

**The application of high-resolution melting
curve analysis for the detection of mutations
in the *BCR-ABL* kinase domain of patients
with chronic myeloid leukaemia**

By

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DECLARATION

I certify that the dissertation hereby submitted by me for the M.Med.Sc. (Molecular Biology) degree at the University of the Free State is my independent effort and had not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

Kirsty Wienand

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“The Sovereign Lord is my strength! He makes me as surefooted as a deer, able to tread upon the heights.”

Habakkuk 3:19

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LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

A	Adenosine
ABI	Applied Biosystems
<i>ABL</i>	<i>Abelson proto-oncogene</i>
AP	Accelerated phase
ASO-PCR	Allele-specific oligonucleotide polymerase chain reaction
ATP	Adenosine triphosphate
B (cells)	Bursa of Fabricus cells
<i>BCR</i>	<i>Breakpoint cluster region gene</i>
<i>BCR-ABL</i>	<i>Breakpoint cluster region Abelson proto-oncogene</i>
bp	Base-pairs
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CCgR	Complete cytogenetic response
CHR	Complete haematological response
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
CP	Chronic phase
Ct	Crossing time
DEPC	Diethyl pyrocarbonate
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
ELN	European LeukemiaNet
<i>et al.</i>	<i>et alia</i> (and others)
e1	Exon 1 (<i>BCR</i> gene)
FISH	Fluorescence in situ hybridization
FLT1	Foetal liver tyrosine kinase 1 receptor
FLT3	Foetal liver tyrosine kinase 3 receptor
<i>FLT3</i>	<i>Foetal liver tyrosine kinase 3 gene</i>
g	Gram
G	Guanine
hOCT1	Human organic cation transporter one
HR	Haematological response
HRM	High-resolution melting
IL-3	Interleukin-3
IRIS	International randomised study of interferon versus STI571
JAK	Janus kinase
kDa	kiloDalton
M	Molar

MAPK	Mitogen-activated protein kinase
M-bcr	Major breakpoint cluster region
m-bcr	Minor breakpoint cluster region
mg	Milligram
MgCl ₂	Magnesium chloride
<i>MHL1</i>	<i>MutL homolog 1 gene</i>
microL	Microlitre
ml	Millilitre
mM	Millimolar
MMR	Major molecular response
MMuLV RT	Moloney murine leukaemic virus reverse transcriptase
MRD	Minimal residual disease
NEG	Negative
ng	Nanogram
NH ₄ Cl	Ammonium chloride
NH ₄ HCO ₃	Ammonium bicarbonate
mRNA	Messenger ribonucleic acid
PI3K	Phosphatidylinositol 3 kinase
PCgR	Partial cytogenetic response
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor

pH	Percentage hydrogen
Ph	Philadelphia chromosome
P-loop	Phosphate binding loop
pmol	Picomole
p190	190kD BCR-ABL oncoprotein
p210	210kD BCR-ABL oncoprotein
p230	230kD BCR-ABL oncoprotein
RNA	Ribonucleic acid
RQ-PCR	Real-time quantitative polymerase chain reaction
rpm	Revolutions per minute
SCT	Stem cell transplantation
STAT	Signal transducers and activators of transcription
SRC	Sarcoma
T	Thymine
TAE	Tris acetate EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
T (cells)	T-lymphocyte
TKIs	Tyrosine kinase inhibitors
Tm	Melting temperature
U	Unit
UV	Ultraviolet

LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

V	Volts
www	World wide web
°C	Degree Celsius
%	Percentage
μ - bcr	Micro breakpoint cluster region
μg	Microgram
μl	Microlitre

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PREFACE

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder that is characterized by the clonal proliferation of myeloid cells in the bone marrow. The development of CML is due to a reciprocal translocation between chromosomes 9 and 22 resulting in the fusion of *BCR* and *ABL* genes that encodes an oncoprotein with constitutive tyrosine kinase activity. The constitutively active tyrosine kinase disrupts normal cellular activity resulting in uncontrolled and poorly differentiated cellular proliferation. The treatment of choice for CML is tyrosine kinase inhibitors such as imatinib, nilotinib and dasatinib.

Despite the success of using tyrosine kinase inhibitors to treat CML, 30% of patients develop acquired resistance. Acquired resistance is primarily due to single base mutations although insertions, deletions and duplications in the *BCR-ABL* kinase domain have also been described in literature. Detection and characterization of mutations in the kinase domain is important, since the type and position of the mutation may affect the patient's response to treatment. DNA sequencing is most commonly used for detecting mutations. However, this technique is costly and time consuming and a screening method to exclude samples without mutations would make mutational analysis more cost-effective. Recently, high-resolution melting (HRM) has been used as a method to screen *BCR-ABL* in the region of the kinase domain for single base mutations followed by sequencing. However, it is not known whether HRM analysis can be used to identify insertions, deletions and duplications in the kinase domain. Thus, the purpose of this study was to determine whether HRM can be used to screen for mutations including insertions, deletions and duplications in *BCR-ABL* in the region of the kinase domain in CML patients, prior to sequencing.

This thesis contains three chapters including a literature review, a research chapter on chronic myeloid leukaemia and two chapters on the detection of mutations in *BCR-ABL* in the region of the kinase domain with sequencing and HRM, respectively. The literature review presents a background to CML and focuses on the detection of mutations including insertions, deletions and

duplications in *BCR-ABL* in the region of the kinase domain. Chapter two and three were carried out over a three year period using 40 CML patients that were selected based on treatment response with a tyrosine kinase inhibitor and *BCR-ABL* mRNA levels. The aim of chapter two was to sequence the region of *BCR-ABL* encoding for the kinase domain of the 40 CML patients being treated with a tyrosine kinase inhibitor to detect single base mutations, insertions, deletions or duplications. The same patient samples were then used in chapter three to determine if HRM could be used as a screening method to detect the single base mutations, insertion and deletion variants identified in chapter two with sequencing. Furthermore, chapter three aimed to determine if HRM could detect mutations previously not identified with sequencing. The tables and figures are numbered according to the chapter in which they occur and have been included within the text where applicable. A summary in both English and Afrikaans is found after chapter three and a complete reference list at the end of the thesis.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction to chronic myeloid leukaemia

1.1.1 Leukaemia

Leukaemia describes a group of heterogeneous cancers that arises in the haematopoietic system (Linnet, 1985; Zeeb and Blettner, 1998). Leukaemia was first recognized in the early 19th century and today comprises the eleventh most common form of cancer, with more than 250,000 new cases per year worldwide (Bennett, 1845; Craigie, 1845; Stewart and Kheihues, 2003). Leukaemia is characterized by uncontrolled proliferation of the neoplastic cells leading to the accumulation of immature white blood cells and blast cells in the bone marrow and peripheral blood (Pillar, 1997). The most common symptoms of leukaemia include anaemia, weakness, leukopenia, increase in infections, thrombocytopenia and enlargement of the spleen (Linnet, 1985; Stewart and Kheihues, 2003). Leukaemia can be classified into different subtypes, which involves either the lymphoid (B and/or T cells) or myeloid lineage (granulocytic, erythroid and megakaryotic cells). The subtypes are further divided into acute or chronic leukaemia depending on the degree of maturation of the malignant cell, age of onset and progression to a fatal clinical stage of the disease with further subdivision based on morphological, immunohistochemical and cytogenetic characteristics (Linnet, 1985; Zeeb and Blettner, 1998; Stewart and Kheihues, 2003).

1.1.2 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a haematological malignancy that accounts for 15% to 20% of all cases of leukaemia with an incidence of one to two per 100,000 people annually (Faderl *et al.*, 1999; Sawyers, 1999; Druker *et al.*, 2006; Agis *et al.*, 2007). The median age of presentation of CML is 45 to 55 years. Less than 10% of patients diagnosed with CML are older than 60 years of age (Faderl *et al.*, 1999; Nardi *et al.*, 2003; Frazer *et al.*, 2007; Heaney and Holyoake, 2007). CML affects slightly more males than females at a ratio of 1.35 to 1 (Laneuville *et*

al., 2006). The most common symptoms of CML include fatigue, weight loss, anorexia, abdominal fullness, bleeding, purpura, splenomegaly, leukocytosis, anaemia and thrombocytosis (Faderl *et al.*, 1999; Sawyers, 1999; Frazer *et al.*, 2007). Approximately 50% of CML patients are asymptomatic in the chronic phase and diagnosis is as a result of routine blood tests, where the patient often has leukocytosis, anaemia or a thrombocytosis (Faderl *et al.*, 1999; Sawyers *et al.*, 1999; Van Etten, 2011).

CML is characterized by the clonal proliferation of haematopoietic stem cells. The myeloid lineage is predominantly affected resulting in an increase in granulocyte precursors in the peripheral blood and the bone marrow (Fialkow *et al.*, 1977; Sawyers, 1999). CML is further characterized by abnormalities in cell differentiation, apoptosis and adhesion of leukaemic cells to the bone marrow, due to the lack of control over signal transduction pathways including the SRC kinase family and JAK/Stat. Abnormalities in cellular differentiation, proliferation and apoptosis lead to the accumulation of immature and undifferentiated white blood cells in the peripheral blood and the bone marrow (Heaney and Holyoake, 2007; Kwong, 2007).

