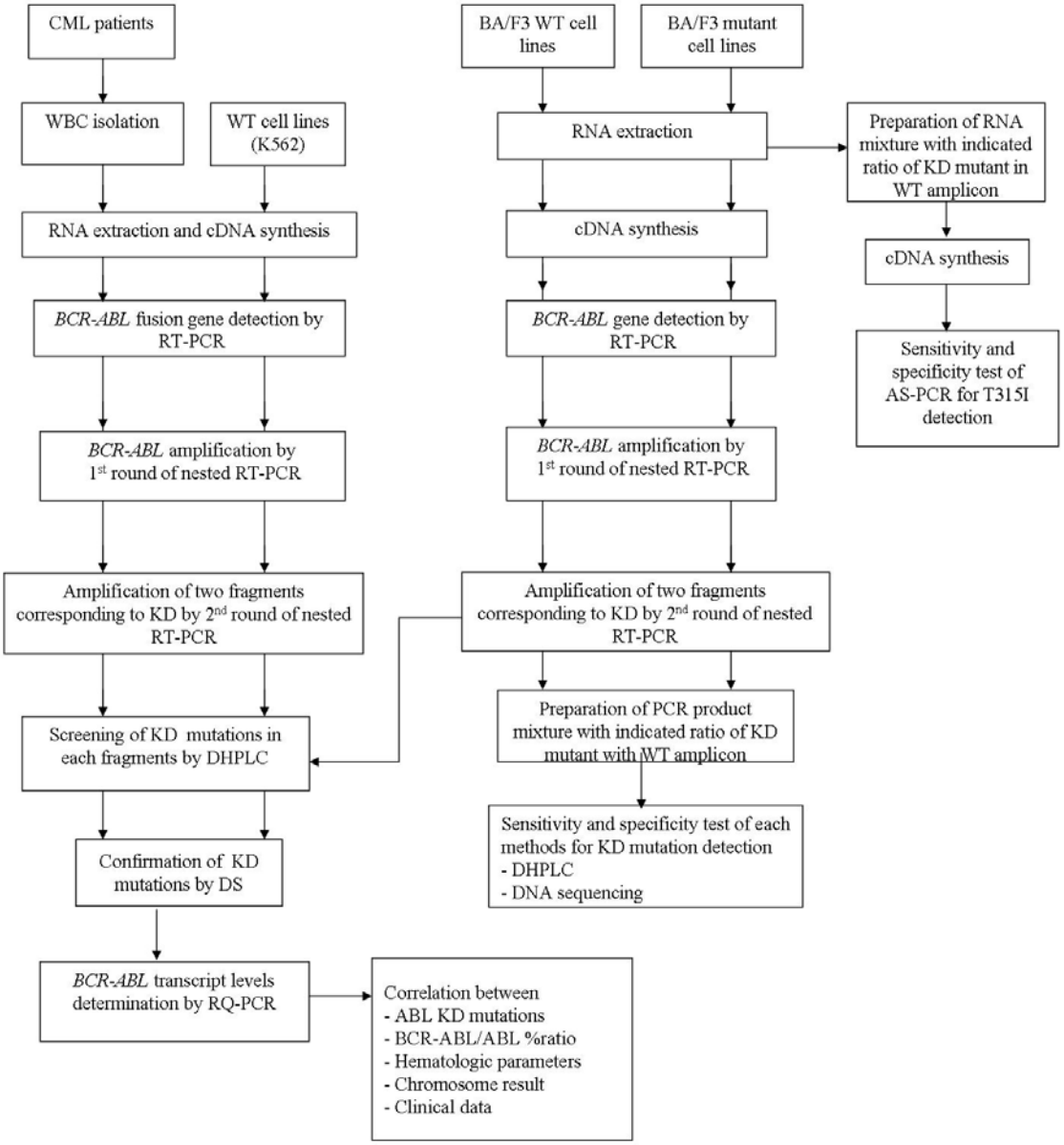


Figure 4.5 Experimental strategies in this study

4.2.2 Cell line subculture

Human cell line (K562) and murine BA/F3 expressing wild type *BCR-ABL* and *BCR-ABL* mutants cell lines were maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% fetal calf serum and incubated at 37°C with 5% CO_2. Each cell line was then subcloned every 3 days or kept between $2x10^5$-$1x10^6$ cells/mL for routine maintenance as recommended by the OHSU.

4.2.3 Sample preparation

EDTA samples were evaluated for complete blood count (CBC): hemoglobin (HB) (g/dL), hematocrit (%), white blood cell (WBC) count (cells/μL), and differential WBC (%), and platelet (PLT) count (cells/μL). The contaminating red blood cells in the PB or BM were lysed with a lysis buffer and the remaining WBC were washed with phosphate buffer saline (PBS) twice, divided into aliquots and then stored at –80°C until RNA extraction process.

4.2.4 RNA preparation and cDNA synthesis

Total RNA was extracted from WBCs by a single-step method using TRIZOL™ reagent (Invitrogen corperation, CA, USA). One mL of TRIZOL™ reagent was added to lyse the WBCs. The homogenized samples were incubated for 5 minutes (min) at 15-30 ºC to permit the complete dissociation of nucleoprotein complexes. Two hundred microlitres (μL) of chloroform were added to 1 mL of TRIZOL™ reagent, gently mixed for 15 seconds (sec) and incubated for 2-3 min, then centrifuged at 12,000g for 15 min at 4 ºC. The mixture was separated into a lower red phenol-chloroform phase, interphase, and a colorless upper aqueous phase. The aqueous phase which contains total RNA was transferred to a fresh tube. The total RNA was precipitated by adding 0.5 mL of isopropyl alcohol and incubated at room temperature for 10 min and then centrifuged at 12,000xg for 10 min at 4 ºC. The supernatant was discarded. The RNA pellet was then washed with 75% ethanol before centrifugation at 7,500xg for 5 min at 4 ºC. The RNA pellet was dried for 5-10 min and dissolved in 10-30 μL in RNase-free water or sterile diethyl pyrocarbonate (DEPC)-treated water,

depending on the amount of RNA. The concentration of the total RNA was estimated by using the following formula:

$$\text{Concentration of RNA } (\mu g/ml) = \frac{OD_{260} \text{ x dilution factor x } 40}{1000}$$

The quantity and purity of RNA was spectrophotometrically measured by 260 nm and 280 nm absorbance. RNA was quantified at 260 nm absorbance and the purity of RNA preparation was determined by the A260 nm/A280 nm ratio using a spectrophotometer (UV-1201, Shumadzu, Japan). A value of 1.8-2.0 indicates that the total RNA was pure. The RNA was kept in DEPC-treated water at -80 ºC. For long-term storage, RNA was precipitated and kept in 75 % ethanol at -80 ºC.

cDNA was synthesized by a reverse transcription reaction with either random hexamer primers or oligo dT primers of the SuperScript III cDNA synthesis kit according to the manufacturer's procedure (Invitrogen, CA). Three micrograms (µg) of total RNA was mixed with 1 µg oligo (dT) primer or random hexamer, followed by a denaturation step in 70 ºC for 10 min. The cDNA was synthesized at 42 ºC for 45 min and 97 ºC for 10 min. cDNA was kept at -20 ºC.

4.2.5 Detection of *BCR-ABL* fusion transcripts by multiplex reverse-transcription PCR (RT-PCR) method

The *BCR-ABL* fusion gene was detected by the multiplex RT-PCR method. cDNA was amplified by the fusion gene primers, bcr_f, abl_r and internal control primers, β-actin_f and β-actin_r, as previously described (3,112). PCR reactions were performed in a total volume of 25 µL composed of 5x PCR buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTPs, 15 pmol primer mix, 1 U Taq DNA polymerase, and 1 uL cDNA. The mixture underwent initial denaturation at 95 ºC for 7 min followed by 35 cycles at 94 ºC for 45 sec, 60 ºC for 1 min, 72 ºC for 1 min, with a final extension of 72 ºC for 10 min. The amplification was performed on the P2 PCR machine or Veriti Thermo cycler (Applied Biosystems, USA). Two variants were separated with different product sizes: e14a2 (168 bp) and e13a2 (93 bp). The cDNA integrity was assessed by amplification of β-actin (540 bp).

4.2.6 Amplification of *ABL* KD by nested RT-PCR

The first round PCR for the amplification of 1,643 bp or 1,719 bp *BCR-ABL* was performed using the following primers: B2A _f (position on BCR exon12) and BA_r (*ABL* exon 11) (19). First round PCR reaction mixture was made up in a total volume of 25 µL with the following constituents, 0.2 U of High-Fidelity DNA polymerase (Phusion^{TM}, FINNZYME), 5X GC Phusion buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 10pmol of each primer in first set (see Table 4.2 for sequences), and 1-2 µL cDNA. The reaction mixture was briefly centrifuged and then placed in a thermal cycler with a PCR profile as follows: an initial denaturation step at 98 °C for 30 sec and 40 cycles of denaturation at 98 °C for 10 sec, annealing at 60 °C for 30 sec and extension at 72°C for 1 min 30 sec with final extension step at 72°C for 10 min. PCR results were visualized on ethidium bromide-stained 2% agarose gel.

For a second round PCR for amplification of KD, internal two primer pairs were designed to separate such domain (codon 206-522) into two partially overlapping fragments, abl_1 (447 bp, codons 206-346) and abl_2(333 bp, codons 293-428,) (Figure 4.6). PCR reaction was performed in a total volume of 25 µL containing 1 µl first round PCR product, 0.2 U of High-Fidelity DNA polymerase (Phusion^{TM}, FINNZYME) , 5X Phusion buffer, 2.5 mM $MgCl_2$, 200 uM dNTPs, and 10 pmol of each primer pair. The PCR profile was as follows: initial denaturation at 98 °C for 30 sec, amplification for 40 cycles (98 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 40 sec) and final extension at 72 °C for 5 min. The control samples were K562 cDNA (*BCR-ABL* positive controls) and normal samples (*BCR-ABL* negative controls).

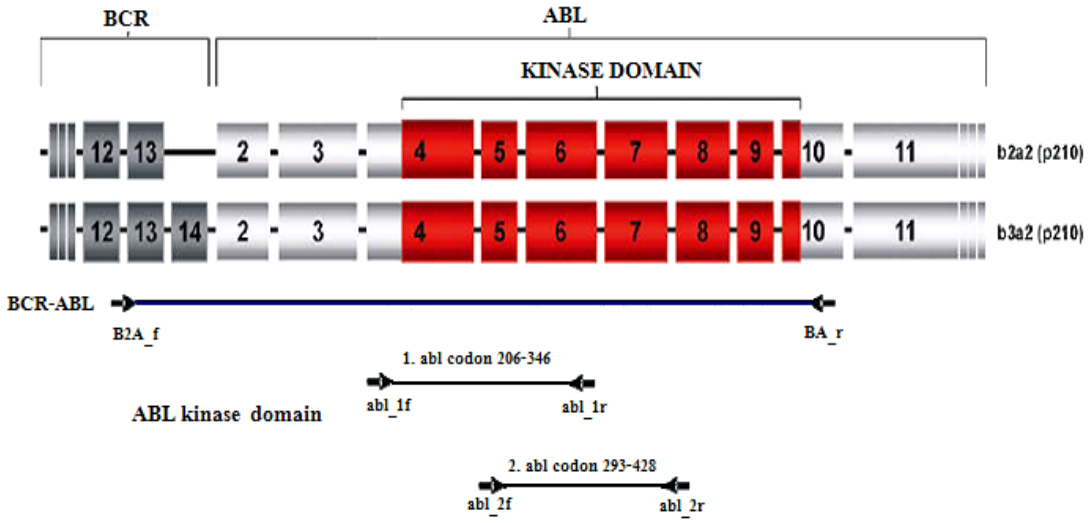


Figure 4.6 A schematic representation of the *ABL* KD amplified fragments. The 1st round PCR was for fusion gene amplification and in the 2nd round PCR, two partial overlapping *ABL* cDNA fragments were analyzed as shown.

4.2.7 *ABL* KD mutation screening by DHPLC

The 447 bp and 333bp PCR products from nested-RT PCR were analysed by DHPLC. PCR products were denatured by heating at 95 °C for 5 min followed by gradual cooling (a decrease at 1°C/min) to 25 °C within 70 min in order to allow the heteroduplex and homoduplex formation. PCR products were then analyzed using a WAVE® Nucleic Acid Fragment Analysis system (Transgenomic™Inc., Omaha, NE, USA) under partial denaturing conditions. Optimal gradient of acetonitrite and column temperature conditions for the separation of heteroduplexes were calculated for each PCR product fragment using Navigator™ Software (Transgenomic, USA). Five to ten µL of each *ABL* KD fragments were injected into a chromatography column (DNASep® HT column, Transgenomic, USA) equilibrated by an ion pairing agent, 0.1 M triethylammonium acetate (TEAA). The heteroduplexes and homoduplexes were then eluted at 59 ºC at a constant flow rate of 1.5 mL/min by a linear acetonitrile gradient achieved by mixing buffer A (0.1 M TEAA, pH 7.0) with buffer B (0.1 M TEAA, pH 7.0, and 25% acetonitrile). The eluted cDNA was detected

plot of absorbance versus time. K562 cells were used as a wild-type control. The DHPLC- positive samples and BA/F3 with common BCR-ABL mutant cell lines (M244V, G250E, Y253F, Y253H, E255K, E255V, T315I, M351T, and F359V) were used as a mutant control.

4.2.8 *ABL* KD mutation detection by sequencing

4.2.8.1 Purification of PCR products

All PCR products with abnormal DHPLC profile were purified and sequenced in forward and/or reverse directions with the primers in the second round PCR to identify the mutation types. After PCR completion, the size and purity of each amplification fragment was assessed by 2.0% agarose gel electrophoresis and stained with ethidium bromide. The product was then purified according to the manufacturer's instructions using the Qiaquick PCR Purification Kit (Qiagen, USA) or purified using ExoSAP-IT® (GE Healthcare Bio-Sciences, USA) by incubation at 37 °C for 15 min and 80 °C for 15 min. The purified product was run on agarose gel electrophoresis and its concentration was estimated from band intensity by comparing to standard DNA markers, Φx174 DNA digested with *Hae*III.

4.2.8.2 Analysis of DNA sequences

An aliquot of the same PCR products analysed by DHPLC were directly sequenced on the ABI3730XL PRISM BigDyeTM analyzer (Applied Biosystems, USA) using ABI PRISM BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, USA) following the manufacturer's protocol. The generated ABL_1 and ABL_2 products encoding amino acids 206-428 of *ABL* KD were sequenced for each fragment. The results were compared with the wild-type *ABL* gene sequence (accession no. NM_005157.4 ; tyrosine-protein kinase ABL1 isoform a) as previously described.

Nomenclature for new sequence variants was performed using the guideline from of the Human Genome Variation Society (HGVS) website (http://www.hgvs.org/). The reliability and properties of new mutations or variations based on the protein identifier from the UniProt database was determined through the Polyphen website (http://genetics.bwh.harvard.edu/pph/). PolyPhen (Polymorphism Phenotyping) is a tool for prediction of possible impact of an amino acid substitution

on the structure and function of a human protein based on physical and comparative considerations which are applied to the sequence, phylogenetic and structural information characterizing the substitution (113). The novelty of any *ABL* KD mutations identified was determined by checking with mutation database available in dbSNPdatabase (http://www.ncbi.nlm.nih.gov/projects/SNP/), COSMIC (Catalogue Of Somatic Mutations In Cancer) (http://www.sanger.ac.uk/genetics/CGP/cosmic/), OMIM (Online Mendelian Inheritance in Man) (http://www.ncbi.nlm.nih.gov/omim), and MoKCa (Mutation of Kinase in Cacer (http://strubiol.icr.ac.uk/extra/mokca/). The nucleotide position was based on the cDNA Gene Bank accession no. NM_005157.4 and the sequence variations were described according to the HGVS system.

4.2.9 Determination of *BCR-ABL* fusion transcript levels by real-time quantitative PCR (RQ-PCR)

BCR-ABL and *ABL* transcripts in cDNA of CML patients who were receiving imatinib or 2nd generation TKI and developed *ABL* KD mutation were determined by RQ-PCR. The PCR reactions were performed in a total volume of 20 µL composed of 16 µL mastermix containing buffer, dNTPs, and *Taq* polymerase (LightCycler DNA Master Hybridisation Probe, Roche diagnostic, Geramany), 0.5 µM of each Fluorescein and LC Red640 fluorescent hybridization probe, 1µM of primers, 1U uracil DNA glycosylase (UDG), and 4 µL cDNA. The mixtures were preincubated at 95 °C for 10 min, and amplified for 50-70 cycles at 95 °C for 15 sec, 55 °C for 20 sec, and 72 °C for 30 sec, then cooled down at 40 °C for 10 sec. Amplification, fluorescence detection and calculation were performed in the LightCycler instrument (LightCycler 480, Roche Diagnostics). The results were reported in terms of the % BCR-ABL/ABL ratio. The principle of method was a relative quantification of the target gene (*BCR-ABL*) normalized over a constant level of an endogenous reference gene (*ABL*) in each sample. The *ABL* copy number indicates that the quality of cDNA was adequate to accurately report this result. The following primers were used for *BCR-ABL* transcript amplification: e14a2, e13a2, e14 a3, and e13a3 variants using primers B2A and NA4- and e1a2 variant using primers PE1+ and NA4-. The control gene *ABL* amplification used primers A2N and NA4- as modified from previously described protocols (70).

4.2.10 AS-PCR for detection of T315I mutation

A single-tube AS-PCR was specifically developed for T315I mutation detection using three pair primers consisting of 1) T315I mutant primers, forward primer (MT_F) and reverse primer (MT_R), which were adapted from previously published primer sets (54), 2) the wild-type primers, WT_F and WT_R, and 3) internal control primers, forward (*β*-actin_F) and *β*-actin_R. First, the AS-PCR was optimized by varying annealing temperature (Ta) (55 °C to 60 °C), $MgCl_2$ concentration (1.5 to 2.5 mmol/L), and a primer ratio (MT primers : WT primer ratio of 9:1, 8:2, 7:3, 6:4, and 5:5). Briefly, the optimized condition was performed in a 25 μL mixture of 1 μL cDNA, 2.5 mmol/L MgCl2, 0.2 mmol/L each of dNTP, 3% DMSO, and 0.625 unit of *Taq* DNA polymerase (Invitrogen, USA) which were subsequently mixed together with 16 pmol of MT primers, 4 pmol of WT primers, and 0.2 pmol of β-actin primers. The PCR conditions comprised an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 45 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products of the T315I mutant, T315 wild type, and *β*-actin were seen as 158 bp, 374 bp, and 540 bp, respectively. The products were assessed on a 2% agarose gel and stained with ethidium bromide. Thirty RNA samples from non-leukemic patients were used as a negative control sample to optimize AS-PCR condition.

4.2.11 Cytogenetic analysis of leukemic samples

Chromosome analysis was performed using the standard G/Q banding techniques and chromosomal abnormalities were described according to the International System for Cytogenetic Nomenclature (ISCN) (114).

4.2.12 Statistical analysis

The Mann-Whitney test was used to compare quantitative variables. Comparison of qualitative variables was performed using Chi-square test or Fisher's exact test. For all analyses, a p-value of less than 0.05 was considered statistically

significant. Statistical significance was set at the level of $P<0.05$. Overall survival (OS) was defined as time from diagnosis to date of last follow-up or death (24).

CHAPTER V
RESULTS

5.1 Development of a molecular method for the detection of *BCR-ABL* fusion gene variants and *ABL* KD mutations

5.1.1 Detection of *BCR-ABL* fusion gene variants by multiplex RT-PCR in CML patients

A multiplex RT-PCR was developed to detect the *BCR-ABL* fusion gene variants in all CML cases recruited into the study. The fusion gene contained the 5'*BCR* gene that fused with the 3' *ABL* gene. The integrity and quality of synthesized cDNA was assessed by RT-PCR amplification of the *β*- actin gene in the same reaction of *BCR-ABL* fusion gene. The PCR products of representative CML patients with *BCR-ABL* fusion gene are shown in Figure 5.1.

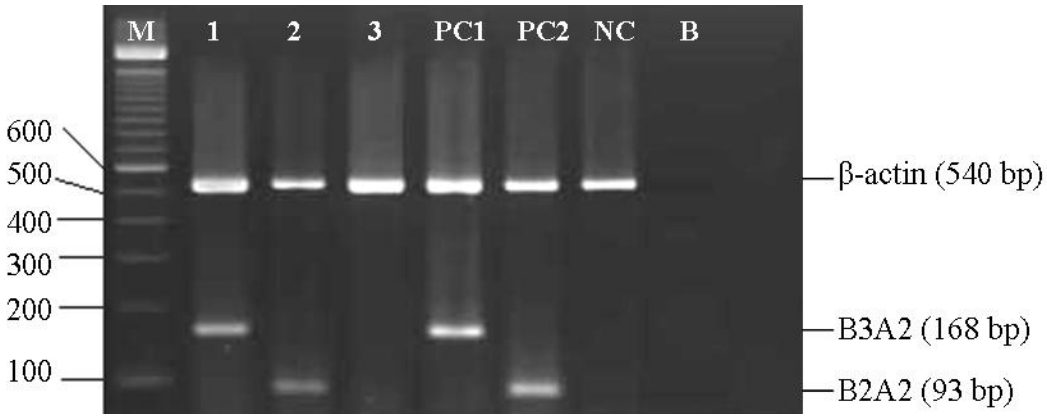


Figure 5.1 Agarose gel electrophoresis of CML patients with *BCR-ABL* fusion gene. The length of the *BCR-ABL* variant B3A2 (e14a2), B2A2 (e13a2), and *β*-actin amplified products were 168 bp, 93 bp, and 540 bp, respectively. Lanes 1, 2, 3 are patient samples. Lanes PC1, PC2, NC and B are B3A2 positive control, B2A2 positive control, negative control, and blank, respectively. Lane M is a 100-bp DNA ladder used as a standard-size marker.

5.1.2 Amplification of two fragment amplicons of *ABL* KD by nested RT-PCR

A total of 162 samples of *BCR-ABL* positive CML patients were available for KD mutation screening. PCR products were generated by a nested-RT PCR to enhance the sensitivity and specificity of *ABL* KD. As the non-rearranged *ABL* transcript not included in to the analysis, the 1st round PCR was performed for the amplification of 1643 bp (B2A2) or 1719 bp (B3A2) *BCR-ABL* using a primer pair which covered the fusion position (BCR exon12 and *ABL* exon 11). Figure 5.2 shows the *ABL* KD products of CML patients with *BCR-ABL* fusion after a 2nd round PCR that amplified two ABL fragments covering the most common KD mutant types. The length of *ABL* fragment 1 and fragment 2 amplified products were 447 bp, and 333 bp, respectively. The cDNA template of *BCR-ABL* positive controls (K562) and *BCR-ABL* negative controls were also amplified in parallel with the patient samples.

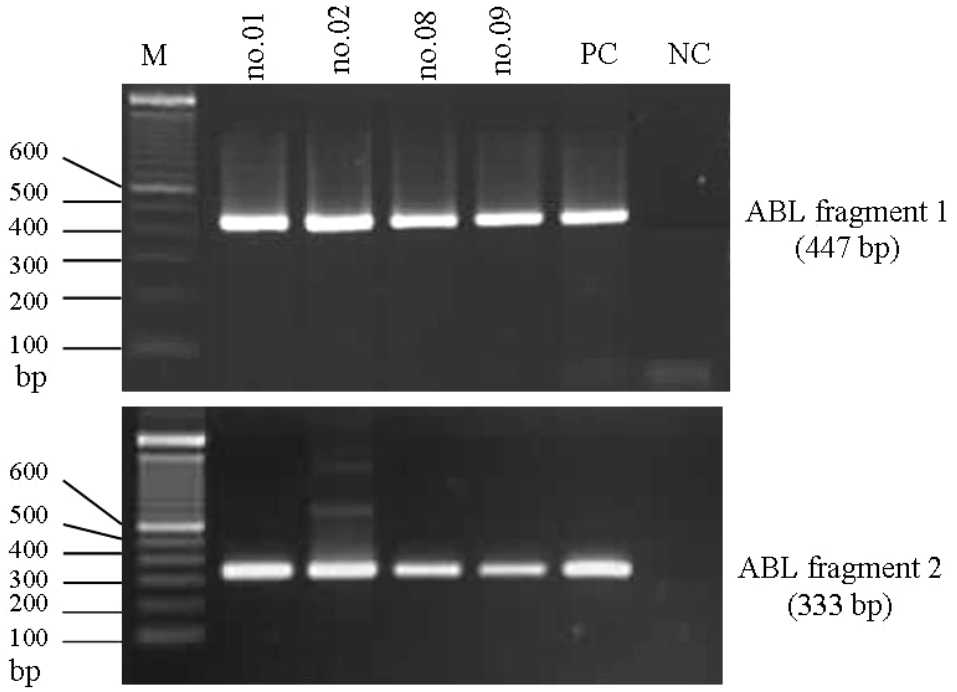


Figure 5.2 Agarose gel electrophoresis of *ABL* KD products of CML patients with *BCR-ABL* fusion gene. Lanes 1-5 are representative patient samples; no.01, no.02, no.08, and no.09. Lanes PC and NC are K562 leukemic cell lines and a normal control, respectively. Lane M is a 100-bp DNA ladder used as a standard-size marker.

5.1.3 Analysis of *ABL* KD mutations by DHPLC and direct sequencing

DHPLC was used to screen for known and unknown KD mutations. The column temperature was optimized (50 ºC - 70 ºC) to allow the formation of 75% - 90% DNA duplexes which could generate the most distinct abnormal peaks. To clearly resolve additional peaks in each KD fragment, at least two levels of column temperature were used as shown in Figure 5.3. Sequencing analysis of the *ABL* KD mutations in the samples with abnormal DHPLC profiles are shown in Figure 5.4.

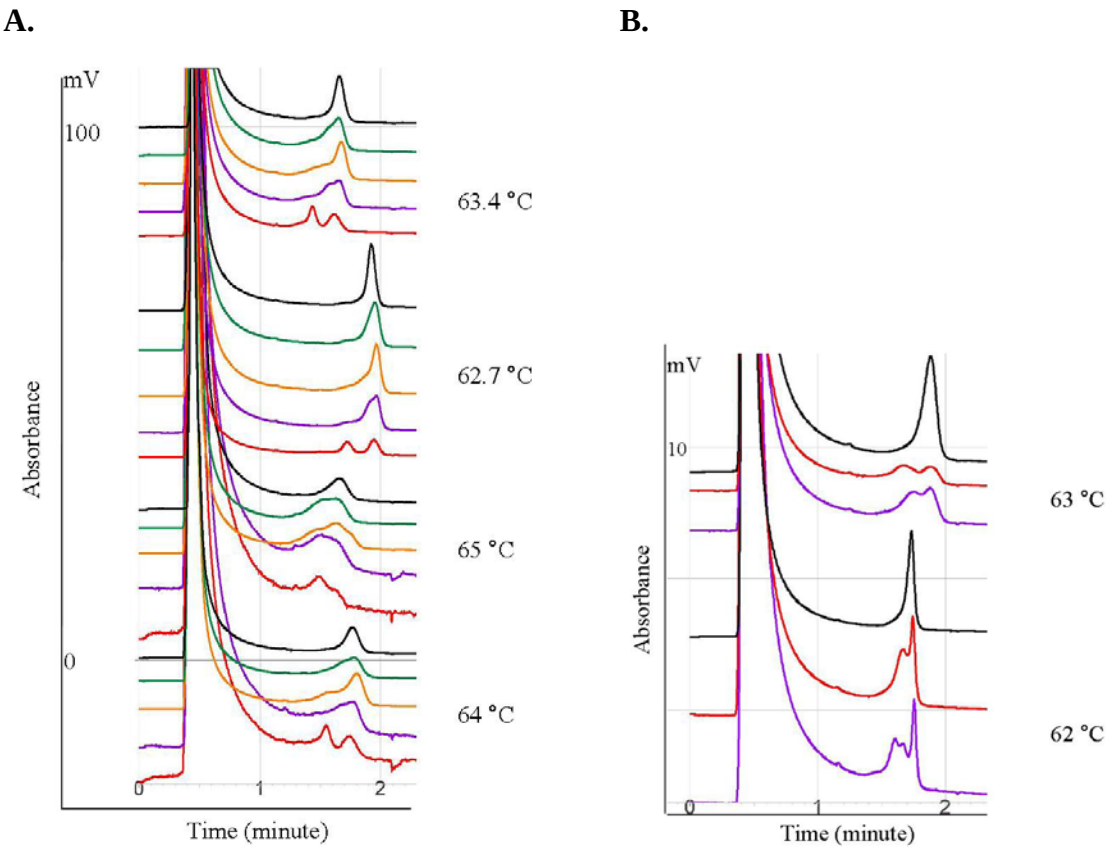


Figure 5.3 The appropriate column temperatures for mutation screening of *ABL* KD fragment. (A) ABL fragment 1 (63.4 ºC, 62.7 ºC, 64.0 ºC) and (B) ABL fragment 2 (63 ºC, 62 ºC), respectively.

Prior to DHPLC analysis, PCR products were mixed with a wild-type (WT) product in a 1:1 ratio to prevent false negative results as the homoduplexes derived from the homozygous mutant cDNA (100% mutants) generated a sharp peak that was quite similar to the homozygous WT peak (0% mutants).

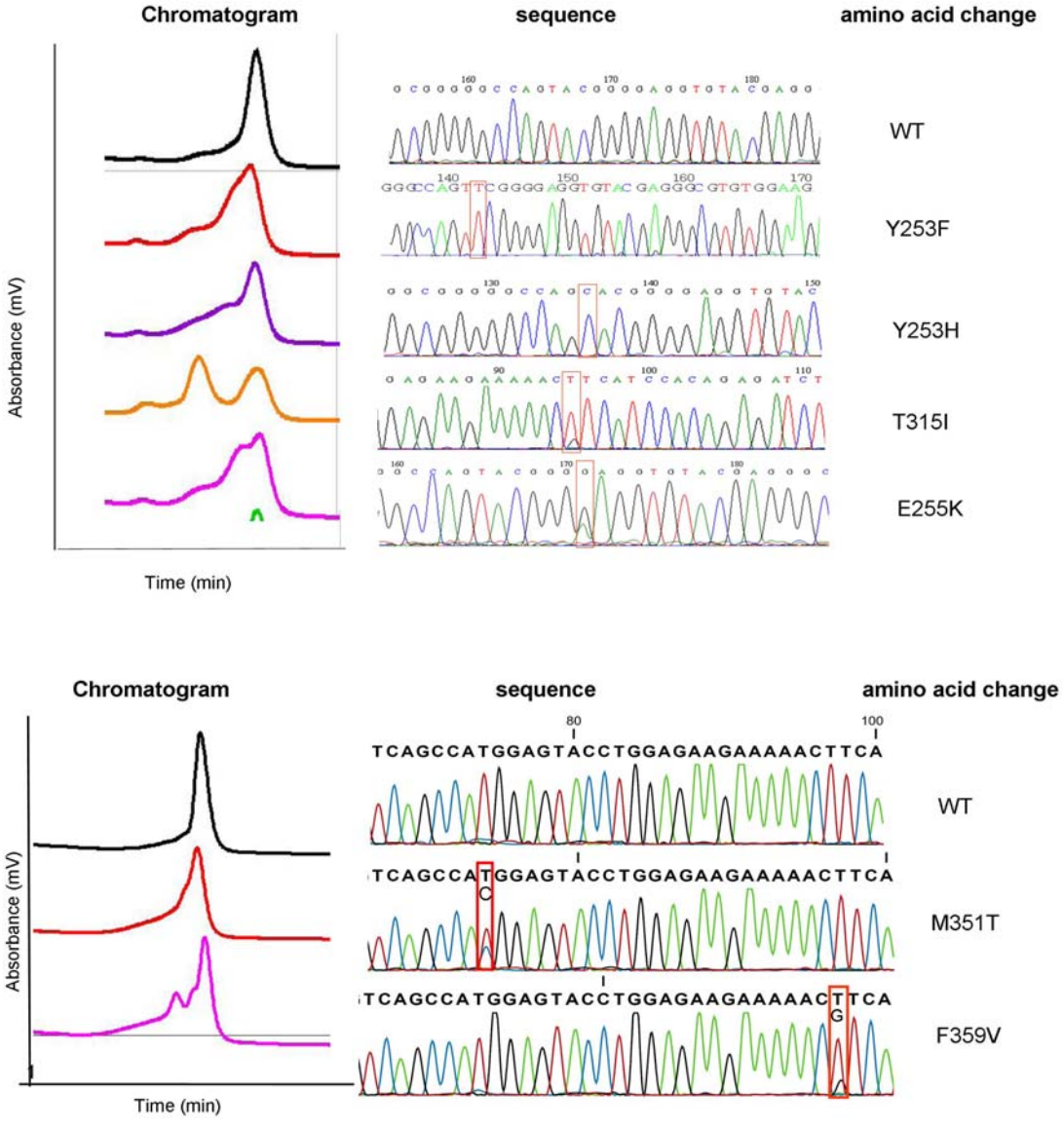


Figure 5.4 Demonstration of representative DHPLC profiles confirmed by sequencing. DHPLC (left panel) and sequencing (right panel) revealed the detection of each mutation in the PCR product.

5.1.3.1 Detection of *ABL* KD mutation in dilution mixtures by DHPLC

To assess the specificity of the DHPLC assay, PCR samples from four cell lines known to be positive for some KD mutations including, Y253F, Y253H, E255K, T315I, M351T, and F359V were tested (Figure 5.5). The results indicated that DHPLC had detection limits or sensitivity of 0.78% - 3.13% for E255K, Y253F, Y253H, T315I and and F359V mutations. However, DHPLC had a poor sensitivity for detecting for M351T mutation (12.5% dilutions). For T315I, the chromatogram exhibited either a single or a double peak with a sensitivity of about 1%. The heteroduplexes generated from the heterozygous products gave a peak (abnormal patterns which were different from the wild type) which could be seen in 90%, 80%, 70%, 60%, 50%, 25%, 12.5% 6.25% 3.13% 1.56%, and 0.78% dilutions.

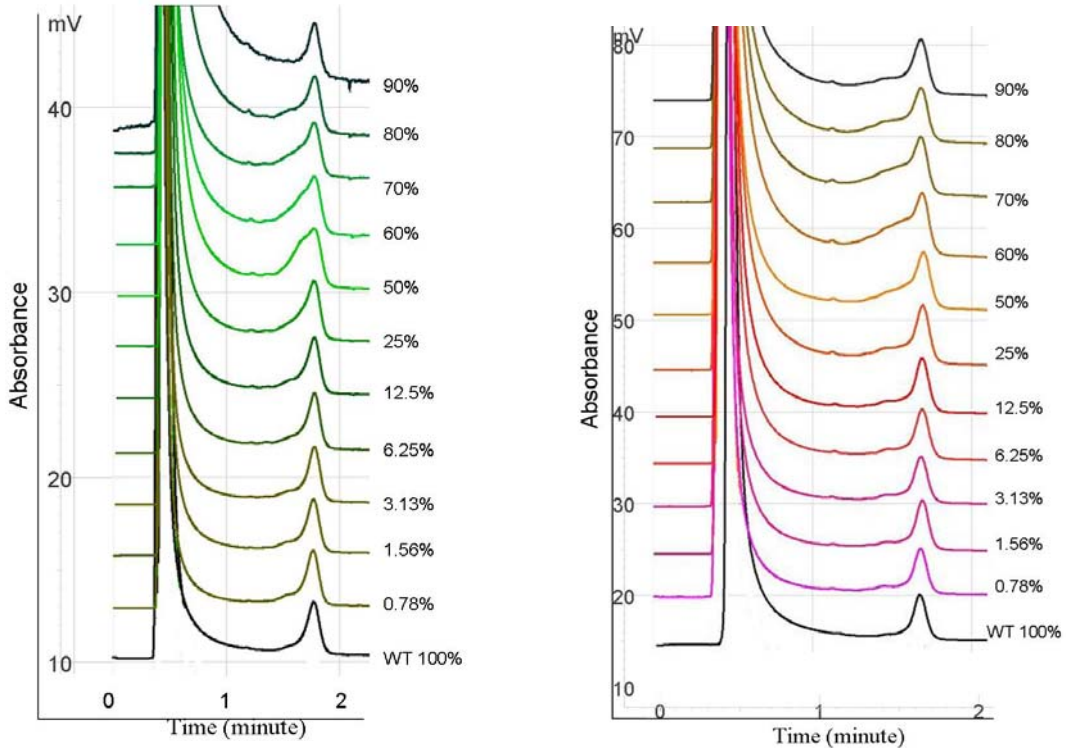


Figure 5.5 DHPLC profiles of each known mutant and WT profile in different dilutions. Each line represents the DHPLC pattern generated by each different mutant allele concentration. For fragment 1 which covers amino acids 206-346 included Y253F (A), Y253H (B), E255K (C), and T315I (D). For fragment 2 which covers amino acids 293-428 included M351T (E), and F359V (F).

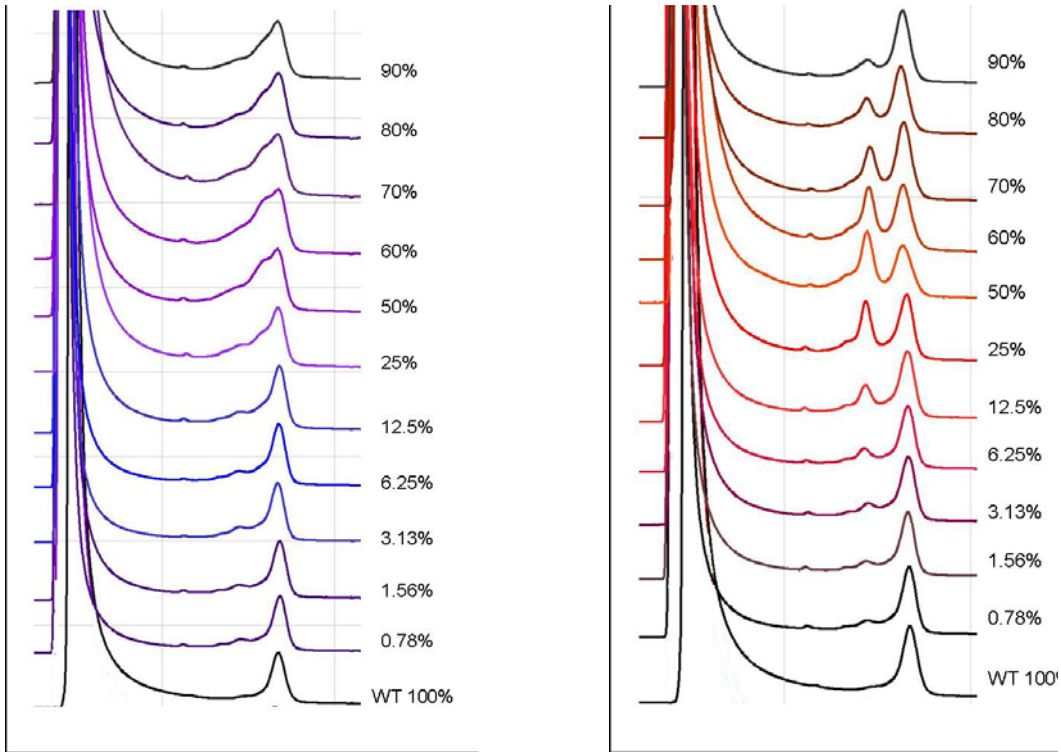


Figure 5.5 DHPLC profiles of each known mutant and WT profile in different dilutions (cont.). Each line represents the DHPLC pattern generated by each different mutant allele concentration. For fragment 1 which covers amino acids 206-346 included Y253F (A), Y253H (B), E255K (C), and T315I (D). For fragment 2 which covers amino acids 293-428 included M351T (E), and F359V (F).