1.1.3 Disease progression

CML can be divided into three phases of progression namely the chronic phase, accelerated phase and blast crisis (Table 1.1) (Sokal *et al.*, 1988; Heaney and Holyoake, 2007). The chronic phase is characterized by leucocytosis and hepatosplenomegaly which arises from an increase in immature blast cells in the myeloid compartment in the bone marrow (Weisberg and Griffin, 2001; Ren, 2005; Talpaz *et al.*, 2006; Heaney and Holyoake, 2007; Kwong, 2007). Although the chronic phase can last several years, if left untreated the disease usually progresses to the accelerated phase and blast crisis (Hochhaus *et al.*, 2002; Calabretta and Perrotti, 2004).

The accelerated phase is an intermediate phase of CML and includes symptoms such as weight loss, bone pain, anaemia and thrombocytopenia (Sokal *et al.*, 1988; Kwong, 2007). The accelerated phase is characterized by an increase in immature blast cells of between 10% to 30% in the peripheral blood and the bone marrow. Eosinophils and basophils are also increased in the accelerated phase (Calabretta and Perrotti, 2004; Willis *et al.*, 2005; Heaney and Holyoake, 2007; Kwong, 2007). If left untreated progression from the accelerated phase to blast crisis typically occurs within two to fifteen months (Sawyers *et al.*, 2002). The blast crisis is characterised by more than 30% immature blast cells, according to the European LeukemiaNet (ELN), in the peripheral blood and/or the bone marrow (Faderl *et al.*, 1999; Sawyers *et al.*, 2002; Druker, 2006). During blast crisis, additional genetic abnormalities often occur and the cancer can resemble acute myeloid or lymphoblastic leukaemia (Faderl, *et al.*, 1999; Deininger *et al.*, 2000; Sawyers *et al.*, 2002; Heaney and Holyoake, 2007). If untreated the median survival of patients in blast crisis is three to six months (Sawyers *et al.*, 2002).

Table 1.1: Phases of chronic myeloid leukaemia according to the World Health Organisation and European LeukemiaNet criteria (Baccarani *et al.*, 2006).

Phase	World Health Organisation Criteria	European LeukemiaNet Criteria
Chronic phase	None of the criteria for accelerated and blast crisis met	
Accelerated phase	<ul style="list-style-type: none"> • Blast cells in blood or bone marrow 10% to 19% • Basophils in blood 20% or more • Persistent thrombocytopenia (platelet count less than $100 \times 10^9/L$) or persistent thrombocytosis (platelet count greater than $1000 \times 10^9/L$), unresponsiveness to therapy • Increasing spleen size and increasing white cell count, unresponsiveness to therapy • Cytogenetic evidence of clonal evolution 	<ul style="list-style-type: none"> • Blast cells in blood or bone marrow 15% to 29% • Blast cells plus promyelocytes in blood and bone marrow more than 30%, with blast cells less than 30% • Basophils in blood 20% or more • Persistent thrombocytopenia (platelet count less than $100 \times 10^9/L$) unrelated to therapy
Blast crisis	<ul style="list-style-type: none"> • Blast cells more than 20% of peripheral white blood cells or nucleated bone marrow cells • Extramedullary involvement (excluding spleen and liver) • Large foci or cluster of blasts in the bone marrow biopsy specimen 	<ul style="list-style-type: none"> • Blast cells in peripheral blood and bone marrow above 30% • Extramedullary involvement (excluding spleen and liver)

1.2 Genetics of CML

1.2.1 The Philadelphia chromosome and BCR-ABL

CML was the first neoplasm ever to be associated with a chromosomal aberration (Nowell and Hungerford, 1960). In 1960, Nowell and Hungerford described a minute acrocentric chromosome in patients with CML that later became known as the Philadelphia (Ph) chromosome as it was discovered in a laboratory in Philadelphia (Nowell and Hungerford, 1960). This was due to a reciprocal translocation between the long arms of chromosomes 9 and 22 (Figure 1.1) (Rowley, 1973). The resulting shortened chromosome 22 is known as the Ph chromosome and is found in approximately 95% of CML patients (Rowley, 1973; Faderl *et al.*, 1999; Sawyers, 1999). Approximately 5% of CML patients are Ph negative and have complex or variant translocations involving chromosomes 9 and 22 that may also result in kinase encoding fusion oncogenes (Sawyers, 1999).

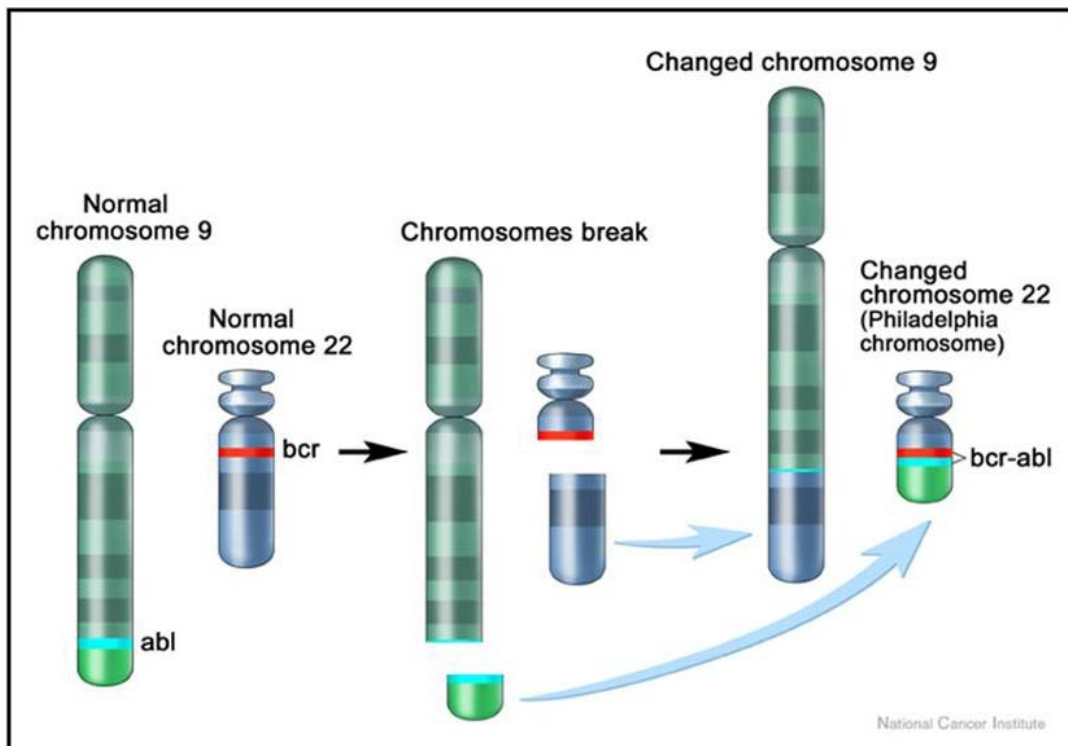


Figure 1.1: The Philadelphia chromosome. The translocation between the long arms of chromosome 9 and 22 results in the shortened Philadelphia chromosome. The *BCR-ABL* fusion oncogene is found on the Philadelphia chromosome (Copied from <http://www.qualityoflife.org/cancer/>).

The translocation involves the *Abelson (ABL) proto-oncogene* and *break-point cluster region (BCR) gene* (Groffen *et al.*, 1984; Shtivelman *et al.*, 1985; Kurzrock *et al.*, 1988). During the translocation, *ABL* is fused in a head-to-tail fashion to *BCR* on chromosome 22 resulting in the *BCR-ABL* fusion oncogene. The oncogene encodes an oncoprotein that is a non-receptor tyrosine kinase with constitutive activity (Shtivelman *et al.*, 1985; Sawyers, 1999).

The *BCR-ABL* oncoprotein can vary in size depending on the breakpoint location within the *BCR* gene (Figure 1.2). The position of the breakpoint in *ABL* is consistent at the 5' end of exon *a2*, while the breakpoint location in *BCR* can occur at one of three positions namely *minor-bcr (m-bcr)*, *major-bcr (M-bcr)* and *micro-bcr (μ -bcr)* (Kurzrock *et al.*, 1988). If the breakpoint occurs at *m-bcr* in exon *e1*, a $p190^{BCR-ABL}$ form of the oncoprotein is encoded (Kurzrock *et al.*, 1988). The most commonly associated breakpoint with CML is *M-bcr* in either exon 13 or exon 14, producing either splice variant *b2a2* or *b3a2*, respectively. Both splice variants encode for oncoprotein, $p210^{BCR-ABL}$ (Kurzrock *et al.*, 1988; Kantarjian *et al.*, 2000). The largest oncoprotein, $p230^{BCR-ABL}$, is encoded when the breakpoint within *BCR* involves the μ -*bcr* region in exon 19 (Kurzrock *et al.*, 1988).