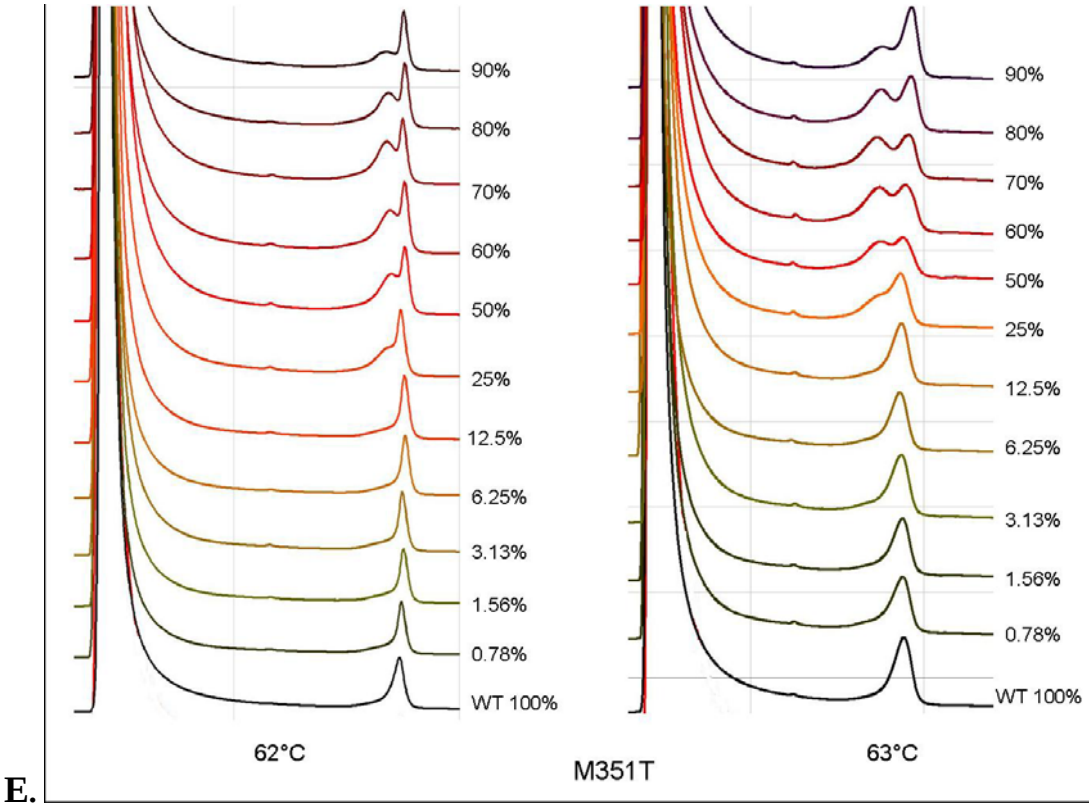
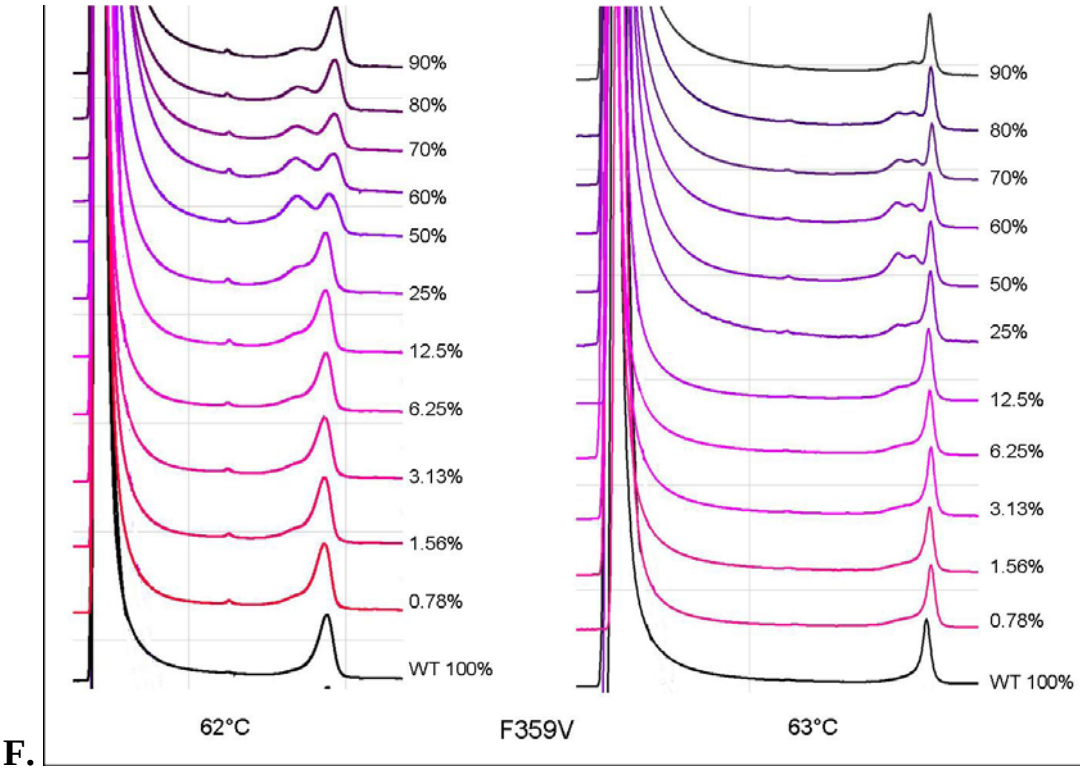


Figure 5.5 DHPLC profiles of each known mutant and WT profile in different dilutions (cont.). Each line represents the DHPLC pattern generated by each different mutant allele concentration. For fragment 1 which covers amino acids 206-346 included Y253F (A), Y253H (B), E255K (C), and T315I (D). For fragment 2 which covers amino acids 293-428 included M351T (E), and F359V (F).



F.

Figure 5.5 DHPLC profiles of each known mutant and WT profile in different dilutions (cont.). Each line represents the DHPLC pattern generated by each different mutant allele concentration. For fragment 1 which covers amino acids 206-346 included Y253F (A), Y253H (B), E255K (C), and T315I (D). For fragment 2 which covers amino acids 293-428 included M351T (E), and F359V (F).

5.1.3.2 Detection of *ABL* KD mutation in dilution mixtures by sequencing analysis

The PCR samples from two cell lines, Y253H and T315I are shown in Figure 5.6. For direct sequencing of Y253H, a "C" peak indicated the presence of Y253H which could be clearly seen in 100%, 50%, 25%, 12.5%, 6.25%, and 5%. A "T" peak which represented the WT *ABL KD* allele could be seen in 50%, 25%, 12.5%, 6.25%, 5%, 3.13%, and 0% dilution (100% WT). For direct sequencing of T315I, a "T" peak which indicated the presence of T315I could be clearly seen in 100%, 50%, 25%, 12.5%, and 6.25%. However, in a 5% dilution, a mutant peak could not be clearly seen as different from the backgrounds. A "C" peak which represented the WT *ABL KD* allele could be seen in 50%, 25%, 12.5%, 6.25%, 3.13%, and 0% dilution (100% WT). For specificity test, the mutant band was absolutely absent in 0% mutant dilution in all experiments. The results indicated that direct sequencing method could detect the *ABL* mutated sequence when the proportion of mutant was more than 5%.

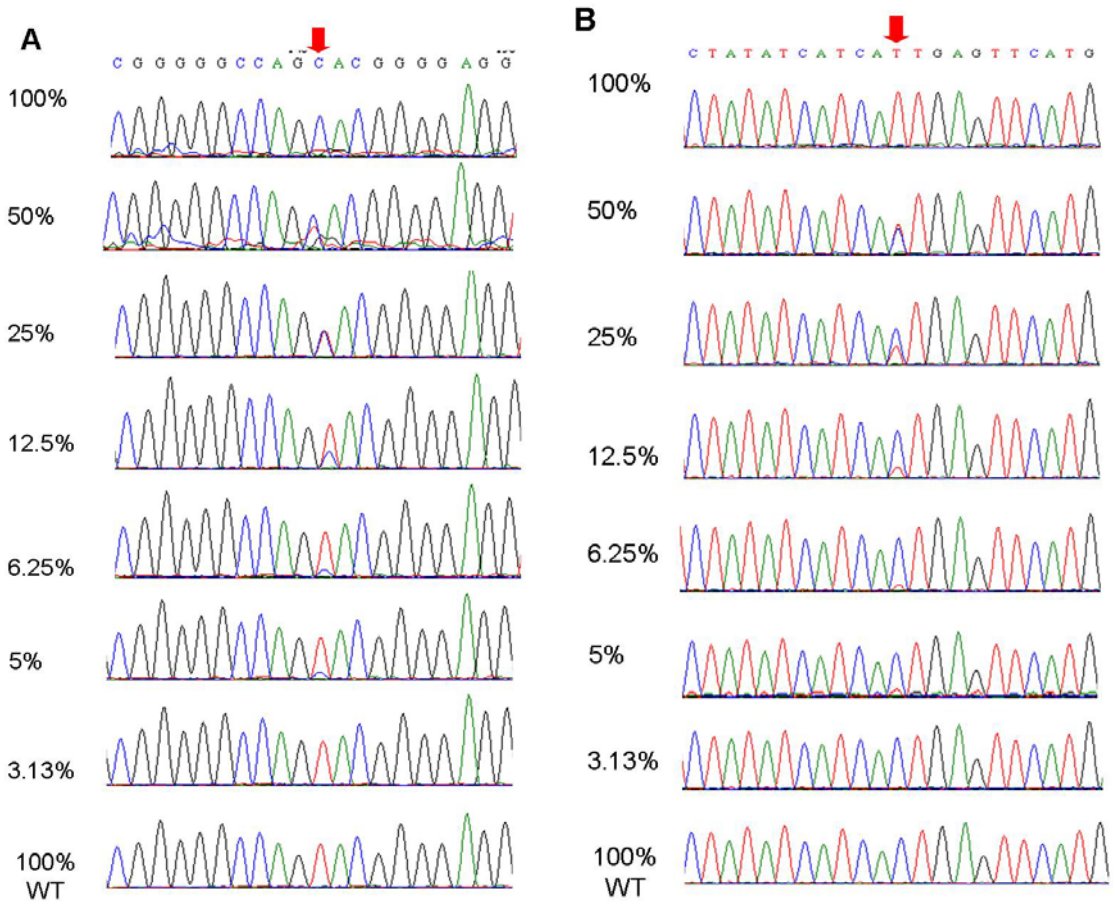


Figure 5.6 Sensitivity studies of direct sequencing to detect Y253H (A) and T315I (B) mutations are demonstrated by the chromatograms of different mixtures. (A) The red arrow indicates c.757T>C mutation; T=Wild-type, C=Mutant, (B) The red arrow indicates c.944C>T mutation; C=Wild-type, T=Mutant. The *ABL* KD mutations were clearly detected in a 5% - 6.25% of mutant dilutions.

5.2 Analysis of ABL KD mutation in Thai CML patients

5.2.1 The incidence of *ABL* KD mutation

One-hundred seventy-one *BCR-ABL* positive CML patients including 80 naïve cases and 91 exposed TKI cases were screened for *ABL* KD mutations covering codons 206-428 by DHPLC analysis. Abnormal chromatogram patterns that were different from the WT profile were detected in 78 samples (45.61%) by DHPLC. Sequencing analysis confirmed the presence of KD mutations in 37 cases (21.64%) (Table 5.1). About 23% of patients receiving first-line imatinib, 62.5% of imatinib-resistant patients receiving 2nd generation TKI, 75% of advanced phase patients being treated with front-line 2nd generation TKI had KD mutations, and 8.8% of naïve cases were found to carry the KD mutations. A marked difference in the incidence of mutations were observed between the naïve cases and cases who were treated with imatinib (p=0.010). Patients who had been exposed to imatinib alone had a lower incidence of mutations as contrasted to those who received 2nd generation TKI (p=0.003).

Table 5.1 Incidence of *ABL* KD mutations in naïve CML patients and TKI-exposed patients (n=171)

Type of CML Patients	No. of cases studied	No. of cases with KD mutations (%)
Overall patients	171	37 (21.6%)
Naïve	80	7 (8.8%)[a]
Exposed to IM only	71	17*(23.9%)[a,b]
Exposed to IM and switch to 2nd TKI	16	10 (62.5%)[b]
Exposed to 2nd TKI without IM	4	3 (75%)

*Coexistence of a known mutation and a SNP in 2 patients; [a]p = 0.010; [b]p =0.003.

5.2.2 Locations and types of *ABL* KD mutations

Twenty-one types of *ABL* KD mutations were found in 37 CML cases including 13 known mutations and 8 previously unidentified mutations (Table 5.2). A known single nucleotide polymorphism (SNP) (L354L) was detected in 2 patients. The most common type of KD mutation was T315I (which has a nucleotide change from C to T at the amino acid position 315, resulting in Threonine changing to Isoleucine) (n=9 cases, 24.3%). Other less common mutations included 13 cases (35.1%) of various P-loop mutations (1 M244V, 3 G250E, 5 Y253H, 2 E255K, 1 E255V, and 1 H246Y), 7 cases (18.9%) of catalytic domain mutations (5 M351T and 2 F359V), 3 cases (8.11%) of activation loop mutations (L387F, M388L, and A399V), and a point mutation in 7 separate cases (E258E, L273L, 2 E279K, H295H, L301H, and V304A), a deletion in 2 cases (p.Asn297ProfsX9 and p.Pro218GlnfsX27), and duplication in 1 case (p.Phe311ValfsX6). About seventy-five percent (28/ 37) of all mutations recognized in this study were among the eight most common types (M244V, G250E, Y253H, E255K, E255V, T315I, M351T, and F359V) and most of them were insensitive and intermediate sensitive types of KD mutations according to the IC50 level. Only two types (M351T and M388L) were considered sensitive to imatinib (Table 5.2). Thirty cases had a single mutation, whereas 7 cases had multiple mutations including T315I+L354L, F359V+L354L, G250E+F359V, L387F+M388L, Y253H+T315I and L301H+E255K. Two cases with a SNP (No.459 and No.504) were found to have coexisting mutations including T315I and F359V.

The location of each identified mutation is depicted in Figure 5.7. Twenty-one types of KD mutations detected in this study are demonstrated according to the sites of mutations on the main regions of KD (exons 4-7). The majority of KD mutations in the naïve cases were localized in the minor region including the C-helix, the SH3 contact site, and other sites whereas most KD mutations in the exposed cases were localized in the main region of KD, the drug contact site, P-loop, and the catalytic domain.

Table 5.2 Type and frequency of known and novel KD mutations in 171 CML cases

***ABL* KD mutations**		**No. of cases with KD mutations****	**Imatinib sensitivity (18,34,81)**
Predicted protein	**cDNA**		
Point mutations			
M244V	c.730A>G	1	intermediate
H246Y*	c.736C>T	1	N/A
G250E	c.749G>A	3	intermediate
Y253H	c.757T>C	5	insensitive
E255K	c.763G>A	2	insensitive
E255V	c.764A>T	1	insensitive
E258E *	c.774G>A	1	N/A
L273L	c.822G>A	1	N/A
E279K	c.835G>A	2	intermediate
H295H*	c.885C>T	1	N/A
L301H*	c.902T>A	1	N/A
V304A	c.911T>C	1	N/A
T315I	c.944C>T	9	insensitive
M351T	c.1052T>C	5	sensitive
F359V	c.1075T>C	2	intermediate
L387F	c.1161G>T	1	intermediate
M388L	c.1162A>T	1	sensitive
A399V*	c.1196C>T	1	N/A
Deletion and duplication			
p.Asn297ProfsX9*	c.888_919del	1	N/A
p.Pro218GlnfsX27*	c.653delC	1	N/A
p.Phe311ValfsX6*	c.929dup	1	N/A
Polymorphism			
L354L (rs1141213)	c.1065G>A	2	N/A

The sensitivity levels of KD mutations were determined by IC50. IC50 (50% inhibitory concentration) is the imatinib concentration for inhibit 50% *BCR-ABL* transfected cell line by in vitro proliferation assay. Sensitive: an IC50 ≤1000nM IM, Intermediate: 1000-3000nM, and Insensitive: IC50>3000nM (18). N/A, not applicable.* These variations had not been previously described; ** Two different point mutations indentified in the same patient.

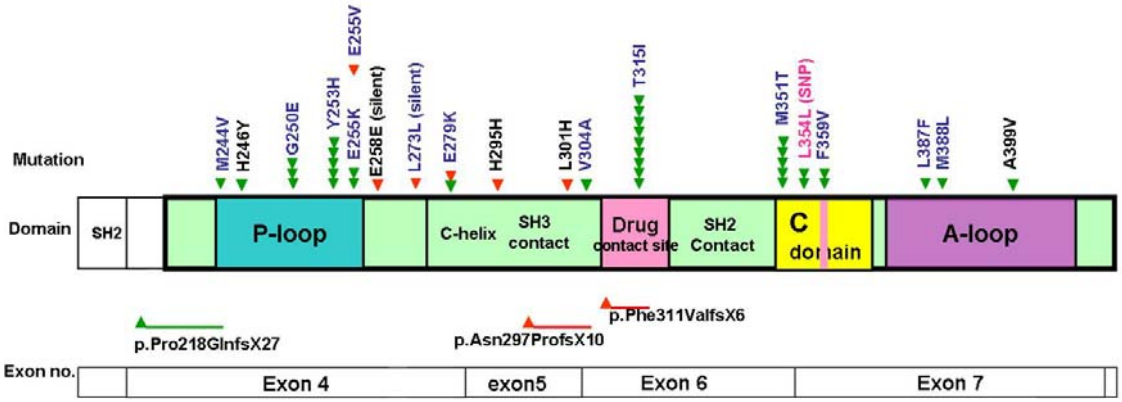


Figure 5.7 Distribution of mutations on the KD region. Thirteen known and 8 novel mutations are printed in blue and black letters, respectively. TKI-exposed and TKI-naïve cases are indicated by green and red triangles, respectively. SNP is shown in pink. The lines indicate a deletion or a duplication resulting in early stop codon which generated a truncated protein.

5.2.3 Types of *ABL* KD mutations in the naïve patients

Among the 80 studied imatinib-naïve patients (54 newly diagnosed cases and 26 cases treated with hydroxyurea), there were 8 different mutation types in 7 patients which were 3 known mutations (E255V, E279K, L273L) and 5 novel KD mutations (E258E, H295H, L301H, p.Asn297ProfsX9, and p.Phe311ValfsX6). Dual mutations, E255V and L301H, were found in Patient no. 080. Figure 5.8 shows the abnormal elution profiles screened by DHPLC and sequencing results of naïve patients with the KD mutations as compared with the WT samples and the *ABL1* reference sequences. A novel 32-bp deletion, p.Asn297ProfsX9, was found in a chronic-phase naïve patient (Patient no.136) who had been on hydroxyurea for 12 years and subsequently developed imatinib intolerance (skin rash) at the initial treatment. p.Asn297ProfsX9 resulted in a novel frameshift mutation which produced a new protein sequence with 8 new residues beginning at position 297 and a stop codon at position 305 within the BCR-ABL KD leading to a truncated BCR-ABL protein (Figure 5.9). A single nucleotide duplication within codon 311 (a nucleotide at cDNA position c.929dup) was found in Patient no. 512 resulted in a frameshift mutation of 5

amino acids that produced a new protein sequence beginning at position 311 and a stop codon at position 316 leading to a truncated BCR-ABL protein (p.Phe311ValfsX6) (Figure 5.10).