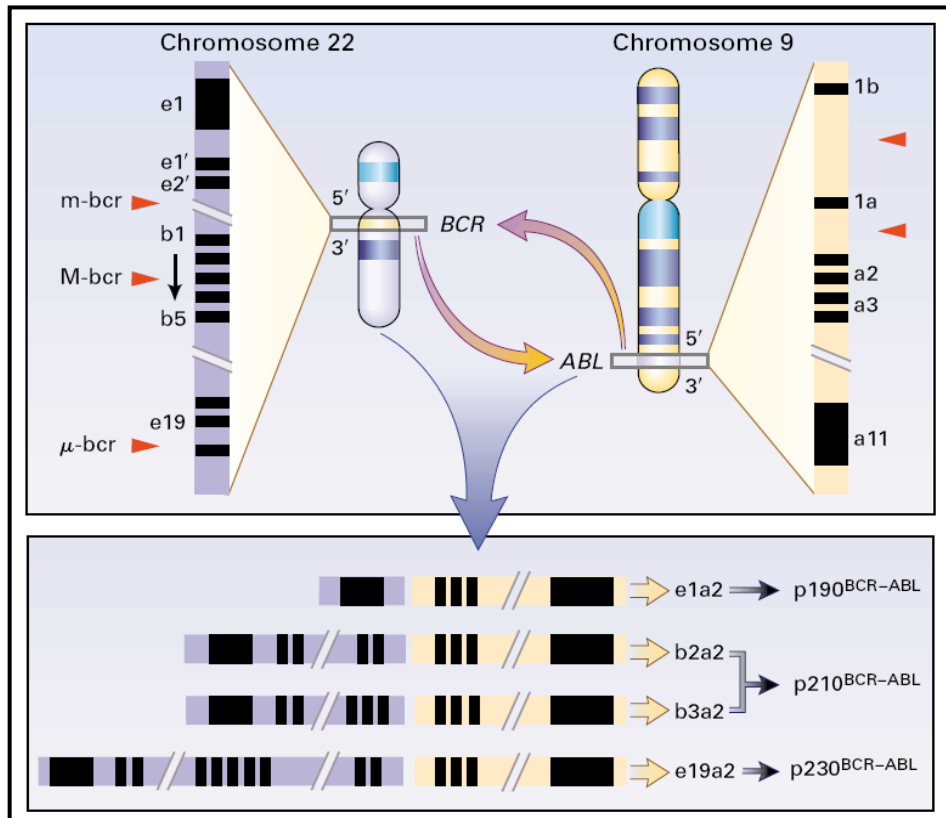


Figure 1.2: The different BCR-ABL oncoproteins. The different BCR-ABL oncoproteins are the result of three breakpoints within BCR namely m-bcr, M-bcr, μ-bcr, resulting in p190^{BCR-ABL}, p210^{BCR-ABL} and p230^{BCR-ABL}, respectively (Copied from Savage and Antman (2002)).

1.2.2 The BCR-ABL tyrosine kinase

Several functional domains in the BCR-ABL oncoprotein contribute to the constitutive activity of the tyrosine kinase. The tyrosine kinase activity of the ABL component of the fusion protein is encoded in the SH1 (SRC homology 1) domain and is controlled by the two adjacent regulatory domains, SH2 and SH3, respectively. In BCR-ABL, a large segment from BCR is situated adjacent to the SH2 domain in ABL affecting the regulation of the SH1 domain. Therefore, the absence of regulation of the SH1 domain, in BCR-ABL, results in uncontrolled and deregulated constitutive activity of the BCR-ABL tyrosine kinase (Figure 1.3) (Fadrel *et al.*, 1999; Goldman and Melo, 2003; Melo *et al.*, 2003). In addition to the lack of regulation of the tyrosine kinase domain of the ABL section of BCR-ABL, other functional domains in the BCR component of BCR-ABL contribute to the tyrosine kinase activity (Melo *et al.*, 2003). The first domain of BCR promotes dimerization of the oncoprotein resulting in autophosphorylation of adjacent BCR-ABL molecules further increasing tyrosine kinase activity (Goldman and Melo, 2003). Thus, the loss of regulation of the SRC homology domains of ABL together with the juxtaposition of the foreign BCR sequence, in BCR-ABL, respectively, results in uncontrolled and deregulated tyrosine kinase activity (Melo *et al.*, 2003).

The constitutive tyrosine kinase activity of BCR-ABL disrupts different signal transduction cascades by interacting with several protein adaptors and substrates. Phosphorylation of alternative adaptor proteins such as CRKL and p62Dok activates signal transduction pathways including Ras/MAPK, JAK/Stat, PI3K as well as Myc and their respective downstream targets (Figure 1.4) (Faderl *et al.*, 1999; Deininger *et al.*, 2000; Sattler *et al.*, 2002; Ren, 2005). Even though the role of some of the individual pathways may seem negligible, the cooperative interplay of pathways results in the proliferation of poorly differentiated haematopoietic cells (Quintas-Cardama and Cortes, 2008).

In CML, poorly differentiated haematopoietic cells are released prematurely from the bone marrow into the peripheral blood. The early release of cells is the result of decreased adhesion of leukaemic cells to the bone marrow and the extracellular matrix due to the influence of BCR-ABL on adhesion proteins like paxillin via $\beta 1$

integrin (Deininger *et al.*, 2000). Furthermore, cellular regulation and apoptosis of leukaemic cells is affected by the uncontrolled BCR-ABL constitutive tyrosine kinase activity (Tauchi and Ohyashiki, 2004; Krause and Van Etten, 2005; Bikker *et al.*, 2008). BCR-ABL affects cellular responsiveness to external stimuli resulting in the loss of cellular regulation which includes the inability to respond to growth factors including interleukin-3 (IL-3), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Savage and Antman, 2002; Ren, 2005; Bikker *et al.*, 2008). The net result of autophosphorylation of the kinase is unregulated cellular proliferation, decreased adherence of leukaemic cells to bone marrow stroma and a reduction in apoptotic responses to mutagenic stimuli (Goldman and Melo, 2003; Melo *et al.*, 2003).

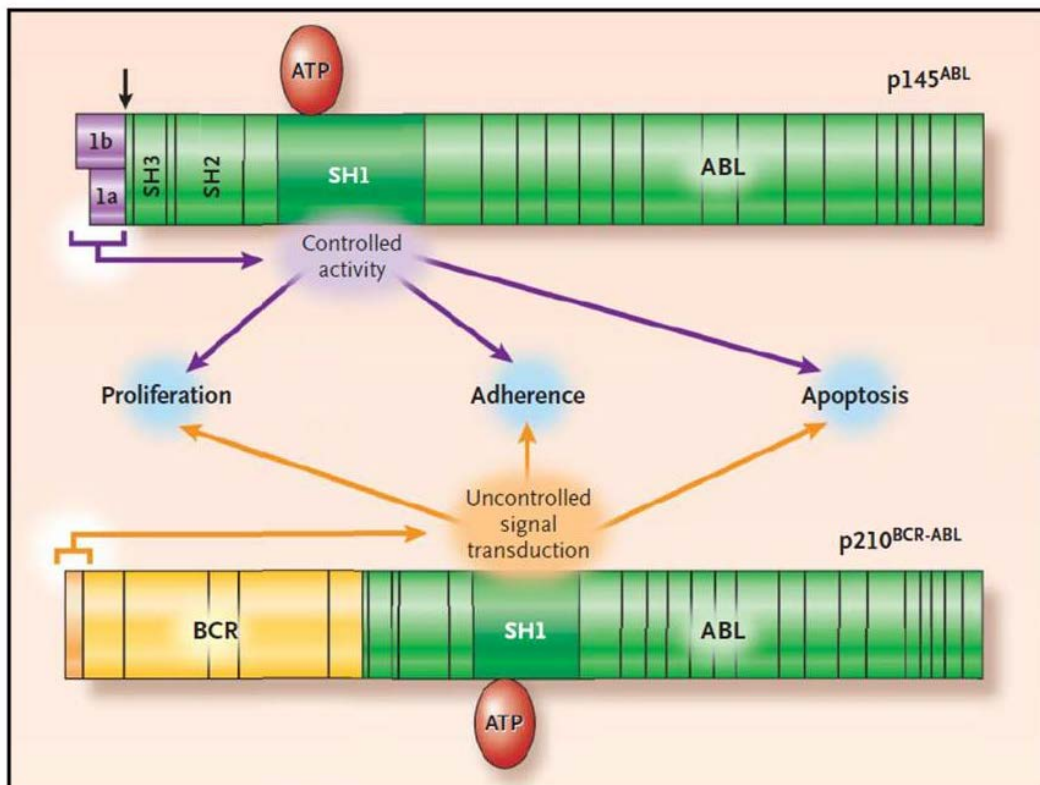


Figure 1.3: The BCR-ABL tyrosine kinase. The SH2 and SH3 domains of ABL are altered during the translocation resulting in activation of uncontrolled constitutive tyrosine kinase activity (Copied from Goldman and Melo (2003)).