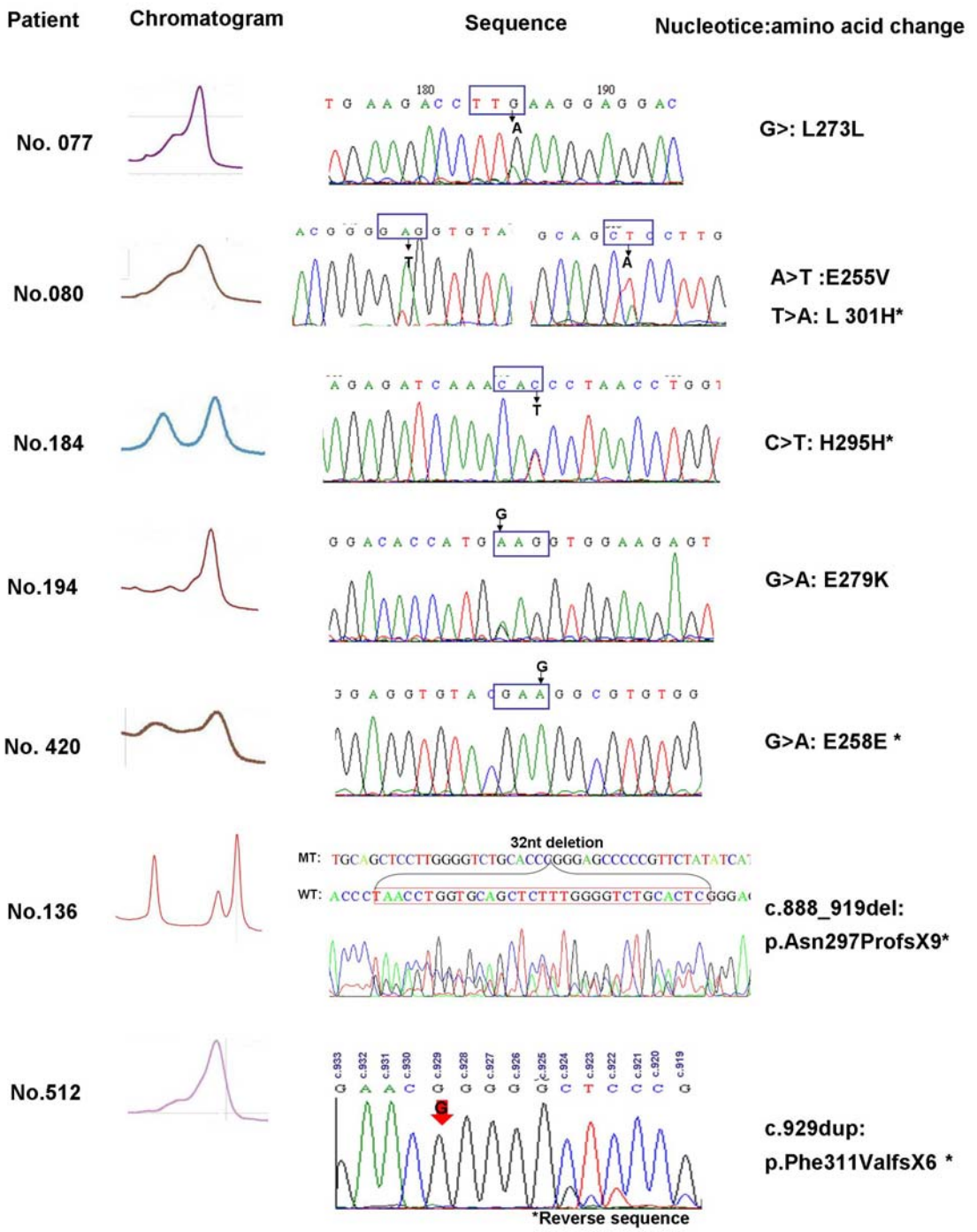
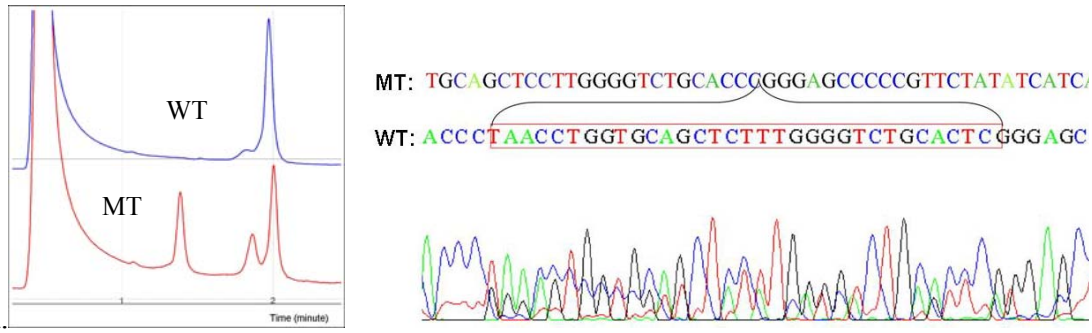


Figure 5.8 Demonstration of the abnormal DHPLC profiles and cDNA sequence of 7 naïve patients. *indicate novel mutations.

A.



B.

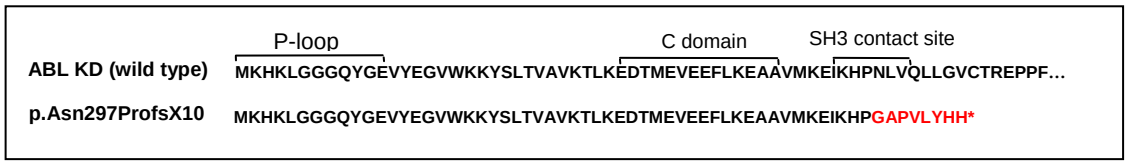
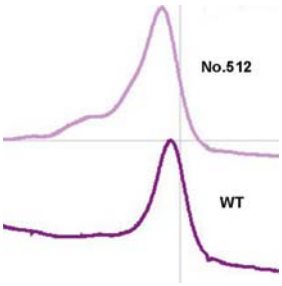
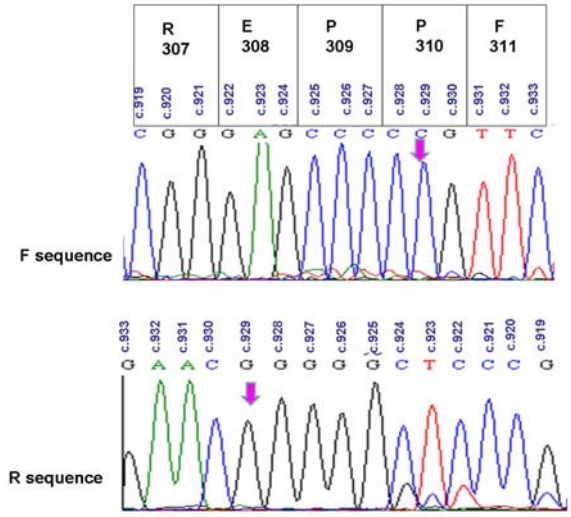


Figure 5.9 Deletion of *ABL* KD in Patient no.136. The deletion, c.888-919del or p.Asn297ProfsX9, was identified by DHPLC (left) and direct sequencing (right) (A). The deletion generates a novel frameshift mutation (red box) that produces a new amino acid (in red) and a premature stop codon (*) within BCR-ABL KD leading to a truncated BCR-ABL protein (B). (Protein reference sequence NP_005148.2).

A.



B.



C.

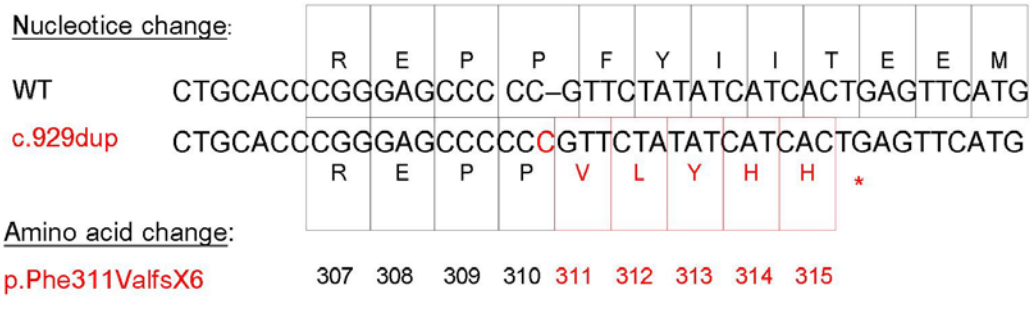


Figure 5.10 Duplication (c.929dup) of *ABL* KD in a naïve patient (Patient no. 512); (A) DHPLC profiles; (B) direct sequencing shows a duplication at cDNA position 929 (pink arrow); (C) This duplication generates a novel frameshift mutation, p.Phe311ValfsX6, that produce new 5 amino acid (in red) and premature stop codons (*) with in BCR-ABL KD leading to truncated BCR-ABL protein. (Protein reference sequence NP_005148).

5.2.4 Types of *ABL* KD mutations in TKI-exposed cases

In the TKI-exposed patients, there were 11 known mutations and 3 novel mutations (H246Y, A399V, and p.Pro218GlnfsX27) (Figure 5.11). H246Y and A399V results from a C to T base substitution at cDNA position c.736 within P-loop (exon 4) and c.1196 within A-loop (exon 7), respectively. The deletion, c.653delC (p.Pro218fsX27), was found in Patient no.439 who had been on imatinib for 3 months before he developed imatinib intolerance. p.Pro218GlnfsX27 is a 1-bp deletion at the position c.653 which generates a frameshift mutation and encodes 26 new amino acids and a premature stop codon leading to truncated BCR-ABL protein (Figure 5.12).

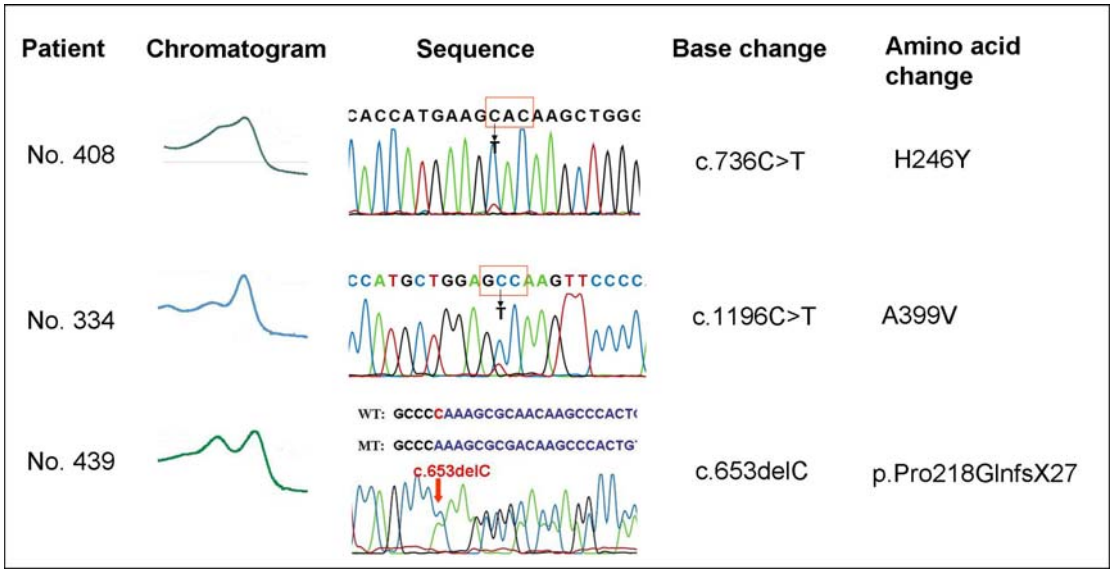
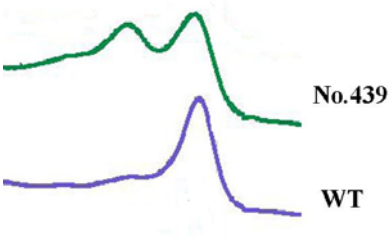
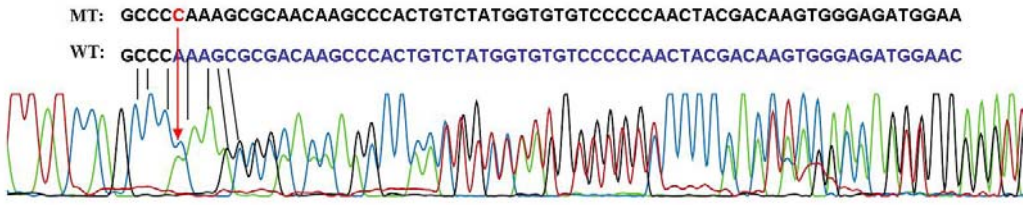


Figure 5.11 Demonstration of the abnormal DHPLC profiles and cDNA sequences of 3 novel mutations in the TKI-exposed patients.

A.



B.



C.

Protein predicted from variant coding sequence:

```
  1  MLEICLKLVG CKSKKGLSSS SSCYLEEALQ RPVASDFEPQ GLSEAARWNS KENLLAGPSE
 61  NDPNLFVALY DFVASGDNTL SITKGEKLRV LGYNHNGEWC EAQTKNGQGW VPSNYITPVN
121  SLEKHSWYHG PVSRNAAEYL LSSGINGSFL VRESESSPGQ RSISLRYEGR VYHYRINTAS
181  DGKLYVSSES RFNTLAELVH HHSTVADGLI TTLHYPAQSA TSPLSMVCPP TTTSGRWNAR
241  TSP*
```

Figure 5.12 A single nucleotide deletion (c.653delC) of *ABL* KD is shown in Patient no.439. Two-peaked DHPLC profiles (A), and sequencing results (B); A red arrow indicates a deletion at cDNA position 653). The results were compared with the *ABL1* reference sequences and shows frameshift mutation affecting 27 amino acid residue (p.Pro218fsX27) (C); Premature stop codons (*)

5.2.5 Incidence of T315I resistant mutation according to the type of TKI drugs

Among 171 cases, 9 cases (5.3%) were found to carry T315I resistant mutation. Two of the 69 cases (2.9%) who received imatinib only, 4 of the 18 cases who received both imatinib and 2nd generation TKI (22.2%), and 3 of the 4 cases who received 2nd generation TKI only (75%) had T315I , respectively (Table 5.3). In this study, the T315I mutation was not observed in naïve patients who had never been treated with TKI. Overall, about 10 % of the patients who received TKI were found to have T315I mutation.

Table 5.3 Frequency of T315I mutation in CML patients according to TKI treatment history

Type of CML Patients	No. of Cases with T315I (%)
Total study (n=171)	9 (5.3%)
Naïve (n=80)	0 (0%)
TKI-exposed patients (n=91)	9 (9.9%)
Exposed to imatinib only (n=69)	2 (2.9%)[a]
Exposed to imatinib and switch to 2nd TKI (n=18)*	4 (22.2%)[a]
Exposed to 2nd TKI without imayinib (n=4)	3 (75%)[a]

[a]$p < 0.0001$, * include two patients who had T315I mutation after receiving 2nd TKI with a different baseline mutation observed while on imatinib treatment

5.3 Characteristics of Thai CML patients with or without *ABL* KD mutations

5.3.1 Overall clinical characteristics of 171 CML patients

The clinical characteristics of all CML patients included in this study are summarized in Table 5.4. The population of CML patients bearing the KD mutations had a slightly higher number of males to females (24:13). The median age of imatinib treated patients with or without KD mutation was 45 and 50 years (p=0.416), respectively, whereas the median age of imatinib-naïve patients with or without mutations was 41 and 44 years (p=0.709), respectively. Most of the patients in both groups with or without KD mutation were in chronic phase. About one-third of mutated cases in TKI-exposed and about half of mutated cases in naïve cases were in advanced phase (accelerated phase plus blastic crisis) whereas about one-fourth of wild-type cases in both groups were in advanced phase. The median WBC and PLT counts were higher ($p<0.0001$ and $p=0.0002$, respectively) and the Hb was lower ($p<0.0001$) in the naïve CML cases (n=80) as compared to the TKI-treated cases (n=91).

5.3.2 Clinical characteristics of cases with *ABL* KD mutations

The characteristics of the 30 TKI-exposed and 7 naïve patients who carried the KD mutations and their follow-ups are shown in Table 5.5 and Table 5.6, respectively, whereas the characteristics of all 8 cases with novel mutations and their responses to imatinib are summarized in Table 5.7. Among 3 novel mutations in the TKI-exposed cases (H246Y, A399V, and p.Pro218GlnfsX27), all of them were intolerant or resistant to imatinib. Only 2 of 4 naïve cases with novel mutations were subsequently treated with imatinib and both of them failed to have a good response. One patient with H295H is still on hydroxyurea and in chronic phase while another patient with p.Pro218GlnfsX27 died at 3 months after allogeneic stem cell transplantation.

Table 5.4 Clinical characteristics of all CML patients in this study

Parameter	TKI-exposed patients (n=91)		P	TKI-naïve patients (n= 80)		P
	MT (n=30)	WT (n=61)		MT (n=7)	WT (n=73)	
Sex (M/F)	18/12	36/25		6/1	39/34	
Median age, range (years)	45 (21-73)	50 (17-82)	0.416	41(19-53)	44 (15-81)	0.220
Median WBC ($x10^{9}/L$)*	8.5	5.6	0.007	53.7[b]	43.2[b]	0.694
Median PLT ($x10^{9}/L$)*	167	186	0.326	492	331	0.637
Median Hb (g/L)*	11.7	11.5	0.870	9.3	9.3	0.733
BCR-ABL transcript						
B3A2	23	36		5	41	
B2A2	5	20		1	22	
n/a	2	5		1	11	
Phase at MT detection	n=30	n=61	0.668	n=7	n=73	0.371
Chronic phase (CP)	19	46		4	55	
Accerated phase (AP)	3	4		2	5	
Blastic crisis (BC)	8	11		1	13	

*p <0.0001 for WBC, Hb , and p=0.0002 for PLT, between TKI-exposed (n=91) and TKI-naïve patients (n=80); MT, mutation; WT, wild type.

Table 5.5 Characteristics of the 30 TKI-exposed CML patients with *ABL* KD mutations and their follow-ups

No.	Mutation	Age/sex	Phase at time of analysis	TKI treatment duration at time of analysis (months)	Treatment response
Imatinib and 2nd TKI					
509	T315I	51/M	AP	HU (33)→IM (24)→NT(10)	IM and NT resistance (no CCyR, pancytopenia, rash, and bleeding) Disease progression to AP after receiving IM
611	G250E	38/M	BC	HU(16)→IM(3)→DT(8)	IM and DT resistance and disease progression to BC
354	E255K	49/M	AP	IM (17)→NT (3)	IM and NT intolerance
459	L354L(SNP), F359V	36/M	CP	IM(20)→ NT(10)	IM resistance (noCCyR) and NT resistance myelofibrosis
403	M351T	36/M	CP	IM (28)→NT(1)	Resistance (no CCyR at 17 mo) and disease progression into BC
334	A399V	63/F	CP	IM(32)→NT(10)	IM resistance and NT intolerance (no any CCyR at 4years)
009	E255K	52/M	CP	IM(36)→NT(11)	IM resistance and NT intolerance and DT resistance (no CCyR at 18mo)
320	T315I	32/M	BC	IM (1)→DT(12)	IM intolerance-->DT resistance and disease progression→ Death
656	Y253H	50/M	CP	HU→IM(32)→NT(16)→D(5)	IM and NT resistance, DT →CR at 8 mo →Still on DT
001	L387F, M388L	58/M	CP	HU(2)→IM (43)→NT(15)	IM intolerance constrictive pericarditis
169	Y253H→T315I	27/M	BC	IM (4)→DT (3)	IM resistance and disease progression to BC → started ALL protocol with DT →Death
360	Y253H→T315I	32/M	CP	IM (34)→ NT (17)	IM resistance and NT resistance
002	Y253H	34/F	AP→BC	IM (14)	IM resistance →NT(6) resistance with focal myelofibrosis→ Death
408	H246Y	49/M	CP	HU(145)→s/p BMT (58)→IM(30)	IM intolerance s/p BMT
014	E279K	37/F	CP	HU→IM (50)	IM resistance

Table 5.5 Characteristics of the 30 TKI-exposed CML patients with *ABL* KD mutations and their follow-ups (cont.)