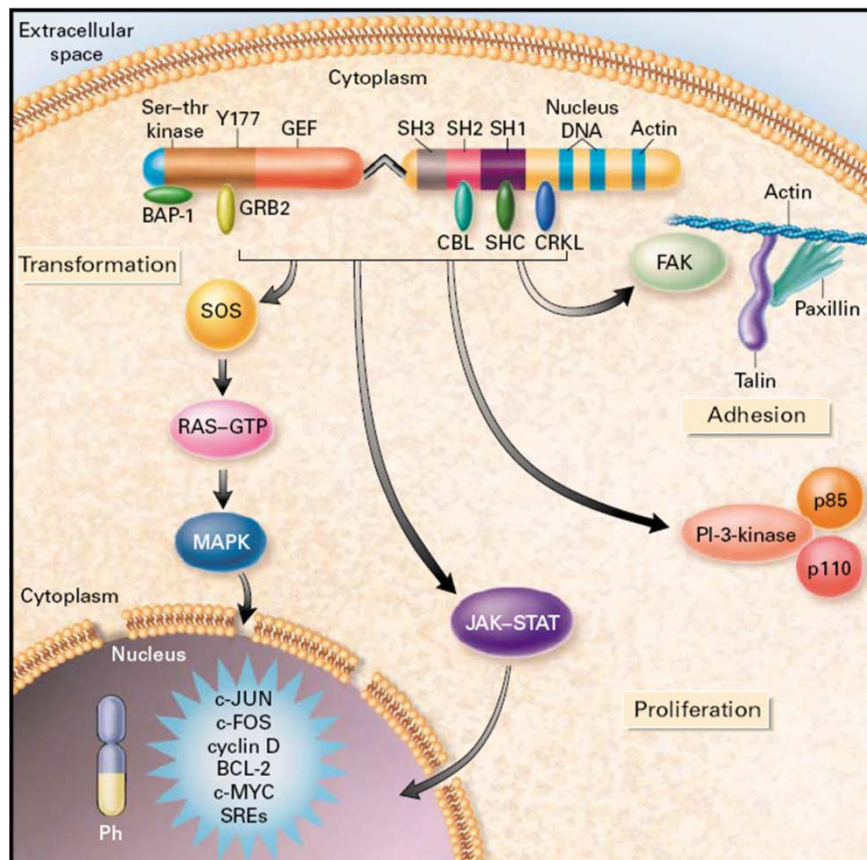


Figure 1.4: The signalling pathways of BCR-ABL. There are different signalling pathways of BCR-ABL, which lead to uncontrollable cellular progression and differentiation as well as a decrease in apoptosis (Copied from Faderl *et al.* (1999)).

1.3 Treatment and monitoring of CML

1.3.1 Treatment of CML

In the past, treatment options for CML have included allogeneic stem cell transplantation (SCT), chemotherapy with hydroxyurea or busulfan as well as interferon alpha (Sawyers, 1999; Druker *et al.*, 2001; Sawyers *et al.*, 2002; Hehlmann *et al.*, 2005). Although SCT has been used since the 1970's for the treatment of CML, it is only accessible to a small number of CML patients due to a limitation in the availability of histocompatible donors (Kantarjian *et al.*, 1999; Sawyers, 1999; Goldman and Melo, 2003). In addition to SCT, treatment with chemotherapy using hydroxyurea or busulfan has been used to control CML (Sawyers, 1999). Busulfan improves quality of life, while hydroxyurea returns blood counts to normal and reduces the size of the spleen (Baccarani *et al.*, 2006; Yokota and Kimura, 2008). In contrast, treatment with interferon alpha, a glycoprotein that has antiviral and antiproliferative properties, controls blood counts in only two thirds of CML patients. However, the side effects of interferon alpha often prevents prolonged usage (Savage and Antman, 2002; Frazer *et al.*, 2007). Unfortunately these treatments, with the exception of SCT, do not have a significant effect on survival as they are aimed at managing symptoms of the cancer and do not address the cause of CML, namely the BCR-ABL tyrosine kinase.

1.3.2 Treatment of CML with tyrosine kinase inhibitors

Tyrosine kinase inhibitors were the first examples of targeted molecular therapy for cancer and have been used as first line treatment for CML since 1998 (Nardi *et al.*, 2003; Frazer *et al.*, 2007; Melo *et al.*, 2007). Tyrosine kinase inhibitors bind to the ATP-binding site of the BCR-ABL kinase domain through several van der Waals interactions (Figure 1.5) (Schindler *et al.*, 2000; Nardi *et al.*, 2003). The interaction of the tyrosine kinase inhibitor with BCR-ABL prevents the binding of ATP and this stabilizes the kinase domain in the inactive conformation (Goldman and Melo, 2001; Nardi *et al.*, 2003). Since ATP cannot bind to the inactive kinase, normal signal transduction in the cell is restored (Nardi *et al.*, 2003; Tauchi and Ohyashiki, 2004). Although the exact mechanism of how the tyrosine kinase inhibitor reduces

levels of BCR-ABL is unknown, it appears that the inhibition of downstream signal transduction pathways suppresses the clonal proliferation of oncogenic cells and induces apoptosis.

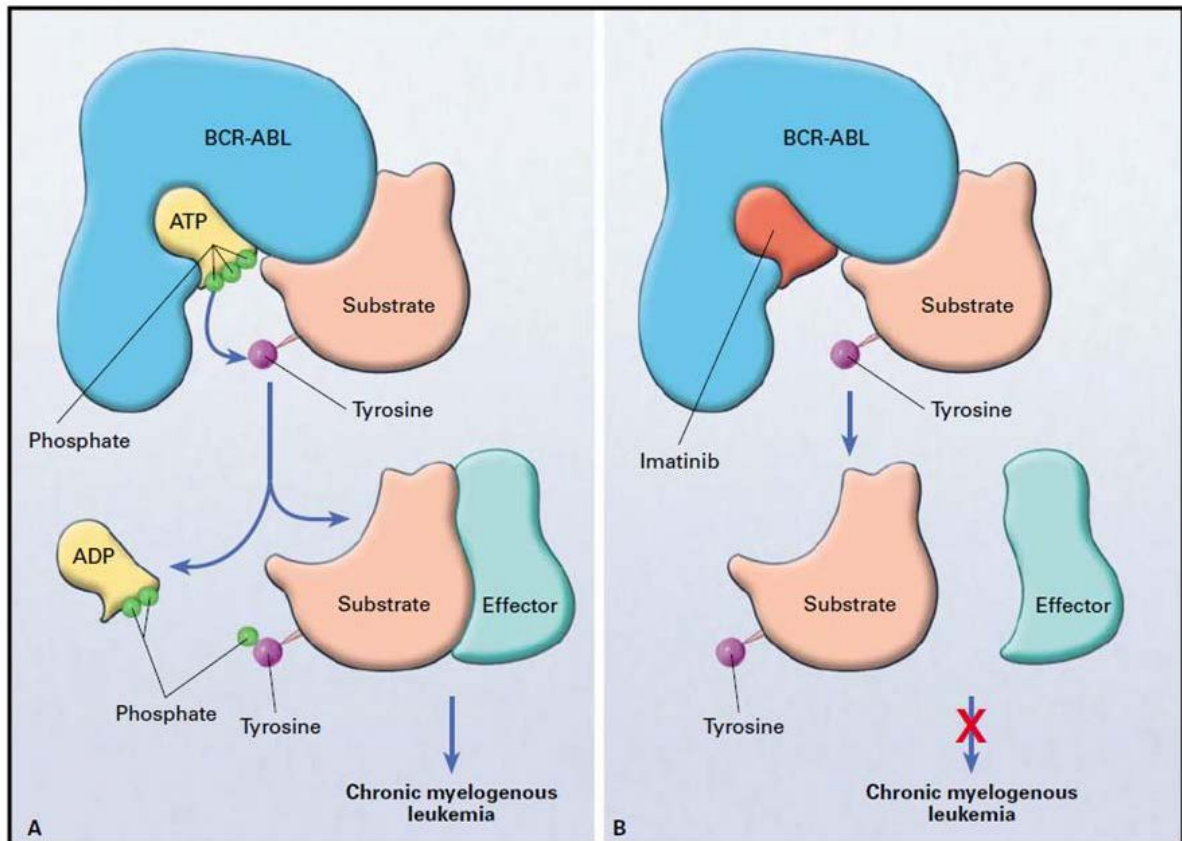


Figure 1.5: Mechanism of action of BCR-ABL and the inhibition by a tyrosine kinase inhibitor. Panel A shows the action of ATP when it is bound to BCR-ABL, resulting in activation of downstream effector molecules. Panel B illustrates the mechanism of a tyrosine kinase inhibitor such as imatinib. Imatinib binds to the ATP binding site of the BCR-ABL protein and inhibits tyrosine kinase activity thus restoring normal cellular signalling, proliferation and apoptosis (Copied from Savage and Antman (2002)).

The first generation of tyrosine kinase inhibitors, imatinib mesylate (STI571), also known as Gleevec® (Novartis, Basel, Switzerland), was the first drug designed to specifically inhibit the kinase activity of BCR-ABL. Imatinib was first identified in the early 1990's by Ciba-Geigy after a compound was identified *in vitro* to inhibit

tyrosine kinase activity and was approved in May 2001 by the Food and Drug Administration for the treatment of CML (Druker *et al.*, 2001; Gambacorti-Passerini *et al.*, 2003; Frazer *et al.*, 2007). Imatinib inhibits several other kinases including platelet-derived growth-factor receptor (PDGFR), ARG, tyrosine kinase receptors FLT1 and FLT3 and C-KIT (Gambacorti-Passerini *et al.*, 2003; O'Brien *et al.*, 2003; Peggs, 2004).

The IRIS (International Randomized Study of Interferon versus STI571) trial, a phase III study, demonstrated the effectiveness of imatinib as first line therapy for CML (O'Brien *et al.*, 2003). The IRIS trial consisted of 1,106 newly diagnosed CML patients who were randomly treated with either 400 mg/d imatinib or interferon alpha with low dose cytarabine (O'Brien *et al.*, 2003). Treatment with imatinib proved highly effective and CML patients had an overall survival of 89% after five years of follow-up (Druker *et al.*, 2006). Imatinib is administered at a dosage of 400 mg per day in the chronic phase and 600 mg to 800 mg per day in the more advanced phases (Sawyers *et al.*, 2002; Goldman, 2004; Hochhaus, 2006). Treatment response varies between patients and side-effects can include gastrointestinal disorders (nausea and vomiting), oedema, muscle cramps and diarrhoea (Druker *et al.*, 2001; Goldman, 2004; Hochhaus, 2006). Although some patients experience some side-effects due to intolerance to imatinib, the adverse effects are considerably lower compared to other drugs previously used to treat CML. In addition, approximately one third of CML patients develop resistance to imatinib (Bixby and Talpaz, 2009). Therefore, second generation tyrosine kinase inhibitors were developed to overcome problems experienced with intolerance and resistance in CML patients being treated with imatinib (Shah *et al.*, 2006; Talpaz *et al.*, 2006; Milojkovic *et al.*, 2010).

Second generation tyrosine kinase inhibitors including dasatinib and nilotinib are more potent than imatinib regarding BCR-ABL inhibition. Studies have indicated that dasatinib is approximately 300 fold more potent than imatinib, *in vitro*, with regards to BCR-ABL inhibition. Similar studies have shown that nilotinib is

approximately 20 fold more potent than imatinib, *in vitro*, concerning the inhibition of BCR-ABL (Goldman, 2007; Rix *et al.*, 2007; Jabbour *et al.*, 2009). Similar to imatinib, second generation tyrosine kinase inhibitors bind to the ATP binding site within the kinase domain. However, the basic thiazole structure of dasatinib has been modified allowing binding to either the inactive or active conformation of BCR-ABL (Frazer *et al.*, 2007; Heaney and Holyoake, 2007; Yokota and Kimura, 2008). Side-effects of second generation tyrosine kinase inhibitors vary from skin rashes and headaches to myelosuppression as well as non-haematological adverse effects, pleural effusion, hypertension and autoimmune disease (Talpaz *et al.*, 2006; Le Coutre, 2007; Rix *et al.*, 2007; Jabbour *et al.*, 2009). Similar to imatinib, some patients experience adverse effects and resistance to second generation tyrosine kinase inhibitors. Thus, third generation tyrosine kinase inhibitors are currently under development for CML patients with intolerance and/or resistance to both first and second generation tyrosine kinase inhibitors (Hehlmann *et al.*, 2011; Mathisen *et al.*, 2011). Despite the success of treating CML patients with tyrosine kinase inhibitors, patients may develop resistance, highlighting the need for patient monitoring.

A series of response criteria to treatment with a tyrosine kinase inhibitor were proposed by the European LeukemiaNet (ELN) to determine if an optimal response, suboptimal response and/or failure to treatment is present (Table 1.2) (Baccarani *et al.*, 2006). An optimal response is characterized by a complete haematological response after three months, a partial cytogenetic response after six months, a complete cytogenetic response after 12 months and a major molecular response after 18 months of treatment with a tyrosine kinase inhibitor (Table 1.2) (Baccarani *et al.*, 2009; Jabbour *et al.*, 2009; Louw *et al.*, 2011). When a patient fails to achieve an expected response within a pre-determined period, the development of a suboptimal response or failure to treatment with a tyrosine kinase inhibitor is suspected (Baccarani *et al.*, 2006; Jabbour *et al.*, 2009). Therefore, close monitoring of CML patient response plays a vital role to determine optimal treatment outcome to tyrosine kinase inhibitors (Goldman and Melo, 2003; Goldman, 2004; Frazer *et al.*, 2007; Hughes and Branford, 2009).

Table 1.2: Response criteria in chronic myeloid leukaemia (Marktel *et al.*, 2003; Druker *et al.*, 2006; Hughes *et al.*, 2006; Hughes and Branford, 2009; Mahon *et al.*, 2010).

Response	Definition
Complete haematological response (CHR)	Normalization of peripheral blood with WBC < 10x10 ⁹ /L with no immature granulocytes and with <5% basophils, platelet count < 450x10 ⁹ /L, and disappearance of all signs and symptoms of the disease.
Partial haematological response	As above, but persistent immature peripheral cells (blast, promyelocytes, myelocytes), persistent splenomegaly or thrombocytosis, but >50% reduction.
Complete cytogenetic response (CCgR)	0% Ph ⁺ metaphases on cytogenetic analysis
Partial cytogenetic response	1% to 35% Ph ⁺ metaphases on cytogenetic analysis
Minor cytogenetic response	36% to 65% Ph ⁺ metaphases on cytogenetic analysis
Minimal cytogenetic response	66% to 95% Ph ⁺ metaphases on cytogenetic analysis
Major molecular response (MMR)	> 3 log reduction of <i>BCR-ABL</i> mRNA
Complete molecular response (CMR)	Negativity by RQ-PCR
Complete molecular response 4.5 (CMR ^{4.5})	4.5 log reduction of <i>BCR-ABL</i> mRNA

1.3.3 Monitoring of CML patients

It is necessary to monitor CML patients being treated with a tyrosine kinase inhibitor in order to determine response to treatment. The aim of monitoring response to treatment with a tyrosine kinase inhibitor is to identify patients who have achieved an optimal response and to recognize a suboptimal response due to the development of acquired resistance or as a result of non-compliance (Hughes and Branford, 2009). According to the ELN recommendations, CML patients must be managed according to the response to treatment at specific time intervals using haematological, cytogenetic and molecular criteria (Table 1.3) (Baccarani *et al.*, 2006; Marin *et al.*, 2008; Hughes and Branford, 2009; Jabbour *et al.*, 2009).

Monitoring of patients using haematological, cytogenetic and molecular criteria determines the level of response of a CML patient to treatment with a tyrosine kinase inhibitor (Table 1.2). Patients are monitored by full blood counts every two weeks until a complete haematological response is achieved, followed by conventional cytogenetics FISH (fluorescence in situ hybridization) every six months until a complete cytogenetic response is reached and then by *BCR-ABL* mRNA quantification every three months until a major molecular response and/or complete molecular response is attained with monitoring for minimal residual disease thereafter (Goldman *et al.*, 2004; Baccarani *et al.*, 2006; Hughes and Branford, 2009; Jabbour *et al.*, 2009). Subsequent monitoring of CML is vital to identify loss of a response due to the possible development of acquired resistance (Baccarani *et al.*, 2006).

Monitoring levels of *BCR-ABL* mRNA has become an integral part of CML patient management since the IRIS trial in 2000 (Baccarani *et al.*, 2006). A decrease in *BCR-ABL* mRNA levels is indicative of treatment response on a molecular level and this can be linked to a haematological or cytogenetic response as well (Branford and Hughes 2006; Jabbour *et al.*, 2009; Foroni *et al.*, 2011). Real-time quantitative polymerase chain reaction (RQ-PCR) is currently considered the most

sensitive method for monitoring CML as it has been shown to predict the likelihood of relapse (Wang *et al.*, 2006; Frazer *et al.*, 2007; Volpe *et al.*, 2009). The loss of an achieved response such as a major molecular response (MMR) indicates the presence of resistance development or can be the result of non-compliance. It is recommended that when a patient experiences a loss of an achieved response, mutational screening of the kinase domain should be performed to determine if resistance is present (Branford, 2007; Hughes and Branford, 2009).

Table 1.3: Definitions of a suboptimal response and failure to treatment with a tyrosine kinase inhibitor in CML patients (Druker *et al.*, 2006; Marin *et al.*, 2008; Baccarani *et al.*, 2009; Hughes and Branford, 2009; Jabbour *et al.*, 2009).