No.	Mutation	Age/sex	Phase at time of analysis	TKI treatment duration at time of analysis (months)	Treatment response
037	F359V, G250E	54/M	BC	IM (8)	IM resistance and disease progression to BC→ Death
439	p.Pro218Glnfs	31/M	CP	IM (3)	IM primary resistance and pancytopenia at initial treatment
452	T315I	46/M	CP	IM(9)	IM resistance (loss of CHR)→started NT with HU →NT resistance (rash, BM suppression, and no CCyR)→Death
410	M351T	50/F	CP	IM (24)	IM intolerance
217	M351T	43/F	CP	IM (13)	IM intolerance (suboptimal response , thrombocytopenia)
504	T315I	70/M	BC	IM (5)	IM resistance (loss of CCyR) with myelofibrosis
600	Y253H	44 /F	CP	IM (48m)	IM resistance (loss of CCyR)
449	V304A	73/F	CP	IM (43)	IM resistance (CHR but noCCyR at 5y)
401	M351T	46/F	BC	IM (n/a)	Disease progression to BC
275	M244V	27/M	CP	IM (21)	CR (CCyR)
337	M351T	44/F	CP	IM(60)	CR (CCyR)
599	G250E	38/F	CP	HU(1)→IM(36)	CR (CHR, CCyR, but no CMR at 3y)
2nd TKI					
461	T315I	59/F	BC	NT (20)	Resistance and disease progression to BC →Death
648	T315I	21/M	CP	Hu (2) →NT (12)	Resistance
550	T315I	35/F	AP	HU (60)→DT (4)	Progressive dyspnea (pneumonia/effusion) BM suppression and disease progression to BC→Death

Table 5.6 Characteristics of the 7 naïve CML patients with *ABL* KD mutations and their follow-ups

No.	Age/ Sex	Phase at analysis	Nucleotide change	Type of KD mutation	Clinical follow-ups	OS (mo)
080	31/M	CP	c.764A>T c.902T>A	E255V L301H	n/a	n/a
077	46/M	BC	c.822G>A	L273L (silent)	Disease progression with thrombocytopenia and anemia→ Death	38
194	19/M	AP	c.835G>A	E279K	HU→ no mutation after receiving IM with CR at 6mo	15
420	53/M	CP	c.774G>A	E258E (silent)	HU →IM→ mutation disappeared after IM 3 mo →suboptimal response at 6 mo	6
184	29/M	AP	c.885C>T	H295H (silent)	HU (11mo) with CHR	11
136	45/F	CP	c.888_919del	p.Asn297ProfsX9	HU (11 y) with pancytopenia →IM with initial resistance but mutation disappeared	13 years
512	41/M	CP	c.929dup	p.Phe311ValfsX6	HU →disease progression and turned to BC →s/p BMT →Death	15

IM, imatinib; HU, hydroxyurea; CR, complete remission; BMT, bone marrow transplantation; n/a, data not available

Table 5.7 Summary of CML patients with 8 novel mutations and their response to imatinib

TKI-exposed cases		**TKI-naïve cases**	
Mutation Type	**TKI Response (cases)**	**Mutation Type**	**TKI Response (cases)**
H246Y	Intolerance (chronic phase)	E258E	Suboptimal response (chronic phase)
A399V	Resistance (chronic phase)	H295H	Still on hydroxyurea with CHR (chronic phase)
p.Pro218GlnfsX27	Resistance (chronic phase)	L301H	Lost follow-up (chronic phase)
		p.Asn297ProfsX9	Resistance (chronic phase)
		p.Phe311ValfsX6	Death after stem cell transplant

5.3.3 Association of the disease phase and *ABL* KD mutations

Table 5.8-5.9 and Figure 5.13 show the incidence and types of KD mutations according to the phase of the disease, i.e. chronic phase (CP), accelerated phase (AP), and balstic crisis (BC). Twenty-three (18.4%) of 125 CP patients, 5 (35.7%) of 14 AP patients, and 9 of 32 (28.1%) BC patients carried the KD mutations. In CP cases, the mutants were distributed in all major KD domains, p-loop, T315I, C domain, A-loop, and also other sites with the most mutation located in the p-loop (8 of 26, 31%). T315I was the most common type of mutation in AP cases (2 of 5, 40%).

Table 5.8 Incidence and types of KD mutations among 171 CML patients with a different phase of disease

Parameter	Phase (%)		
	Chronic phase	**Accelerated phase**	**Blast crisis**
Naïve cases	4/59	2/7	1/13
TKI-exposed cases	19/66	3/7	8/18
Total cases	23 /125 (18.4%)	5/14 (35.7%)	9/32 (28.1%)
Mutation type	**Point mutation** 4 M351T, 2 T315I, 2 Y253H, H246Y, E279K, M244V, E255K, V304A, G250E, E258E(silent), F359V, A399V, **Deletion and duplication** c.653delC (p.Pro218GlnfsX27, c.888_919del (p.Asn297ProfsX9), c.929dup (p.Phe311ValfsX6) **Multiple mutations** L387F+M388L, Y253H+T315I, E255V+L301H	**Point mutation** 2T315I, E279K, H295H (silent), E255K	**Point mutation** 3T315I, G250E, Y253H, L273L(silent), M351T **Multiple mutations** Y253H+T315I, G250E+F359V

Table 5.9 Incidence and distribution of mutations on the *ABL* KD according to the disease phase

KD location	**Disease phase (n=37)**		
	CP (n=26)	AP (n=5)	BC (n=11)
P-loop	8[1,2]	1	4[3, 4]
T315I	3[2]	2	4[4]
C domain	5	0	2[3]
A-loop	3[5]	0	0
Other	7[1]	2	1

*[1, 2, 3, 4 and 5] were three cases carrying 2 different types of mutations.

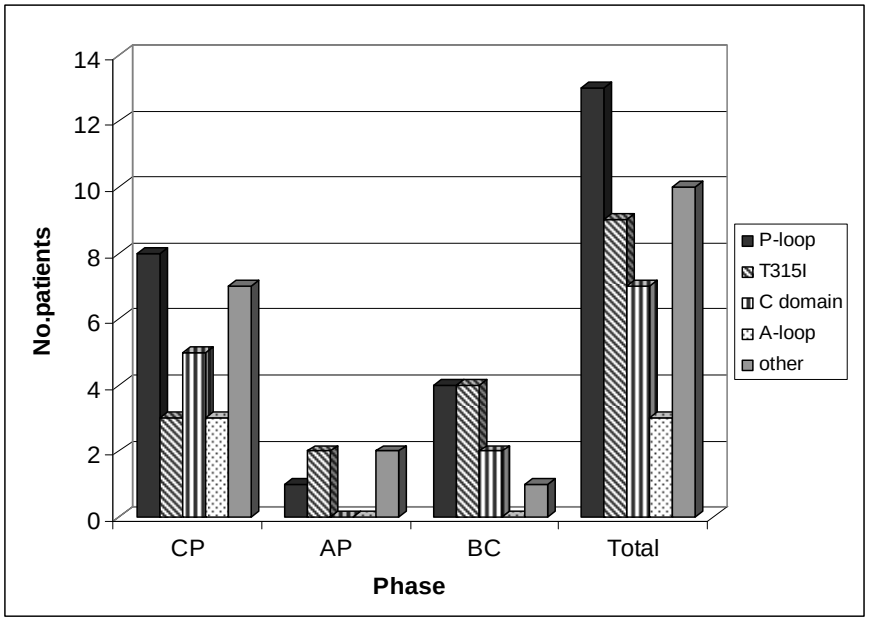


Figure 5.13 Position and type of *ABL* KD mutations according to the disease phase

5.3.4 Frequency of *ABL* KD mutations in imatinib-treated patients with or without drug resistance

About 45% (23 of 51) of imatinib-resistant patients and 11% (4 of 36) of imatinib-treated patients without drug resistance were found to carry the KD mutations (Table 5.10). The incidence of KD mutations in cases with resistance to imatinib was three-fold higher than cases without resistance to imatnib.

Table 5.10 Frequency of KD mutations in patients with or without TKI resistance

Imatinib response	Frequency of KD mutations	
	Total case studied	No. of cases (%mutation)
Total imatinib-exposed cases	87	27 (31%)
Cases with imatinib resistance	51	23 (45%)
Cases without imatinib resistance	36	2 (11%)

5.3.5 Time to occurrence of *ABL* KD mutations and overall survivals of patients with the mutations

KD mutations were detected at a median time of 24 months (range, 3-60 months) after TKI initiation as shown in Table 5.11. The median overall survival (OS) in TKI-exposed cases harboring KD mutations was 42 months (range, 8-156 months). With respect to 4 major KD positions, T315I had the shortest duration of treatment before mutation arose (12 months with a range of 4-51 months) (Figure 5.14) and the shortest OS of 28 months (range, 14-90 months) (Figure 5.15) as compared to other mutations.

Table 5.11 Median times from the start of TKI treatment to the detection of *ABL* KD mutations and overall survivals according to mutant type

Mutation location	Median time (months)	
	Time after TKI initiation (range)	Overall survival (range)
P-loop	25.5 (4-53)	51 (8-156)
T315I	12 (4-51)	28 (14-90)
C domain	26.5 (8-60)	36 (15-95)
A-loop	58 (42-58)	75 (36-75)
others	43 (3-50)	77 (15-120)
Total	**24 (3-60)**	**42 (8-156)**

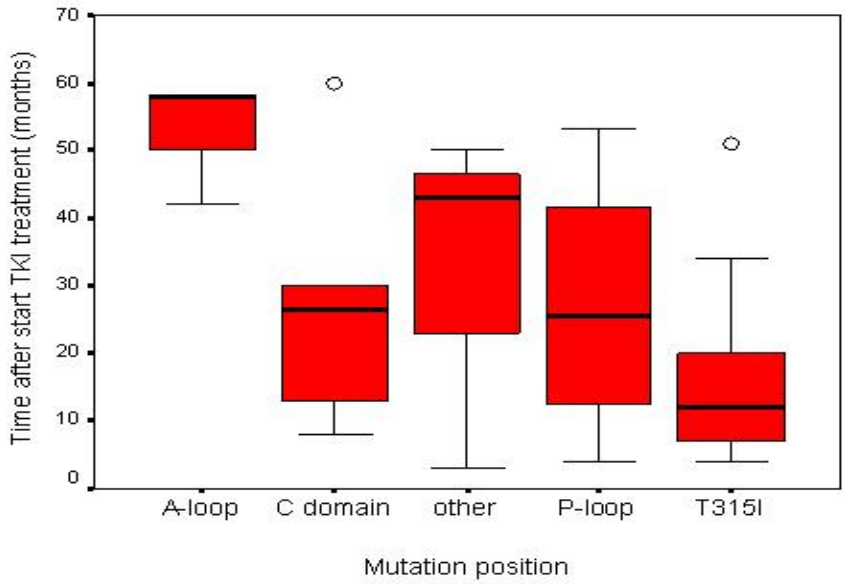


Figure 5.14 Time intervals from the TKI initiation to the detection of mutations

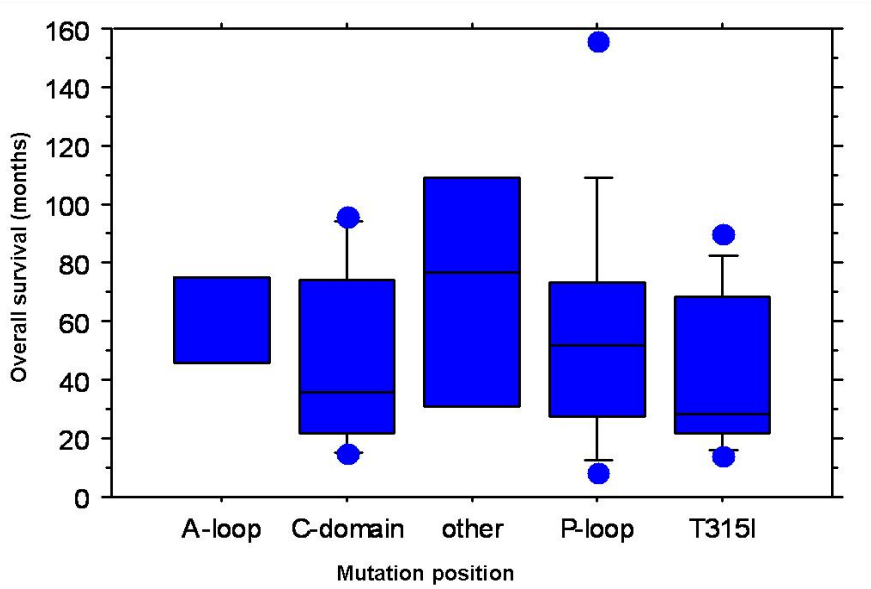


Figure 5.15 Association of mutation in different KD regions and overall survivals

5.3.6 Associated chromosome abnormalities and outcome

Cytogenetic data were completely available in 131 patients. Thirteen cases (9.9%) of mutated patients who received TKI treatment had clonal chromosome abnormality (CCA), i.e. -7, -Y, t(9;22;12), +8 with inv(13), t(2;14), +8, +19, t(3;7), t(5;9;22), -22, and -19 (Table 5.12). Two imatinib-resistant patients (No.002, No.014) had -8. Twenty-eight (21.4%) of WT patients had chromosome abnormalitie, i.e. t(12;22), t(6;9;22), t(7;9;22), t(8;21), del(22)(q11), t(11;17), (+14,+15, +19), t(9;22;11), (+6,+8), t(4;22), t(2;12) with -13, inv7, inv9, t(17;22), t(11;17), t(22:11), -22 with t(11;17), t(6;11), and -7.

The OS of the mutated patients with Ph chromosome and CCA (Ph+/ CCA+) were shorter than that of the mutated patients with Ph chromosome and without CCA, implicating the negative impact of *ABL* KD mutation, Ph chromosome, and an additional chromosome abnormality. However, statistical analysis could not observe a major difference in the OS of the Ph+/CCA+ (median 36, range 15-156) and Ph+/CCA- (median 43, range 8-96) patients (p=0.760).

Table 5.12 Chromosome abnormalities in TKI-exposed patients with *ABL* KD mutation

No.	Sex	Age	Mutation	Treatment history	Karyotype
461	F	59	T315I	2nd TKI resistance	46,XY, t(9;22)(q34;q11.2)
648	M	21	T315I	2nd TKI resistance	46,XY, t(9;22)(q34;q11.2)
550	F	35	T315I	2nd TKI resistance	46,XY, t(9;22)(q34;q11.2)
509	M	51	T315I	IM resistance and NT intolerance	46,XY, t(9;22)(q34;q11.2)
611	M	38	G250E	IM and DT resistance	Ph+ , -7(q22;q32)
354	M	49	E255K	IM intolerance	46,XY, t(9;22)(q34;q11.2)
459	M	36	F359V, L354L	IM resistance	46, XY, t(7;9;22)(q11.2;q34;q11.2)
403	M	36	M351T	IM resistance	Ph+/ 45,X,-Y, t(9;22)(q34;q11)
334	F	63	A399V	IM resistance	46,XY, t(9;22)(q34;q11.2)
009	M	52	E255K	All TKI resistance	46,XY, t(9;22)(q34;q11.2)
320	M	32	T315I	IM intolerance	46,XY, t(9;22)(q34;q11.2)
656	M	50	Y253H	IM , NT resistance	46,XY, t(9;22)(q34;q11.2)
001	M	58	L387F, M388L	All TKI resistance	46,XY, t(9;22)(q34;q11.2)
169	M	27	Y253H ,T315I	IM, DT resistance	46, XY, t(9;22;12)(q34;p11;q11)
360	M	32	Y253H, T315I	IM and NT resistance	46,XY, t(9;22)(q34;q11.2)
002	F	34	Y253H	IM resistance	Ph+/47,XX,+8,t(9;22),inv(13)(13q22)
408	M	49	H246Y	IM intolerance	46, XY, t(2;14), t(9;22)
014	F	37	E279K	IM resistance	47,XX, +8,t(9;22)(q34;q11.2), i(17)(q10)
037	M	54	G250E, F359V	IM resistance	47,XY,+19,t(9;22)(q34;q11),t(3;7)(q29;q 22)
439	M	31	p.Pro218Glnfs	IM resistance	46,XY,t(5;9;22)(q31;q34;q11.2)
452	M	46	T315I	IM resistance	Ph+/ -22
410	F	50	M351T	IM intolerance	46,XY, t(9;22)(q34;q11.2)
217	F	43	M351T	IM intolerance	46,XY, t(9;22)(q34;q11.2)
504	M	70	T315I, L354L	IM resistance	46,XY, t(9;22)(q34;q11.2)
600	F	44	Y253H	IM resistance	47, XX, +12
449	F	73	V304A	CR	46,XY, t(9;22)(q34;q11.2), del(19)(p13.1)
401	F	46	M351T	CR	47, XX,+21
275	M	27	M244V	CR	46,XY, t(9;22)(q34;q11.2)
337	F	46	M351T	CR	46,XY
599	F	38	G250E	CR	46,XY, t(9;22)(q34;q11.2)

*IM, imatinib; DT, dasatinib; NT, nilotinib; CR, complete remission

5.4 Dynamics of *BCR-ABL* transcripts and *ABL* KD mutations during TKI treatment

5.4.1 *BCR-ABL* transcript levels in imatinib-treated CML patients with *ABL* KD mutations

The complete results were available in 16 mutated patients. The median *BCR-ABL* transcript levels are shown in Table 5.13 and Figure 5.16. The median %BCR-ABL/ABL ratio in T315I mutation and the other types were 35.2 (15.3-305.3) and 34.5% (5.6-91.5), respectively, (p=0.480) (Figure 5.17).