Assessment method	Initial monitoring	Subsequent monitoring	Suboptimal response	Failure
Haematological	Every two weeks	Every three months once CHR is achieved	Less than CHR within three to six months	No haematological response within three to six months Loss of CHR at any time
Cytogenetic	At three months, then six months	Every six months once CCgR is achieved followed by every 12 months	No CCgR at 3 months Less than PCgR within six months No PCgR within 12 months	No cytogenetic response within six months Less than a PCgR within 12 months Less than a CCgR within 18 months Loss of CCgR at any time
Molecular	Every three months	Every three months, monthly if <i>BCR-ABL</i> mRNA levels increase	Less than MMR within 18 months Loss of MMR at any time	Not applicable

CCgR: Complete cytogenetic response

CHR: Complete haematological response

MCgR: Major cytogenetic response

MMR: Major molecular response

PCgR: Partial cytogenetic response

1.4 Resistance to tyrosine kinase inhibitors

Despite the success of tyrosine kinase inhibitors, 30% of CML patients experience resistance to treatment (Hughes and Branford, 2009). Primary resistance is defined as the failure to achieve a significant haematological, cytogenetic or molecular response from the start of treatment with a tyrosine kinase inhibitor (Table 1.3) (Gambacorti-Passerini *et al.*, 2003; Branford *et al.*, 2003; Wei *et al.*, 2006; Melo and Chuah, 2007; Diamond and Melo, 2011). Development of primary resistance can be as a result of several factors including defects in the P-glycoprotein export protein and/or hOCT1 protein and/or α 1-acid glycoprotein. (Gambacorti-Passerini *et al.*, 2000; Mahon *et al.*, 2000; Thomas *et al.*, 2004; Bixby and Talpaz, 2009). Acquired resistance is defined as the loss of a haematological, cytogenetic or molecular response and/or the progression to the accelerated phase and/or blast crisis (Branford *et al.*, 2002; von Bubnoff *et al.*, 2002; Branford *et al.*, 2003; Melo and Chuah, 2007; Diamond and Melo, 2011).

One of the primary reasons for CML patients treated with a tyrosine kinase inhibitor developing acquired resistance is due to single base mutations in the BCR-ABL kinase domain. Single base mutations in *BCR-ABL* in the region of the kinase domain alter the amino acid sequence of BCR-ABL changing the conformation of the kinase domain (Branford *et al.*, 2003; Goldman *et al.*, 2004; Frazer *et al.*, 2007; Ernst *et al.*, 2008). Single base mutations in the kinase domain either reduce the binding of a tyrosine kinase inhibitor, resulting in partial resistance or directly prevent the binding of a tyrosine kinase inhibitor resulting in absolute resistance (Branford *et al.*, 2003; Branford and Hughes, 2006).

There are currently approximately 100 known single base mutations in the BCR-ABL kinase domain of which approximately 70 have prognostic value (Figure 1.6) (Wei *et al.*, 2006; Branford, 2007; Ernst *et al.*, 2008; Volpe *et al.*, 2009; Hanfstein *et al.*, 2011). Single base mutations have been found to occur in all the regions of the BCR-ABL kinase domain including the P-loop, the gatekeeper domain (tyrosine kinase inhibitor binding site), the catalytic domain and the activation loop (Corbin *et al.*, 2003; Wang *et al.*, 2006; Cowen-Jacob *et al.*, 2007).

The position of a mutation within the BCR-ABL kinase domain often determines the severity of its effect:

- The P-loop (ATP phosphate binding loop) is a conserved glycine-rich region of the kinase domain and interacts directly with a tyrosine kinase inhibitor through hydrogen bonds and van der Waal's forces (Schindler *et al.*, 2000; Hochhaus *et al.*, 2002). Single base changes G250E, Q252F/H, M244V and L248V modify the flexibility of the P-loop, destabilizing the conformation of the kinase domain required for tyrosine kinase inhibitor binding with resistance to treatment as a result (Shah *et al.*, 2002; Branford *et al.*, 2003). Depending on the type of mutation resulting in partial resistance, it can be overcome with a dose increase of imatinib or by using a tyrosine kinase inhibitor with higher potency such as nilotinib or dasatinib (Branford *et al.*, 2003; Nardi *et al.*, 2003; Branford, 2007; Hehlmann *et al.*, 2007; Volpe *et al.*, 2009). Studies have indicated that patients who have single base changes, E255K and Y253H, in the P-loop have a poor prognosis, when second generation tyrosine kinase inhibitors are not available, since these mutations result in absolute resistance to imatinib (Branford *et al.*, 2003; Volpe *et al.*, 2009). However, single base mutations E255K and Y253H can be treated with the second generation tyrosine kinase inhibitor such as dasatinib (Diamond and Melo, 2011).
- Single base mutations in the gatekeeper domain directly impair binding of a tyrosine kinase inhibitor to the BCR-ABL kinase domain. Several studies have indicated that the T315I mutation in the gatekeeper domain confers absolute resistance to tyrosine kinase inhibitors imatinib, nilotinib and dasatinib (Gorre *et al.*, 2001; Chomel *et al.*, 2009). In normal BCR-ABL, threonine at position 315 forms a hydrogen bond between the tyrosine kinase inhibitor and BCR-ABL, which sterically controls the binding of the tyrosine kinase inhibitor to the kinase domain (Schindler *et al.*, 2000; Branford *et al.*, 2002; Soverini *et al.*, 2006). However, when a mutation occurs at amino acid 315, an isoleucine is encoded in the place of threonine, preventing the bond between the tyrosine kinase inhibitor and

BCR-ABL from forming and resulting in absolute resistance (Gorre *et al.*, 2001; Gambacorti-Passerini *et al.*, 2003; Yamamoto *et al.*, 2004). Third generation tyrosine kinase inhibitors are being developed for treatment of resistance due to the T315I mutation. However, these tyrosine kinase inhibitors are currently still in clinical development and therefore alternative treatments to tyrosine kinase inhibitors are suggested (Hehlmann *et al.*, 2007; Hehlmann *et al.*, 2011; Mathisen *et al.*, 2011).

- The activation loop consists of a highly conserved motif of three amino acid residues that can occur in an active or inactive conformation (Schindler *et al.*, 2000; Shah *et al.*, 2002; Melo and Chuah, 2007). Mutations in the activation loop change the stabilization of the kinase domain resulting in an active conformation that a tyrosine kinase inhibitor is unable to bind to (Shah *et al.*, 2002). Examples of single base mutations in the activation loop that result in partial resistance to tyrosine kinase inhibitors include Y393F, H396R and L387M. These mutations can be treated with a dose increase of imatinib or alternative tyrosine kinase inhibitors such as nilotinib or dasatinib (Hochhaus *et al.*, 2002; Roumiantsev *et al.*, 2002; Shah *et al.*, 2002; Corbin *et al.*, 2003; Hehlmann *et al.*, 2007; Volpe *et al.*, 2009).
- The catalytic domain has a close topographic involvement with the activation loop and stabilizes the activation loop in an inactive conformation required for binding of a tyrosine kinase inhibitor (Schindler *et al.*, 2000; Shah *et al.*, 2002; Melo and Chuah, 2007). Single base mutations including S417Y/F, E459K/Q and F486S in the catalytic domain influence the binding of the tyrosine kinase inhibitor resulting in partial resistance (Branford *et al.*, 2003; Gambacorti-Passerini *et al.*, 2003; Melo and Chuah, 2007). These single base mutations can be treated with a dose increase of imatinib or by using second generation tyrosine kinase inhibitors nilotinib or dasatinib (Branford *et al.*, 2003; Nardi *et al.*, 2003; Branford, 2007; Hehlmann *et al.*, 2007; Volpe *et al.*, 2009).

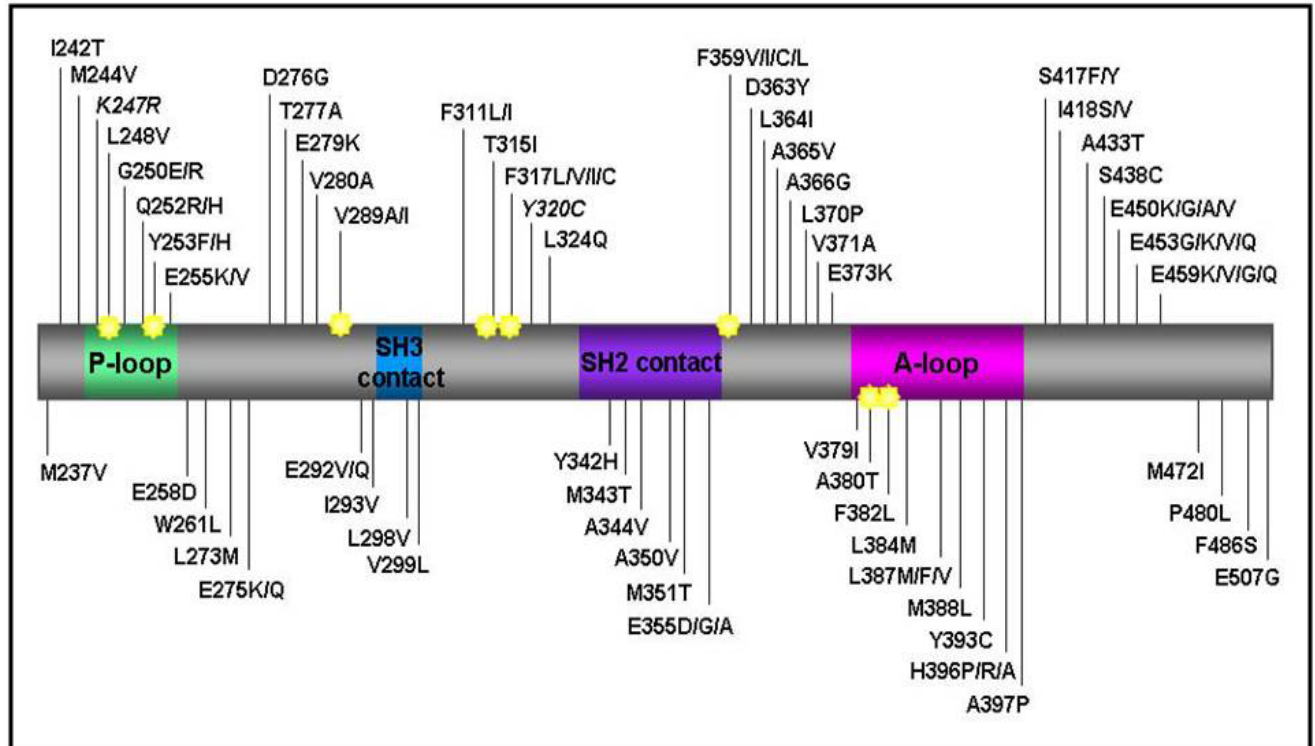


Figure 1.6: Single base mutations in the BCR-ABL kinase domain that are associated with resistance to treatment with a tyrosine kinase inhibitor. The BCR-ABL kinase domain consists of four different domains namely the P-loop, the gatekeeper domain, the catalytic domain and the activation loop. Mutations found in these domains result in either partial or absolute resistance (Copied from Soverini *et al.* (2011))

Although single base mutations in the BCR-ABL kinase domain are considered the primary cause of resistance to tyrosine kinase inhibitor, insertions, deletions and duplications are also being reported in the literature (Melo and Chuah, 2007; Sherbenou *et al.*, 2008; Hanfstein *et al.*, 2011). Studies by Laudadio *et al.* (2008), Sherbenou *et al.* (2008) and Ma *et al.* (2009) suggest that insertions and deletions in the BCR-ABL kinase domain can result in partial resistance to treatment with a tyrosine kinase inhibitor. For example, a 35 bp insertion in the BCR-ABL kinase domain results in a splice variant that has conformational changes in the C-terminal of the kinase domain. The 35 bp insertion results in a truncated form of the BCR-ABL kinase domain that may also result in absolute resistance to tyrosine kinase inhibitor (Laudadio *et al.*, 2008; Quigley *et al.*, 2008; Bixby and Talpaz, 2009; Ma *et al.*, 2009). However, based on research using an *in vitro* system, O'Hare *et al.* (2011) suggested that resistance associated with the 35 bp insertion

is due to other mutations and does not on its own confer resistance to tyrosine kinase inhibitor. It has been suggested that deletions in the central parts of the kinase domain involving amino acids 184 to 274, 248 to 274 and 362 to 444 are thought to result in an inactive kinase due to the loss of the activation loop and catalytic domain. A study by Sherbenou *et al.* (2008) suggested that there is no association between the patient's response to treatment with a tyrosine kinase inhibitor and the presence of Δ 248 to 274 and Δ 184 to 274 in the kinase domain. However, they found that when the deletion variant occurs in conjunction with a single base mutation L248V, in the BCR-ABL kinase domain, the patient experienced partial resistance to treatment with a tyrosine kinase inhibitor (Sherbenou *et al.*, 2008; Ma *et al.*, 2009; Gruber *et al.*, 2011). The impact that other deletion variants in the kinase domain might have on the treatment with tyrosine kinase inhibitors is currently unknown. However, deletion variants in the kinase domain are being identified in a significant percentage of patients experiencing resistance to tyrosine kinase inhibitors. Furthermore, studies by Le Coutre *et al.* (2000) and Gorre *et al.* (2001) demonstrated that duplications in BCR-ABL influenced the tyrosine kinase activity both *in vitro* and *in vivo*. They suggested that duplications in BCR-ABL could result in acquired resistance to tyrosine kinase inhibitor, although the mechanism thereof is unknown (Litz *et al.*, 1993; Le Coutre *et al.*, 2000; Gorre *et al.*, 2001; Saglio *et al.*, 2002). Thus, the role of insertions, deletions and duplications in the BCR-ABL kinase domain in the development of resistance towards tyrosine kinase inhibitors is not clearly understood. Therefore, detection and characterization of insertions, deletions and duplications in *BCR-ABL* in the region of the kinase domain is important, since these mutations may possibly result in resistance to tyrosine kinase inhibitors impacting the patient's response to treatment.

1.5 Methods of mutation detection

Several methods have been described to detect mutations in *BCR-ABL* in the region of the kinase domain. Methods include enhanced polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), denaturing high-performance liquid chromatography (DHPLC) and allele-specific oligonucleotide -

polymerase chain reaction (ASO-PCR) (Liu *et al.*, 2003; Willis *et al.*, 2005; Ernst *et al.*, 2008). Enhanced PCR-RFLP analysis involves the restriction digestion of a specific PCR segment of DNA using restriction enzymes where the loss of the restriction site indicates the presence of a mutation (Lui and Cordes, 2004). PCR-RFLP is a relatively inexpensive and simple technique but is not suitable to detect all mutations in *BCR-ABL* in the region of the kinase domain and as a result is not routinely used. Lui and Markrigiorgos (2003) used this approach to detect the T315I and Y253F mutations in *BCR-ABL* in the region of the kinase domain. DHPLC involves the separation of heterozygous and homozygous sequence alterations in a solution over a resin column (Lui *et al.*, 1998; Sivakumaran *et al.*, 2003). Heterozygous sequence alterations move more slowly through a column than that of homozygous sequence changes, thus through the elution profile the presence of mutations can be determined. A study by Ernst *et al.* (2008) used DHPLC to detect 22 known single base mutations in *BCR-ABL* in the region of the kinase domain. However, a drawback of this specialized technique is that routine DHPLC does not always detect homozygous sequence alterations and therefore some mutations can be missed. ASO-PCR involves conventional PCR with the use of allele specific PCR primers that are complementary to either the wild-type or mutation at the 3' end (Twyman, 2005). Willes *et al.* (2005) used ASO-PCR to detect eight common mutations including E255K and M351T in *BCR-ABL* in the region of the kinase domain. However, the assay is considered to be too sensitive and can result in false positives that are not clinically relevant (Willis *et al.*, 2005; Jones *et al.*, 2009).

The most commonly used technique to currently detect mutations is DNA sequencing (Branford *et al.*, 2002; Branford *et al.*, 2003). Sequencing is used to identify single base mutations, insertions and deletions in *BCR-ABL* in the region of the kinase domain (Hochhaus *et al.*, 2002; Laudadio *et al.*, 2008; Sherbenou *et al.*, 2008). However, a drawback of sequencing is that the technique is costly as well as time-consuming since it requires the sequencing of the entire region of the kinase domain of approximately 900 bp (Branford *et al.*, 2003). The ELN recommends sequencing of the region of the kinase domain in cases of a suboptimal response to treatment with a tyrosine kinase inhibitor and/or the loss of

an achieved response (Baccarani *et al.*, 2006; Branford, 2007; Hughes and Branford, 2009). A suboptimal response includes a lack of achieving a complete haematological or cytogenetic response at three months, a less than a partial cytogenetic response at six months, lack of a partial cytogenetic response at 12 months and a lack of a major molecular response at 18 months (Table 1.3) (Baccarani *et al.*, 2009). In addition to this, a tenfold increase in the *BCR-ABL* mRNA level is considered indicative of a mutation in *BCR-ABL* in the region of the kinase domain (Baccarani *et al.*, 2006). However, a suboptimal response to tyrosine kinase inhibitor may also be due to non-compliance to treatment. This makes mutation monitoring expensive since the region of *BCR-ABL* encoding for the kinase domain may be sequenced unnecessarily. Therefore, it would be more cost-effective to screen the kinase domain for mutations prior to sequencing (Wang *et al.*, 2006; Ernst *et al.*, 2008).

A technique which has the potential to screen DNA for mutations is high-resolution melting (HRM) (Reed and Wittwer, 2004). HRM is based on the principle that mutational changes in a nucleic acid will alter the melting profile of that sequence compared to the wild-type (Ririe *et al.*, 1997; Reed and Wittwer, 2004). HRM follows normal PCR amplification of the region of interest in the presence of an intercalating double-stranded DNA-binding dye (Herrmann *et al.*, 2006; Taylor, 2009). Following amplification, the double-stranded DNA is denatured with increasing temperature increments over time. During dissociation of the double-stranded DNA, a melting profile is generated based on the reduction in fluorescence as the intercalating double stranded DNA-binding dye is released (Ririe *et al.*, 1997; Gundry *et al.*, 2003; Jeong *et al.*, 2007; Poláková *et al.*, 2008; Doi *et al.*, 2009). Each melting profile for a specific DNA segment has a unique melting temperature (T_m) which is defined as the point in the melt curve where 50% of the DNA is double-stranded (Reed *et al.*, 2007). Each segment of DNA has a characteristic T_m which is dependent on several factors including guanine/cytosine (GC) content and the length of the fragment (Herrmann *et al.*, 2006; Seipp *et al.*, 2007; Taylor, 2009). While studies have shown that differences in T_m between samples can be indicative of a mutation, some mutations do not result in a sufficient shift in T_m to be detected. For example, studies have shown

that changes from A to T and C to G do not result in a sufficient difference in T_m to indicate a mutation (Liew *et al.*, 2004; Reed *et al.*, 2007; Gundry *et al.*, 2008).