Table 5.13 Median *BCR-ABL* transcript levels in 16 *ABL* KD mutated patients

Patients	Transcript levels (%BCR-ABL/ABL ratio)	KD Mutation
No.320	305.34	T315I
No.360	91.51	T315I
No.452	34.40	T315I
No.504	15.29	T315I
No.550	36.08	T315I
No.648	22.16	T315I
No.1	5.56	L387F+M388L
No.2	49.43	Y253H
No.9	35.33	E255K
No.14	33.62	E279K
No.169	59.29	Y253H
No.194	29.63	E279K
No.354	91.51	E255K
No.459	8.03	F359V
No.497	41.64	F359V
No.656	14	Y253H

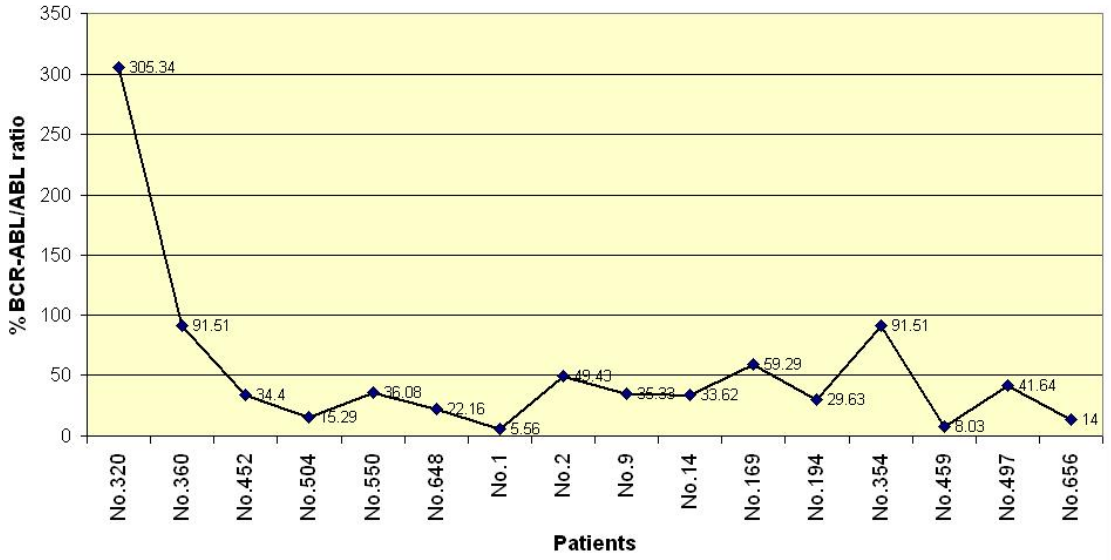


Figure 5.16 Levels of *BCR-ABL* transcripts in 16 *ABL* mutated patients

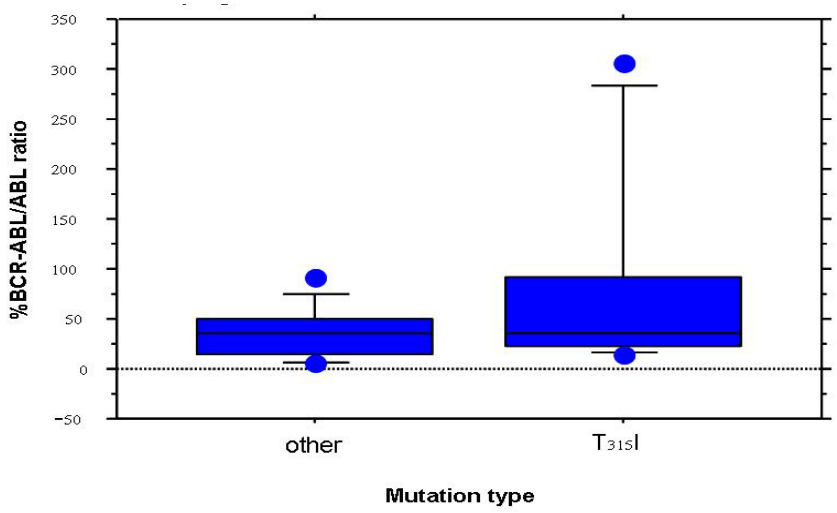


Figure 5.17 Comparison of *BCR-ABL* transcripts levels in T315I mutation and other mutation types

5.4.2 Dynamics of *ABL* KD mutations in the naïve patients

The dynamics of KD mutations were available in 4 naïve patients as shown in Figure 5.18. Patient no.512 initially had a WT but subsequently developed a frameshift mutation of 6 amino acids (p.Phe311ValfsX6) during BC. A novel frameshift mutation (p.Asn297GlyfsX9) was found in a chronic-phase CML patient (no. 136) who had been on hydroxyurea for about 12 years. This mutation subsequently disappeared during disease progression to AP. For Patient no.420 and no.194, the mutations disappeared after inatinib treatment. DHPLC and sequencing results from the sequential samples of this naïve patient (no.194) are shown in Figure 5.19. The first sample was demonstrated with G to A substitution in cDNA position 835 (c.835G>A) and the WT sequence was found in the second sample. The levels of *BCR-ABL* transcripts at the initial diagnosis before imatinib treatment and after 6 months of treatment when mutation disappeared were 29% and 2%, respectively, in Patient no.194. A silent mutation, E258E, which was located next to the P-loop, was found in a CP patient (Patient no.420) before treatment but was undetectable about 3 months after imatinib treatment.

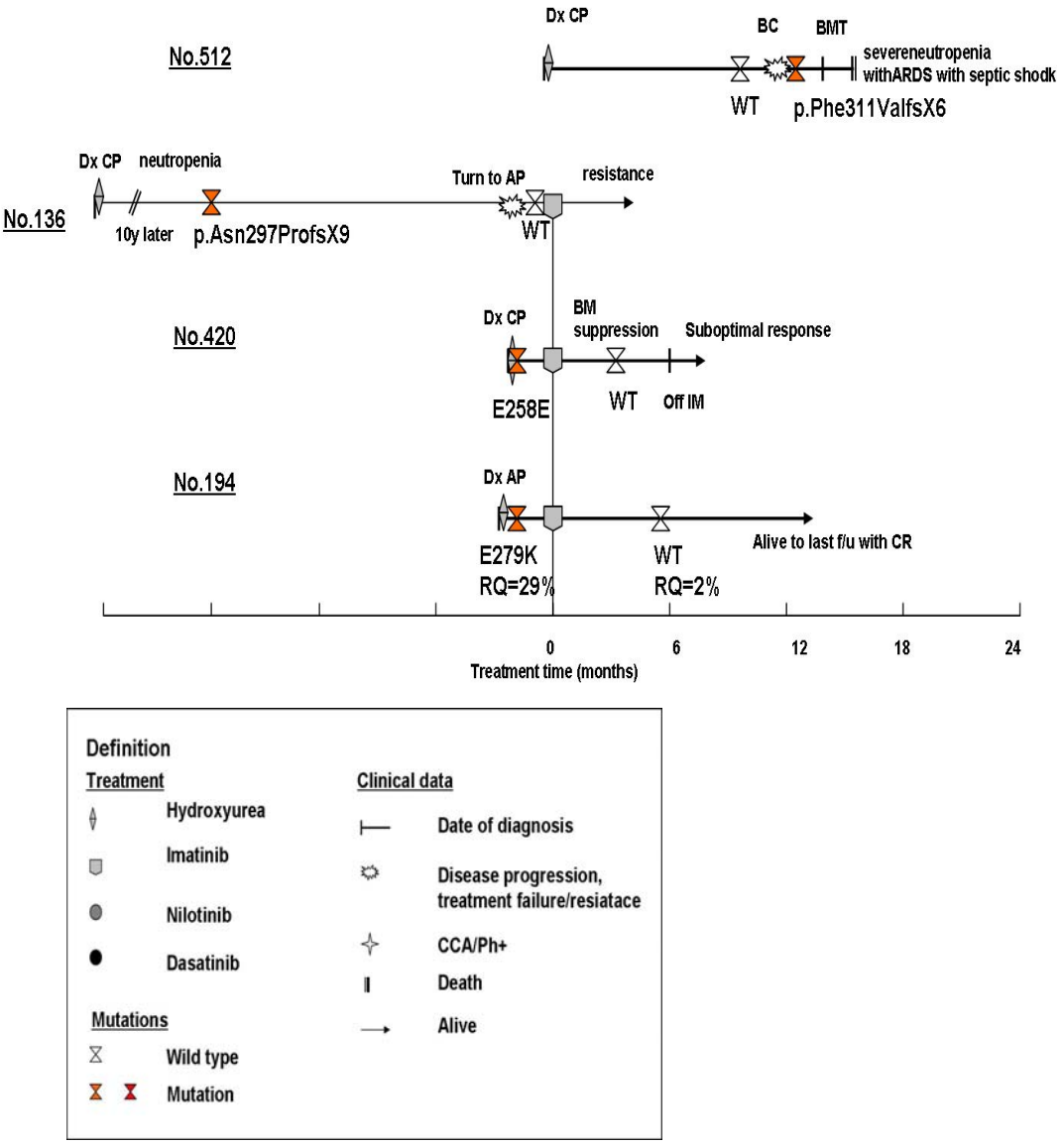


Figure 5.18 A schematic representation of the dynamics of KD mutation in 4 naïve patients

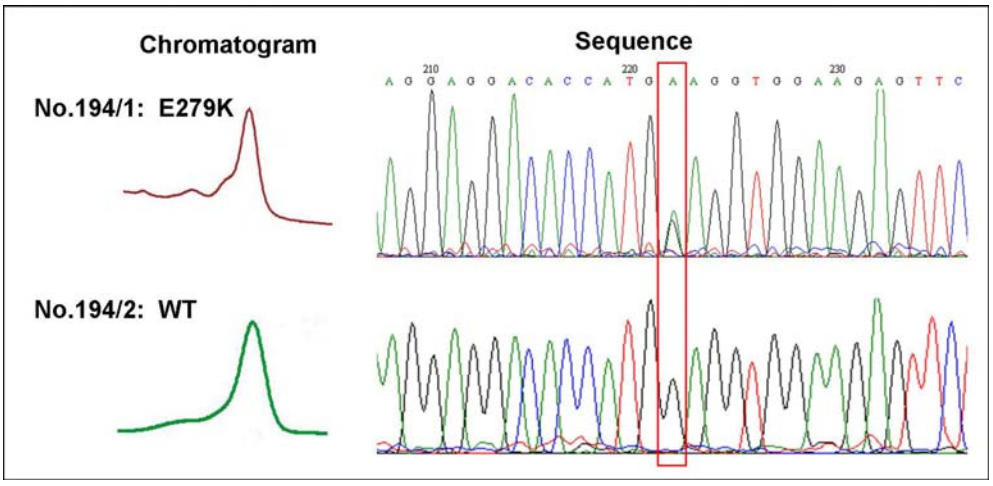
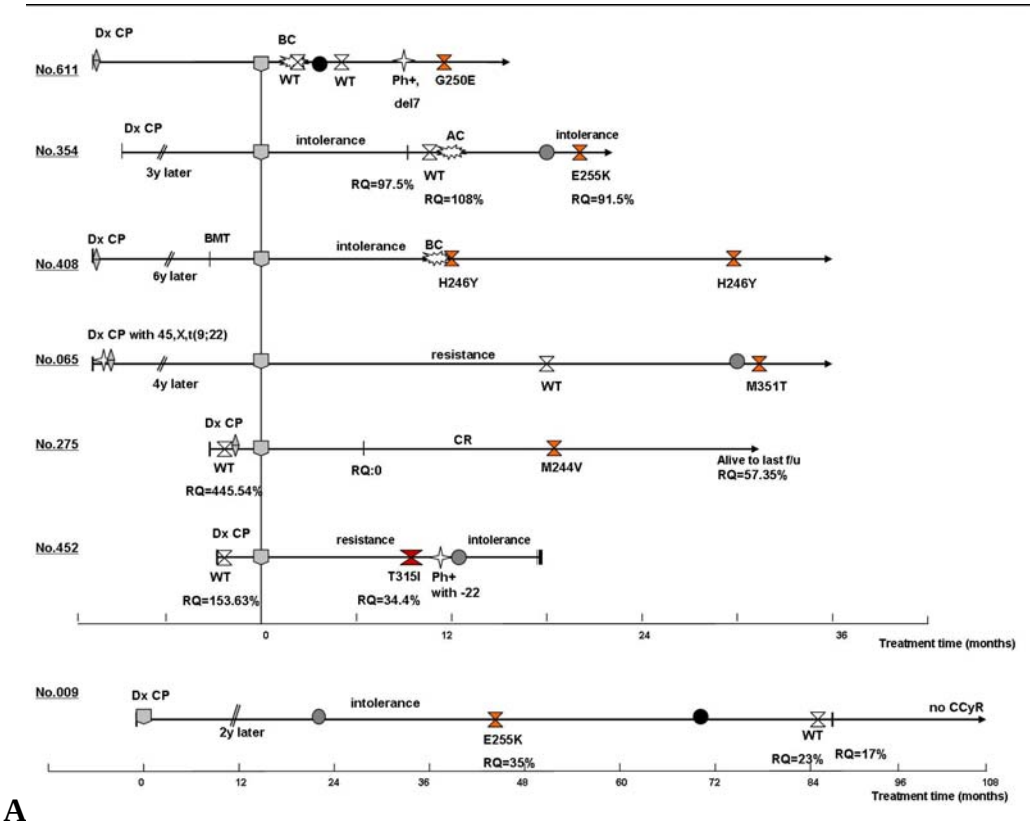


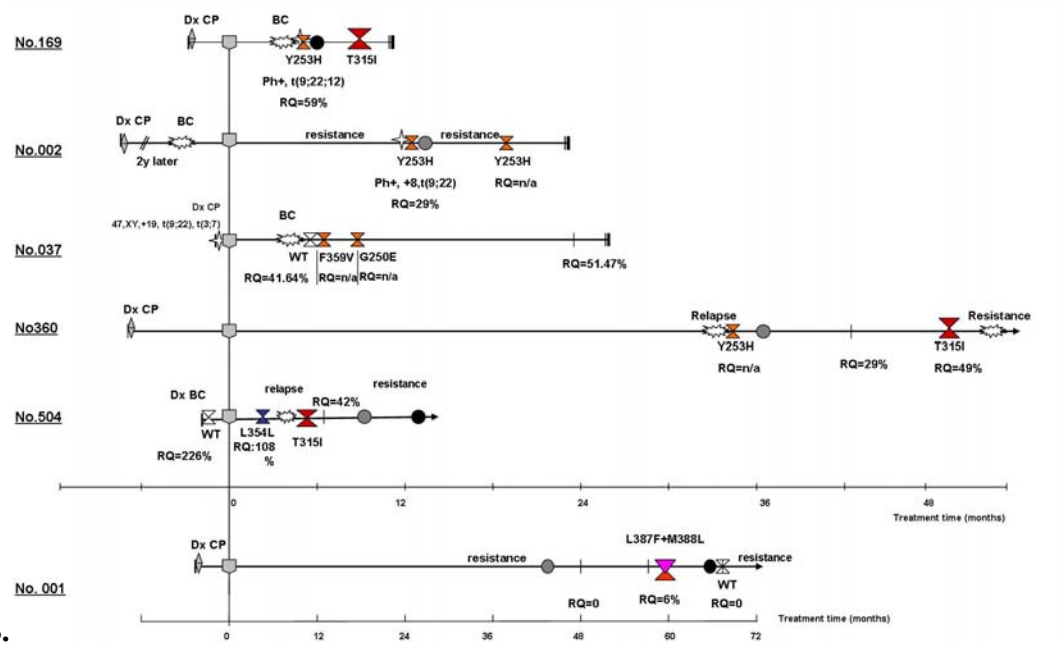
Figure 5.19 DHPLC and sequencing results from sequential samples of a naïve CML patient (no.194). The first sample was demonstrated with G to A substitution in cDNA position 835 (c.835G>A) and the WT sequence was found in the second sample.

5.4.3 Dynamics of *ABL* KD mutations during TKI treatment

Sequential analysis was available in 13 samples of CML patients with *ABL* KD mutations. Among 13 patients, a single mutation, a multiple mutation of sequential samples, and a multiple mutation in the same sample was detected in 7 cases, 5 cases, and one case, respectively (Figure 5.20). Two patients (no.169 and no.360) who had Y253H mutation during imatinib treatment developed T315I after 2nd generation TKI treatment (Table 5.14). Three patients (no.275, no.452, and no.504) had a WT before imatinib treatment. T315I arose developed after resistance to imatinib treatment in No.452 and no.504 before the initiation of 2nd generation TKI resulting in further resistance. E255K was detected in Patient no.009 after nilotinib failure with a *BCR-ABL* transcript level of 35% and then disappeared after dasatinib treatment with a *BCR-ABL* transcript level of 23% and 17%. Patient no. 001 who had been on imatinib treatment for over 12 months before he became imatinib intolerance had a dual mutation, i.e. L387F and M388L. Among the 37 patients with KD mutations at the last follow-up, 7 patients had subsequently died (no.002, no037, no.169, no.320, no.452, no.461, and no.550). Six of these seven cases contained KD mutations in the direct drug contact site (5 T315I and 1 F359V) and one had Y253H.

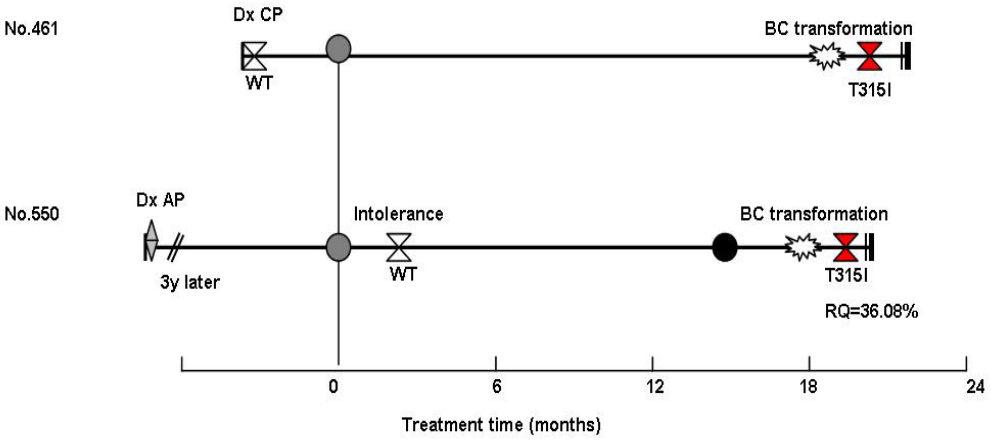


A



B.

Figure 5.20 Dynamics of mutations in the TKI exposed patients. Patients who received imatinib with (A) a single mutation, and (B) multiple mutations; and patients who received the 2nd generation TKI with T315I mutation (C)



C.

Figure 5.20 Dynamics of mutations in the TKI exposed patients (cont.). Patients who received imatinib with (A) a single mutation, and (B) multiple mutations; and patients who received the 2nd generation TKI with T315I mutation (C)

Table 5.14 Four CML patients who developed T13I mutation during the course of treatment

Patient no.	Phase at initial treatment	Duration of treatment (months)	Clinical status	Type of mutation
169	CP	IM (4)	BC	Y253H
		DT and ALL protocol (3)	BC	T315I
360	CP	IM (34)	CP	Y253H
		NT (17)	CP	T315I
461	CP	Naïve	CP	No mutation
		NT (20)	BC	T315I
504	BC	Naïve	BC	No mutation
		IM(2)	BC	L354L(SNP)
		IM (5)	BC	T315I

5.5 Detection of T315I mutation in TKI-treated patients by AS-PCR assay

5.5.1 Optimization of AS-PCR to investigate T315I mutation

The efficiency of the T315I AS-PCR was determined using the cDNA templates synthesized from RNA with known percentages of the mutant allele. Mutants, WT, and internal controls were detected in a single reaction. I first optimized the annealing temperature and the ratio of each primer pair. The results of the triplicate independent experiments demonstrated that the optimal annealing temperature was 57°C with the primers ratio of 7:3:0.5 of mutants, WT, and internal control primer pairs, respectively. A 158-bp PCR product was derived from the mutant allele whereas a 374-bp PCR product could represent a heterozygous allele or no mutant allele and a 540-bp product was an internal control. In three independent experiments, a strong T315I mutant band was detected in as low as 1% dilution and a faint band was observed in 0.5% dilution (Figure 5.21). In addition, 30 samples from non-leukemic patients and WT BA/F3 cell lines were also tested to ensure the AS-PCR's specificity; all of which were found negative for T315I. Therefore, BA/F3 cell lines were subsequently utilized as a T315I negative control.

DHPLC was performed first as a screening to detect abnormal profiles. The heteroduplexes generated from the heterozygous products gave a peak that was distinctive from the WT. Abnormal peaks could be clearly detected in 90%, 80%, 70%, 60%, 50%, 25%, 12.5% 6.25%, 3.13%, and 1.56% dilutions. An ambiguous peak was also seen at 0.78% dilution. Prior to DHPLC analysis, PCR products were mixed with a WT product in a 1:1 ratio to prevent the false negative results as the homoduplexes derived from the homozygous mutant cDNA (100%MT) generated a sharp peak that was quite similar to the homozygous WT peak (0% MT). T315I peak had a different peak pattern from other common mutations (Y253F, Y253H, E255K, M351T, and F359V). For direct sequencing, a "T" peak indicated the presence of T315I which could be clearly seen in 100%, 50%, 25%, 12.5%, and 6.25%. A "C" peak which represented the WT *BCR-ABL KD* allele could be seen in 50%, 25%, 12.5%, 6.25%, 3.13%, and 0% dilution (100% WT) (Figure 5.6).

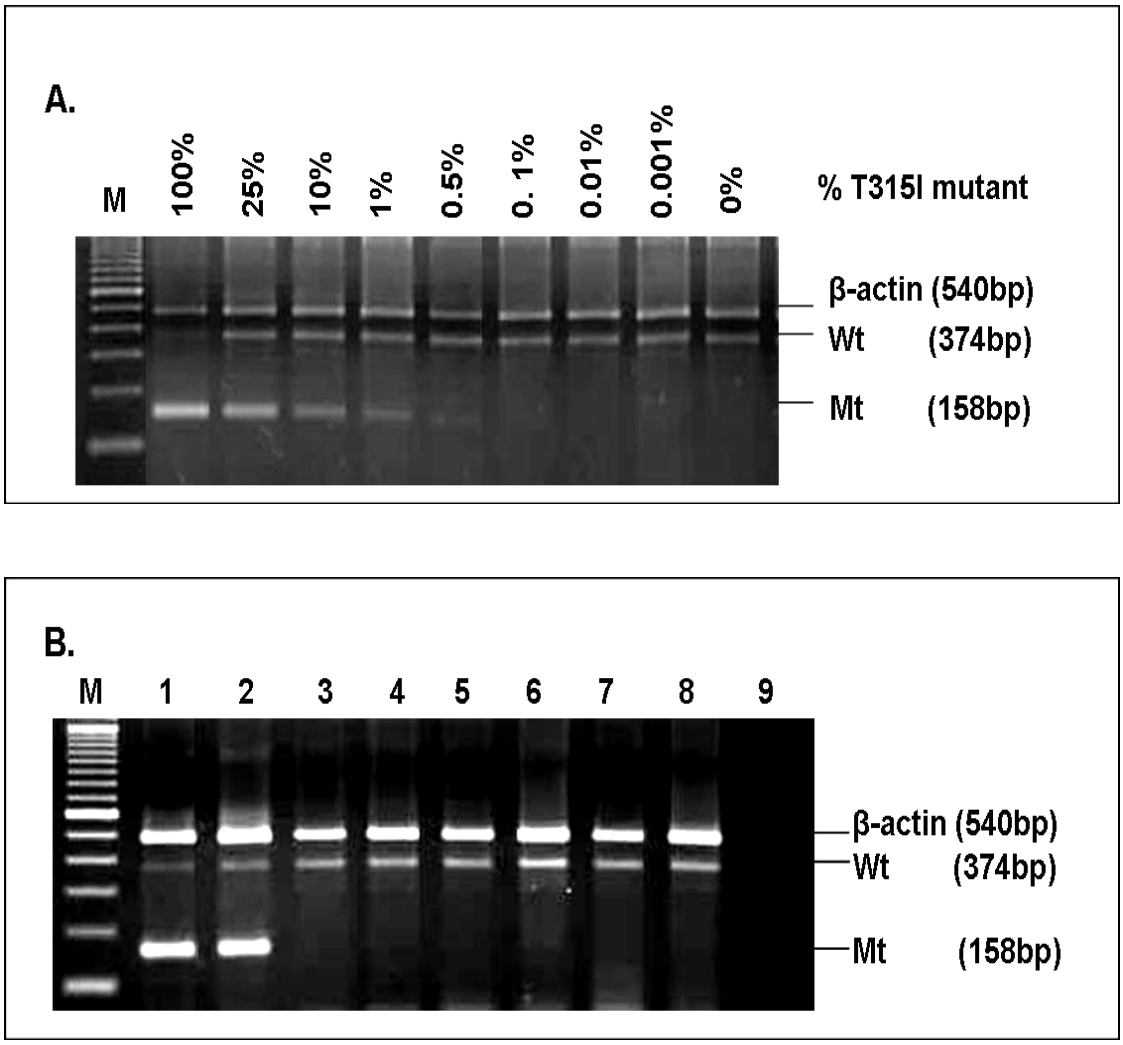


Figure 5.21 Sensitivity and specificity of T315I mutation detection by AS-PCR method. Serial dilutions of T315I mutants with wild-type cells samples demonstrates 158-bp mutant bands in 100%, 25%, 10%, 1%, and 0.5% mixtures (A); Representative samples of T315I mutated cell lines (Lane 1), T315I mutated CML case, (Lane 2), five non-mutated non-leukemic cases (*BCR-ABL* negative patients) (Lanes 3-7), BA/F3 cell lines (Lane 8), and blank (Lane9) (B**)**. Lane M is a 100-bp DNA ladder used as a standard-size marker.

5.5.2 Detection of T315I mutation by AS-PCR, DHPLC and direct sequencing

Nine CML patients were tested for T315 mutation using DHPLC followed by sequencing and AS-PCR. Abnormal DHPLC patterns strongly supportive of T315I mutation were observed and all were confirmed by sequencing. Mutant bands were comparably seen by AS-PCR (Figure 5.22).

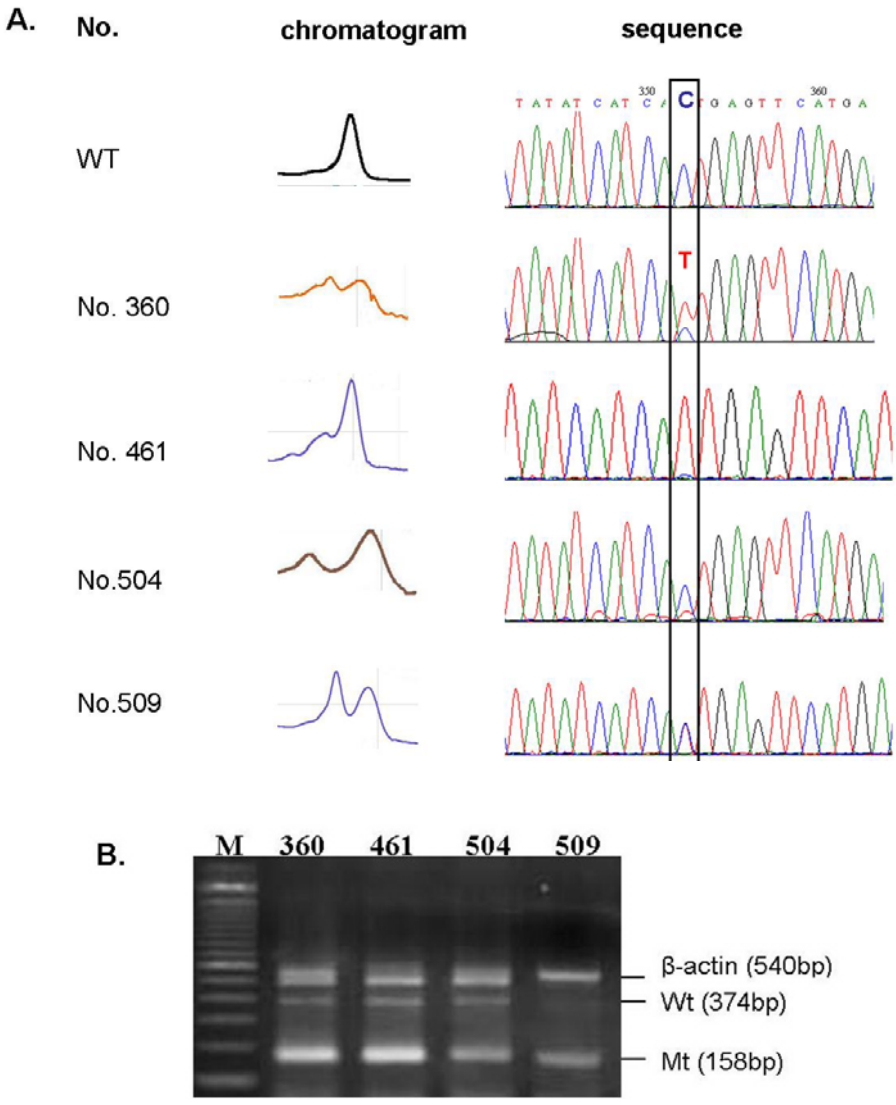


Figure 5.22 Detection of T315I in CML patient samples by DHPLC, sequencing and AS-PCR; DHPLC patterns are shown followed by sequencing analysis if a suspicious peak was observed in (A) and representative AS-PCR results of four CML cases with abnormal DHPLC and sequencing results are demonstrated in (B) (Patients no.360, no.461, no.504, and no. 509).

CHAPTER VI
DISCUSSION

Of all worldwide studies, *ABL* KD mutations were mainly reported in the TKI- resistant CML patients. Very few studies explored the frequency and types of KD mutations in the TKI-naïve cases who never received the drug (38,40,42,44). In this study, two CML populations, TKI-exposed and TKI-naïve, were analyzed using a screening DHPLC followed by sequencing analysis. DHPLC was selected as my screening tool because it has a higher sensitivity than sequencing alone to detect the presence of KD mutations. I first looked at the overall incidence of KD mutations in our Southeast Asian CML population (n=171) (TKI exposed, n =91 and IM naïve) as previous reports from the Asia continent were available from only three countries, i.e. Korea (n=68, n=111) (28,54), China (n=127) (59), and India (n=69, n=65)(43,60) while the Western studies were available from USA (n=297, n=95, n=171, n=150)(29,33,61,62), Germany (n=66, n=141) (19,42), France (n=24)(38), and Italy (n=43)(44) (Table 6.1). The frequency of KD mutations in the TKI-exposed cases of 33% (30/91) was slightly lower than the figures reported from the Western series (43%, 36%, 35.3%, and 55.8%) and the Asian series such as Korea (54%, 44%, and 63%), and China (58%), but was comparable to India (32%) (60). This study represents the largest study ever reported from Asia and the first one from the Southeast Asian ethnic region.

Table 6.1 The incidence of *ABL* KD mutations in CML patients from various countries

Country	No. of cases in each study	No. of mutated cases (%)	Detection method
North America and Europe			
USA Soverini S *et al* (2006) (33)	297 (IM-resistance)	127 (43)	DHPLC and DS
Soverini S *et al* (2009)(62)	95 (IM-resistance)	51 (54)	DS
Jabbour E *et al (*2006) (29)	171 (IM-resistance)	62 (36)	DS
Press RD *et al* (2009) (61)	50 (IM-exposed)	53 (35.3)	DS
Germany Willis SG *et al* (2005) (42)	66 (Naïve patients)	14 (21.2)	AS- PCR
Ernst T *et al* (2008) (19)	95 (IM-resistance) 46 (Naïve patients)	53 (55.8) 9 (19)	DHPLC and DS
France Roche-Lestienne C *et al* (2002) (38)	24 (Naïve patients)	3 (12.5)	AS-PCR
Italy Carella AM *et al* (2010) (44)	43 (Naïve patients)	9 (20.9)	DS
Asia			
Korea Kang HY *et al.* (2006) (54)	68 (IM-resistance)	37 (54) 30 (44)	AS-PCR DS
Kim SH *et al* (2009) (28)	111 (IM-resistance or intolerance)	70 (63)	AS-PCR and DS
China Qin YZ *et al* (2010) (59)	127 (IM-resistance)	74 (58)	DS
India Kuila N *et al* (2009) (43)	44 (IM-naïve) 25 (IM-exposed)	2 (5) 4 (16)	DS
Rajappa S *et al* (2010) (60)	65 (IM-resistance)	29 (32.2)	DS
Thailand ***This study***	80 (IM-naïve) 91 (TKI exposed)	7 (8.75) 30 (33)	DHPLC and DS

This study particularly focused on the frequency and types of KD mutations in the non-TKI exposed CML cases in order to explore if such mutations had already existed in this naïve population. Very few small series previously existed with respect to the incidence of KD mutations in naïve cases in the literature (19,38,42-44). The overall reported incidence was 0-20% in the pre-treatment newly diagnosed samples (Table 6.1), although most studies had a lower number of cases than my study. In this study, naïve cases could be separated in two groups, i.e. newly diagnosed cases (54/80, 65%) and chronic cases who were being treated with hydroxyurea (26/80, 35%). The incidence of KD mutations in the hydroxyurea-treated group was two-fold higher than the newly diagnosed cases (5/26, 19.2% versus 2/54, 3.7%, respectively) (p=0.034). This study is comparable to a small study from India which found 2 cases of KD mutations in 19 hydroxyurea-treated cases (10%) and no mutations in 24 newly diagnosed cases (43). My patients who were chronic-phase cases who had been on a conventional chemotherapy for several months to years (median 12 months) and most of them still had not been offered the drug due to limited insurance coverage in the country or they themselves refused to switch to a TKI drug. The higher frequency of KD mutations in the hydroxyurea-treated group as compared to the newly diagnosed group could reflect the direct effect of hydroxyurea on leukemic stem/progenitor cells. On the other hand, it could be argued that KD mutations could spontaneously develop due to leukemic cell genomic instability during the natural course of the disease. This could be supported by the fact that 18.4 % of my chronic-phase patients had KD mutations whereas 29.8% of advanced-phase patients did, which is also consistent with most other reports.

Among the 21 types of mutations identified in these 37 CML patients, thirteen were known mutations and eight were novel mutations. Different mutations may have a different degree of TKI resistance depending on their locations and effects on the tyrosine kinase. The KD mutations in this study could be classified according to their potential sensitivities to imatinib as determined by in vitro cell proliferation assay (Table 6.2). The known mutations in this study could be categorized into 3 groups based on the degree of reported imatinib sensitivity, i.e. sensitive (M351T, M388L), intermediate (M244V, G250E, F359V, and L387F), and insensitive (Y253H, E255K, E255V, and T315I) (4,18,34,115-117). The known mutations in my naïve

patients, E255V, E279K, and L273L, were also discovered at a low level in newly diagnosed CML patients as reported by Soverini *et al* (33) and Press *et al* (61). The known mutant V304A in 1 imatinib response patient had been previously discovered in $CD34^+$ cells from imatinib-treated CML patients by Chu *et al* (118) and Jiang *et al* (66). They also indicated that V304 was associated with imatinib resistance in their patients. Recently, Jones *et al* also described the loss of V304A in an imatinib-treated patient who underwent a switch from imatinib to a newer TKI with a major molecular response (119). Among the reported Chinese cases, the most frequent mutation was M244V (12/74, 16%) followed by Y253H, F359C/V/I, G250E, E255K, T315I, and M351V whereas the most frequent mutation in the Indian and Korean series was T315I (31% and 23%, respectively). T315I was also the most frequent mutation in our Thai cohort (24.32%) followed by Y253H (13.5%), M351T (13.5%), and G250E (8.1%), but M244V which was common in the Chinese and Indian cohorts was only found in one case in this study. The variations in the KD mutation frequency and types may reflect geographic heterogeneity among various ethnic populations.

Eight different types of novel mutations were found among naïve and exposed cases. They were clustered in 4 regions: 3 located within exon 4 (p.Pro218Glnfs, H246Y, and E258E), 3 located in exon 5 (H295H, p.Asn297ProfsX10, and L301H), 1 located within exon 6 (p.Phe311ValfsX6), and one (A399V) within exon 7. Based on the Polyphen website analysis (http://genetics.bwh.harvard.edu/pph/), these novel point mutations could be categorized into 3 groups, i.e. 1) the variants were predicted to be probably damaging and might be a mutation (H246Y and L301H), 2) the variants were predicted to be benign (A399V), which might be a SNP, and 3) the silent mutation (E258E and H295H), which may not have any effect on the ABL KD protein structure and function.

Table 6.2 The incidence and TKI resistance of common mutation in CML patients

Known mutations	Frequency of mutations in this study	Previously reported incidence (25,34,83)	Reported TKI sensitivity (in vitro) (4,18,34,81,117)		
			Imatinib	Nilotinib	Dasatinib
M244V*	2.7%	4%	Intermediate	Sensitive	Sensitive
L248V*	-	2%	Intermediate	Intermediate	Sensitive
G250E*	8.1%	5%-9%	Intermediate	Sensitive	Sensitive
Q252H*	-	2-3%	Intermediate	Sensitive	Sensitive
Y253F*	-	6%	Insensitive	Intermediate	Sensitive
Y253H*	13.5%	5%	Insensitive	Intermediate	Sensitive
E255K*	5.4%	9%-14%	Insensitive	Intermediate	Intermediate
E255V*	2.7%	2 %-3%	Insensitive	Intermediate	Intermediate
L273L	2.7%	n/a	n/a	n/a	n/a
D276G	-	2%	Intermediate	Intermediate	n/a
E279K	5.4%	5.5%	Intermediate	Sensitive	Sensitive
V299L	-	n/a	Sensitive	Sensitive	Insensitive
F311V*	-	1%	Insensitive	Intermediate	Sensitive
T315I*	24.3%	13%-16%	Insensitive	Insensitive	Insensitive
F317L*	-	3%-4%	Intermediate	Intermediate	Intermediate
M351T*	13.5%	10%-13%	Sensitive	Sensitive	Sensitive
E355G*	-	2%-3%	Intermediate	n/a	n/a
F359V*	5.4%	4%-5%	Intermediate	Intermediate	Sensitive
L384M	-	1%	Sensitive	Intermediate	Intermediate
L387F	2.7%	n/a	Intermediate	n/a	n/a
M388L	2.7%	1%	Sensitive	Sensitive	n/a
V304A	2.7%	n/a	n/a	n/a	n/a
H396R*	-	4%	Intermediate	Sensitive	Sensitive
F486S*	-	2%	Insensitive	Sensitive	Intermediate

*indicate the common mutant residues in imatinib-resistant patients; n/a; the in vitro sensitivity data not available

Among the naïve cases, the first type of mutation was a deletion of 32 nucleotides (c.888-919del), leading to a novel frameshift mutation (p.Asn297GlyfsX9) an premature stop codon resulting in a truncated BCR-ABL protein. This type of deletion was found in a chronic-phase CML patient who had been on

hydroxyurea for 12 year and had never been treated with imatinib. The second type of mutation was a T to A substitution at codon 301 (CTC>CAC) causing the change of leucine to histidine (L301H). L301H was found in a chronic-phase CML patient who also had another known mutation, E255V. The third type of mutation was a single nucleotide duplication within codon 311 (a nucleotide at cDNA position c.929dup) resulting in a frameshift mutation of 6 amino acids (p.Phe311ValfsX6) which occurred within the drug-binding domain. The fourth type of mutation was a silent H295H which was found in an accelerated-phase CML patient who had been on hydroxyurea for 12 months. The fifth type of mutation was a silent mutation as described above (E258E) was found the homozygous mutants peak, G>A substitution (Figure 5.8, no.420).

There were three novel mutations in the TKI-exposed cases, the first of which was identified as H246Y which was a C to T substitution at codon 246 (CAC>TAC), which the result was confirmed by reverse direction of resuencing, causing the change of histidine to tyrosine. H246Y was found in a chronic-phase CML patient who developed imatinib intolerance after a 30-month therapy and progress into advance phase. The H246Y mutation may result in imatinib not response in the no.408 patients, however, the a variant Philadelphia chromosome (46, XY, t(2;14), t(9;22)) was found in this patient. The same amino acid position with a different amino acid type to H246H, E258D, had been reported in a previous study by Ernst *et al* (97). The second mutation was a C to T substitution at codon 399 (GCC>GTC) causing the change of alanine to valine (A399V) and was detected in a chronic-phase CML patient who did not achieve complete cytogenetic response after 32 months of imatinib therapy and was switched to nilotinib for about 10 months. The third type was a deletion of a single nucleotide (c.653delC), resulting in a frameshift mutation of 27 amino acids (p.Pro218GlnfsX27), which was located outside the KD and was detected in a young chronic-phase CML patient who had a primary imatinib resistance and a variant Philadelphia chromosome (46,XY,t(5;9;22)(q31;q34;q11.2)).

In this study, the KD mutations were mainly clustered in the SH3 contact and C-helix domain in the naïve cases (4/7 cases). On the other hand, most of the mutations in the TKI-exposed cases were localized in the drug contact site, P-loop, and catalytic domain with the most frequent mutation being T315I (9/91). The

insensitive type of mutation was also found in the naïve cases such as E255V. However, the most resistant mutation, i.e. T315I could not be detected in any of the 80 naïve CML cases in this series. A high incidence of T315I was noted in cases with exposure to both imatinib and 2nd generation TKI (27.3%) and cases with advanced phase who received 2nd generation TKI only (100%), suggesting that T315I is strongly associated with TKI exposure and/or advanced phase of the disease. Kim *et al* showed that T315I preferentially occurred in the Korean patients with advanced phase as contrasted to chronic phase, suggesting that the frequency of resistant mutation increased as the disease progressed (28). The Y253H mutation is located on ATP binding loop (p-loop) of KD and associated with a true imatinib resistant phenotype and its occurrence would favor alternative treatment strategies. However, it is still not clear whether p-loop mutations are truly the poor outcome predictors (29).

The fact that naturally occurring mutations were found in the naïve cases were consistent with most previous small reports. I am, however, intrigued by the fact that T315I could not be demonstrated in any naïve cases, some of whom were also exposed to conventional chemotherapy. This study was in agreement with Carella *et al* who did not find T315I in a naïve cohort from Italy (44). E255V and C330G without T315I were also reported in a small naïve cohort receiving hydroxyurea from India (43). Contradictory results came from Roche-Lestienne C *et al* who found resistant mutations such as T315I and F311L in a cohort of 24 TKI-unexposed CML cases from France (38). T315I, Y253F/H, Q252H, M351T, M244V, and F359V had also been reported in TKI-unexposed cases from Germany who had a history of hydroxyurea, interferon, and cytarabine treatment (42). The fact that the naïve cases had a milder resistant mutation such as E255V but lack T315I and M351T could indicate the differential occurrence of types of mutations at various stages of disease that were precipitated by different types of TKI exposure.

The clinical characteristics and laboratory parameters of patients with and without KD mutations were also explored. A slightly higher number of males to females was found in the patients bearing the KD mutations although this result may reflect a higher proportion of males in the Thai CML population as previously reported by Udomsakdi-Auewarakul C *et al* (3). The median age of imatinib treated patients with or without KD mutation was comparable, whereas the median age of

imatinib-naïve patients with or without mutations was slightly younger. Overall, CML patients in this study were relatively younger than those in the West, which again was consistent with previous reports by Jootar S *et al* (120). Most of the patients in both groups with or without KD mutation were in chronic phase as expected from the natural history of the disease. About one-third of mutated cases in TKI-exposed and about half of mutated cases in naïve cases were in advanced phase (accelerated phase plus blastic crisis) whereas about one-fourth of wild-type cases in both groups were in advanced phase. The median WBC and PLT counts were higher and the Hb was lower in the naïve CML cases as compared to the TKI-treated cases which could be simply explained by the fact that the naïve cases included newly diagnosed patients who never received any treatments.

With respect to additional chromosomal abnormalities, it was found that about a third of mutated TKI-exposed patients (11/30) had additional clonal chromosome abnormalities (CCA), including i.e. -7, -Y, t(9;22;12), +8 with inv(13), t(2;14), +8, +19, t(3;7), t(5;9;22), -22 , and -19. Some patients with KD mutations had very complex aberrations such as 47,XY,+19,t(9;22)(q34;q11),t(3;7)(q29;q22) which coexisted with a dual mutation of G250E and F359V in 1 case and 47,XX,+8,t(9;22),inv(13)(13q22) which coexisted with Y253H. Both patients developed imatinib resistance. Interestingly, 46,XY, t(9;22)(q34;q11.2), del(19)(p13.1) was found in 1 patient with V304A but did not seem to have any impact as this patient is still in a CR. The overall survivals of the mutated Thai patients with CCA were shorter than that of the mutated patients without CCA, implicating the negative impact of *ABL* KD mutation and CCA. However, the statistical significance could not be reached. Soverini S *et al* recently reported that the Ph^1-positive CML patients who harbored imatinib-resistant *ABL* KD mutations had a higher likelihood of developing additional mutations associated with resistance to second- or third-line TKI (62). Marin D *et al* also reported that KD mutations combined with CCA/Ph^1 predicted a shorter progression-free survival and OS (109). The ELN group thus issued a new recommendation for the management of CML which stated that CCA/Ph^1 is a warning sign as well as a marker of treatment failure in patients undergoing TKI therapy (81). Therefore, all patients receiving TKI therapy should be screened for CCA in addition to KD mutations.

This study also intended to correlate the *BCR-ABL* transcript levels with KD mutations although a limited number of cases were available for complete analysis by RQ-PCR. Although Patients no.320 and no.360 with T315I had a strikingly high *BCR-ABL* level when KD mutations were detected, most T315I patients appeared to have a comparable level of *BCR-ABL* transcripts to other patients with non-T315I mutations. The *BCR-ABL* transcript levels have also been used to signify the development of KD mutations. In this study, the *BCR-ABL* transcript levels did not seem to correlate with the KD mutations indicating that KD mutations may have occurred even before the molecular relapse is fully demonstrated. However, the mutated clones present at a low level at the initial diagnosis may not have a clinical significance as such clones detected when patients are in AP or BC. It was also of interest to find multiple types of mutations in the same patients, some occurred simultaneously, and some occurred in a sequential manner. For example, two Y253H patients developed a new mutation, T315I, when they developed TKI resistance but Y253H was already gone at the time of the treatment change to 2nd generation TKI. Some patients had persistent types of mutations such as Patient no.408 and Patient no.002. It was also observed that the mutant frequency increased in association with a history of receiving 2nd generation TKI after exposure to imatinib. The most common mutation that occurred late in the course was T315I as shown in this study. Three of the ten mutations in the patients who received both imatinib and 2nd generation TKI and all three mutations in the patients with advanced phase who received only 2nd TKI were T315I. Patients with T315I mutation had a shorter OS as compared with other mutations which could reflect the true drug resistance phenotype associated with the advanced phase of the disease. Kim W S *et al* reported that T315I was detected in 16% (9 of 55) of patients during imatinib therapy as well as in dasatinib or nilotinib therapy (24%, 11 of 44) (121). Interestingly, my patients with C-domain mutations (M351T and F359V) also had the OS time closely to the T315I patients which may be explained by their similar drug contact site.

Several technique have been utilized to detect the existence of *ABL KD* mutations such as direct sequencing, DHPLC, RFLP, pyrosequencing, double–gradient denaturing electrophoresis, AS-PCR, AS real-time PCR, array based assays, and HRM, with varying sensitivity and specificity (34,42,48,51,53,54,102,105,122).

In this study, we established an AS-PCR-based method to detect T315I which is the most resistant genotype associated with the highest impact on clinical outcome of CML patients. The sensitivity of my AS-PCR method was better than the sensitivities reported from most previously reported detection techniques and was slightly better than DHPLC and direct sequencing analysis in my hands. By AS-PCR, T315I mutant bands were observed in the mixtures containing as low as 0.5% of mutant alleles whereas DHPLC was unable to detect the mutants below 1.56% dilution and sequencing was unable to detect below 6.25%.

The detection sensitivity of DHPLC in my study was in the range of previously published articles (1-5%) (51,102). Although DHPLC is considered a useful tool to screen for the presence of either known or unknown mutations, chromatograms generated from DHPLC were sometimes difficult to interpret and sequencing analysis is always needed to confirm their results. In this study, sequencing analysis was not able to detect mutant alleles below the 6.25% dilution. Therefore, it is the least sensitive method in my hands. Direct sequencing is recognized as a confirmation method for any screening tests because of its high specificity (34). However, its disadvantage is its high costs and low sensitivity (15-25%) (54), rendering it unsuitable for routine clinical use. In addition, both methods require nested–PCR steps to produced enough PCR products. In this study, 41 patient samples with aberrant DHPLC profiles had no detectable abnormalities by DNA sequencing which could be due to lower sensitivity level of DNA sequencing as compared to DHPLC. Low level of mutant alleles could be enriched further in order to improve the mutation detection rate by using various techniques such as COLD-PCR (123) and fraction collection of individual DHPLC peaks for reamplification (124).

In terms of specificity, all methods showed no false positive results even in the triplicate experiments (0% mutant in the samples or 100% wild type). My AS-PCR method has a high specificity due to the utilization of internal mismatch primers which were designed to specifically target the mutated sequences, therefore, none of the 30 non-leukemic patient samples were falsely found to be T315I positive.

The advantage of AS-PCR is that it does not require additional post-PCR product preparations for the next step as contrasted to the multiple steps such as the DNA purification step in sequencing method and the preparation of a 1:1 mixture of

mutant and wild-type PCR products followed by generation of heteroduplexes and homoduplexes in the DHPLC method which is much more time-consuming. AS-PCR technique can be applied in any general laboratory worldwide. Moreover, this present AS-PCR method could be performed in a single tube containing all three primers for a control gene, a wild-type gene, and a mutant gene; therefore, the cDNA quality could be simultaneously assessed at the same time of the detection of the wild type and the mutant genes. AS-PCR is also suitable to perform in cases with a low DHPLC peak and its shape looks like a known mutation such as T315I. The disadvantage of AS-PCR is its ability to detect only known mutations using specific primer sets and optimized PCR condition for each type of mutant allele. The sensitivity of my AS-PCR method was lower than that of Roche-Lestienne C *et al* (38) and Kang HY *et al* (54), both of which did not use internal *β*-actin control primers. Nevertheless, I believe that the cDNA quality should be simultaneously assessed at the same time of the detection of the wild type and the mutant genes, especially in the homozygous T315I mutant cases, therefore all three primers were utilized in a single-tube reaction. Current clinical practice accepts a detection method with a sensitivity of at least 0.5-1% since a higher sensitivity may detect a clone that may not be of clinical relevance (34,81). Changes of TKI therapy based on a very sensitive molecular test may have an adverse impact if the clinical outcome is not truly affected by the presence of a minute amount of leukemic cells with that particular genetic defect.

Future studies should focus on the design of other primer sets to cover other mutations associated with 2nd generation TKI resistance such as V299L/F317L in dasatinib and E255K/E255V/Y253H in nilotinib. Larger clinical studies are needed to delineate the impact of the known and novel mutations identified in this population. Alternative mechanisms to explain TKI resistance should also be entertained if KD mutations were not detected such as mutations outside the KD in the neighboring regions, clonal chromosomal evolution, *BCR-ABL* amplification, and pharmacogenomic variations.

CHAPTER VII
CONCLUSION

This study was set out to determine the incidence and types of *ABL* KD mutations in the Thai CML patients with *BCR-ABL* rearrangement as previous data existed mainly in the Western countries. Moreover, it was specifically aimed to explore if *ABL* KD mutation may have already existed in the naïve CML patients without prior exposure to TKI drugs. Various techniques were developed including RT-PCR, RQ-PCR, DHPLC followed by sequencing, and a single-tube AS-PCR. This study represents the largest series ever reported from Asia whereby 21 types of mutations were discovered for the first time in the Southeast Asian population which included 8 *novel* mutations. Various types of sensitive and resistant mutations were detected in the leukemic cells without prior TKI exposure and the mutation frequency appears to increase with TKI therapy. The presence of such mutations in the TKI-naïve CML cases suggested that naturally occurring KD mutations are present in the leukemic cells even before drug exposure. T315I as the most resistant mutation leading to therapeutic failure was not observed in any of the naïve cases, suggesting that T315I is potentially induced to arise by 1st and 2nd generation TKI drugs. Moreover, the dynamic studies of KD mutations did not find a strong correlation between the *BCR-ABL* transcript levels and the occurrence of KD mutations, indicating that some mutations may have already occurred before a molecular relapse. The single-tube AS-PCR method which I developed is a simple, rapid, and easy to perform test which requires only simple PCR reagents and a PCR machine leading to overall lower costs as compared to other more complicated and more expensive screening methods. Detection of the most resistant leukemic clone in CML patients undergoing TKI therapy, especially those who reside in the developing worlds, should be feasible with this method. *ABL* KD mutation analysis should always beincorporated into the treatment paradigm of CML in order to facilitate appropriate treatment decisions.

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APPENDIX

List of chemicals, instruments, enzyme, DNA markers, and reagents

1. Chemicals

Chemicals	**Molecular weight (g/ml)**	**Source**
Absolute ethanol (C_2H_5OH)	46.07	E.Merck, Darmstadtt, F.A., Germany.
Agarose ($(C_{12}H_{18}O_9)_n$)		Seakem, Rockland, USA.
Ammonium Chloride (NH_4Cl)	53.49	Sigma chemical, St, Louis, USA.
Boric Acid (H_3BO_3)	61.83	USB, Cleveland, USA.
Chloroform ($CHCl_3$)	119.38	E.Merck, Darmstadtt, F.A., Germany.
3′-Deoxyadenosine 5’-triphosphate or dATP ($C_{10}H_{12}N_5O_{12}P_3Na_4$)	579.2	Promega, Madison, USA.
3′-Deoxycytosine 5’-triphosphate or dCTP ($C_9H_{12}N_3O_{13}P_3Na_4$)	555.1	Promega, Madison, USA.
3′-Deoxyguanosine 5’-triphosphate or dGTP ($C_{10}H_{12}N_5O_{13}P_3Na_4$)	595.1	Promega, Madison, USA.
3′-Deoxythymidine 5’-triphosphate or dTTP ($C_{10}H_{13}N_2O_{14}P_3Na_4$)	570.1	Promega, Madison, USA
Ethidium bromide	394.3	Research Organics, Cleveland, USA.
Ethylenediamine tetra acetic acid tetrasodium salt or EDTA ($C_{10}H_{12}N_2Na_2O_8$)	380.2	E.Merck, Darmstadtt, F.A., Germany.
Hydrochloric acid (HCl)	36.50	E.Merck, Darmstadtt, F.A., Germany.

Phenol (C_6H_5OH)	94.11	E.Merck, Darmstadtt, F.A., Germany.
Potassium hydrogen carbonate ($KHCO_3$)	100.12	E.Merck, Darmstadtt, F.A., Germany.
Potassium hydrogen phosphate (KH_2PO_4)	136.09	E.Merck, Darmstadtt, F.A., Germany.
Sodium chloride (NaCl)	58.44	E.Merck, Darmstadtt, F.A., Germany.
Sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	294.10	E.Merck, Darmstadtt, F.A., Germany.
Sodium dodecyl sulphate or SDS ($C_{12}H_{25}O_4SNa$)	288.38	Bio Basic Inc., Toronto, Canada.
Sodium hydrogen phosphate (Na_2HPO_4)	141.96	E.Merck, Darmstadtt, F.A., Germany.
Sodium hydroxide (NaOH)	40.00	E.Merck, Darmstadtt, F.A., Germany.
Tris (Hydroxymethyl aminomethane) ($NH_2C(CH_2OH)_3$)	121.14	USB, Cleveland, USA.

2. Instruments

ABI3730XL PRISM BigDyeTM analyzer, Applied Biosystems, CA, USA

DHPLC WAVE® system, Transgenomic, Omaha, Nebraska, USA

Gel documentation system, UVItec, Cambridge, UK

Gel electrophoresis apparatus, Gelmate 2000, Toyobo, Osaka, Japan

LightCycler instrument , LightCycler 480, Roche Diagnostics, Geramany

Veriti® 96-Well Thermal Cycler, Applied Biosystems, CA, USA

UV-visible spectrophotometer, Shimadzu UV-160A, Kyoto, Japan

3. Enzymes

ExoSAP-IT® Exonuclease, USB corporation, Cleveland, Ohio, USA

High-Fidelity DNA polymerase , Phusion™, Finnzymes, Finland

SuperScript III reverse transcriptase, Invitrogen, Canada

Taq DNA polymerase, Invitrogen, Brazil

4. DNA markers

100 bp DNA Ladder, New England BioLabs Inc., Beverly, USA.

5. Reagents

5.1 10X Phosphate buffered saline (PBS)

NaCl	76.5	g
Na_2HPO_4	9.95	g
KH_2PO_4	4.08	g
Added distilled water to	1000	ml

The solution was autoclaved for 15 minutes at 121 °C, 15 lb/ inches^2 and stored at room temperature. It was 10-fold diluted to 1xPBS before use.

5.2 10X RBC lysis buffer

NH_4Cl	82.9	g
$KHCO_3$	10	g
EDTA	0.37	g
Distilled water to	1000	ml

The solution was autoclaved for 15 minutes at 121 °C, 15 lb/inches^2 and stored at room temperature. It was 10-fold diluted to 1xRBC lysis buffer before use.

5.3 75% Ethanol

Absolute ethanol	75	ml
Sterile distilled water to	100	ml

The solution was mixed and stored at –20 °C.

5.4 10 mM Tris-HCl, pH 8.0

Tris base	12.11	g

Dissolved in distilled water and adjusted pH 8.0 with HCl

Added distilled water to	100	ml

The solution was sterilized by autoclaving for 15 min at 121 °C, 15 lb/inches^2 and stored at room temperature.

5.5 0.5 M EDTA pH 8.0

EDTA	18.60	g

Dissolved in distilled water and adjusted pH to 8.0 with NaOH

Added distilled water to	100	ml

The solution was steriled by autoclaving for 15 min at 121 °C, 15 lb/inches^2 and stored at room temperature**.**

5.6 TE 20-5 (20 mM Tris-HCl and 5 mM EDTA) buffer

20 mM Tris –HCl, pH 8.0	1	volume
5 mM EDTA	1	volume

The solution was sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/inches^2 and stored at room temperature.

5.7 0.5X Tris-borate buffer (TBE buffer)

Tris-base (Sigma)	5.389	g
Boric acid	2.751	g
0.5 M EDTA (pH 8.0)	2.0	ml
Added distilled water to	1000	ml

The solution was mixed and stored at room temperature.

5.8 Chloroform-isoamyl alcohol (24:1)

Chloroform	24	volume
Isoamyl alcohol	1	volume

The reagents were mixed and stored in the sterile bottle at 4ºC.

5.9 10 mM dNTPs solution (containing 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), to prepare 100 μl, mix

100 mM dATP (Promega)	10	μl
100 mM dTTP (Promega)	10	μl

100 mM dCTP (Promega)	10	µl
100 mM dGTP (Promega)	10	µl
DEPC-treated water	60	µl

The solution was kept at -20°C.

5.10 1.5% (w/v) Agarose gel in 0.5X TBE buffer

Agarose gel	1.5	g
0.5x TBE buffer	100	ml

The slurry was heated in microwave oven until the agarose completely dissolved and then poured the warm agarose solution into the gel box. The gel was between 3 mm and 5 mm thick. It was checked to ensure that there were no air bubbles under or between the teeth of the comb.

5.11 6x gel loading buffer

0.25%	bromophenol blue
0.25%	xylene cyanol
15%	Ficoll (Type 400; Sigma) in water

The reagents were mixed them together and stored in aliquots at 4 °C.

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