Gundry *et al.* (2003) suggested the use of difference plots to detect the presence of mutations following HRM. A difference plot is used to exaggerate the difference between melting profile of a sample compared to a wild-type (Krypuy *et al.*, 2006; Tajiri-Utagawa *et al.*, 2008). Prior to generating a difference plot, the normal variation in fluorescence and temperature between different sample melting profiles needs to be corrected in order to standardize the interpretation of results (Krypuy *et al.*, 2006). Variation in normal fluorescence between samples is due to factors such as differing levels of amplicon and is corrected through a process of normalization between the pre-melt and post-melt region on the normalization plot (Figure 1.7) (Taylor, 2009). Normal temperature variation is due to temperature offsets between samples that are not due to mutations and can be excluded by a process known as temperature shifting (Taylor, 2009). The difference plot is a graphical representation of the difference between melting profiles of a sample compared to a wild-type, over a temperature range (Krypuy *et al.*, 2006; Taylor, 2009). Thus, samples with similar melting profiles are grouped as the same variant, on the difference plot, compared to samples with mutations which are grouped as different variants (Figure 1.8) (Wittwer *et al.*, 2003; Herrmann *et al.*, 2006; Seipp *et al.*, 2007; Taylor, 2009).

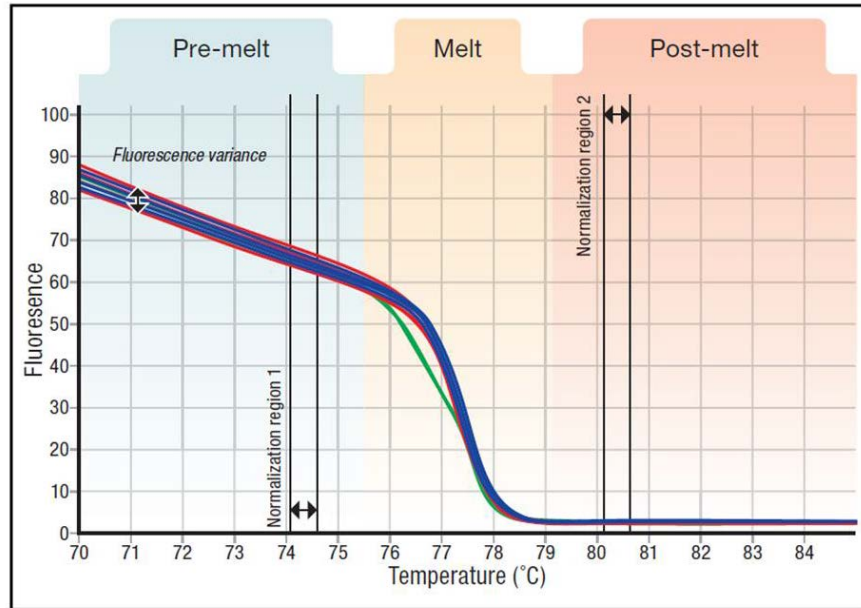


Figure 1.7: Example of selection of the pre-melt and post-melt regions in the generation of the difference plot. Pre-melt and post-melt regions are selected for each primer set and the raw melt curve data are normalized to eliminate fluorescence variation (Copied from KAPABIOSYSTEMS version 1.10).

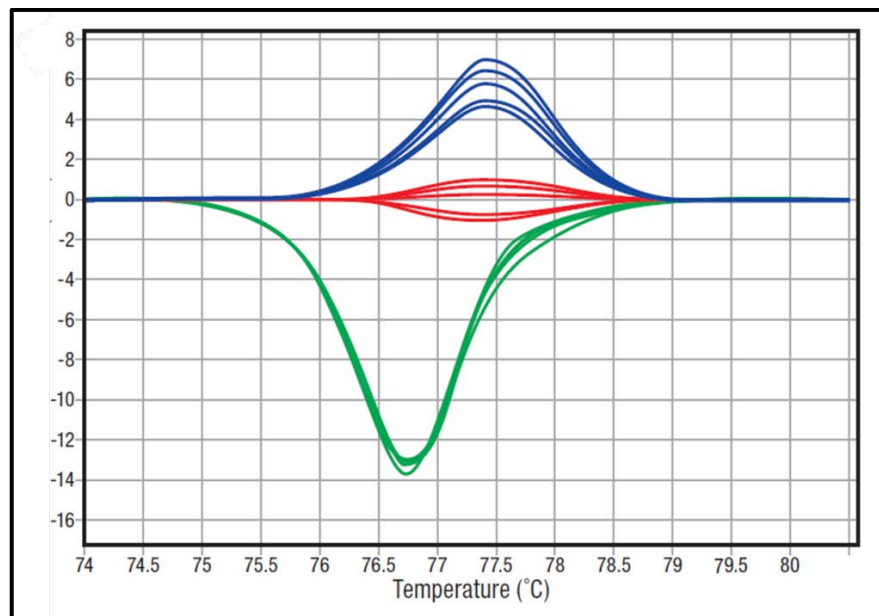


Figure 1.8: Example of results from high-resolution melting. Samples in blue or green deviate away from the wild-type (red), indicating the presence of a potential mutation (Copied from KAPABIOSYSTEMS version 1.10).

There are several considerations for the use of HRM including amplicon length, the absence of non-specific amplification as well as the detection dye used. The recommended amplicon length for HRM is between 100 and 300 bp (Taylor, 2009). It is recommended to use a shorter amplicon to ensure that there is only one melting domain (Wittwer *et al.*, 2003). However, studies have shown that HRM can successfully be performed using amplicons of up to 1,000 bp, with a sensitivity and specificity of 97% and 99%, respectively, compared to sequencing (Reed and Wittwer, 2004; Reed *et al.*, 2007; Taylor, 2009). Furthermore, the use of saturating fluorescent double-stranded DNA dyes such as LCGreen is preferred above the use of non-saturating dyes (Montgomery *et al.*, 2007; Reed *et al.*, 2007; Erali *et al.*, 2008).

Several studies have used HRM to identify the break-point cluster region in *BCR* as well as detect the presence of single base mutations in *BCR-ABL* in the region of the kinase domain (Wittwer *et al.*, 2003; Gutiérrez *et al.*, 2005; Poláková *et al.*, 2008; Doi *et al.*, 2009). Gutiérrez *et al.* (2005) used HRM to determine the type of *BCR-ABL* transcript (b2a2 or b3a2) in terms of the break-point cluster region in *BCR*. In 2008, Poláková *et al.* used HRM to screen the region of *BCR-ABL* encoding for the kinase domain for single base mutations. Poláková *et al.* (2008) used PCR amplicon, spanning the kinase domain, as template for HRM. A study by Doi *et al.* (2009) also used the method of Poláková *et al.* (2008) to detect single base mutations in the region of *BCR-ABL* encoding for the kinase domain. However, Doi *et al.* (2009) used the PCR amplicon from four sub-sections of the kinase domain as template for HRM. They also spiked assays with wild-type DNA to exaggerate the difference in the melting curve through the use of heteroduplex formation. To date HRM has not been used to screen for insertions, deletions or duplications in *BCR-ABL*.

Several studies have used HRM to detect tandem duplications, deletions or insertions in genes (Vaughn and Elenitobe, 2004; Tan *et al.*, 2008; Heideman *et al.*, 2009; Rouleau *et al.*, 2009). Vaughn and Elenitobe-Johnson (2004) used HRM to detect tandem duplications in *FLT3*, a gene important in the growth of

hematopoietic progenitors, in 13 samples with results having a 100% concordance to sequencing. These results were confirmed by Pan *et al.* (2008) in patients with acute myeloid leukaemia. In a study by Rouleau *et al.* (2009), HRM was used to detect five deletion events in addition to single base mutations in the *MHL1* gene, responsible for the development of Lynch syndrome. In 2009, a study by Heideman *et al.* reported the use of HRM in detecting sequence insertions responsible for cancer in *k-ras*, a gene involved in signal transduction. Therefore, HRM is considered to have the potential to screen and detect not only single base mutations, but also insertions, deletions or duplications in the region of *BCR-ABL* encoding for the kinase domain.

1.6 Conclusions

CML is a haematological malignancy due to the reciprocal translocation between chromosomes 9 and 22 resulting in the *BCR-ABL* oncogene. *BCR-ABL* affects signalling pathways that results in uncontrolled proliferation of poorly differentiated *BCR-ABL* cells that have a decreased ability to respond to external stimuli, leading to a loss of regulation and inhibition of apoptosis.

The treatment of choice for CML is a tyrosine kinase inhibitor since *BCR-ABL* kinase activity is decreased and normal cellular function is restored. Nevertheless, some patients experience a suboptimal response to treatment with a tyrosine kinase inhibitor either due to non-compliance or resistance to treatment. Resistance is primarily due to single base mutations in the *BCR-ABL* kinase domain. However, there has been an increase in reports of insertions, deletions and duplications associated with the *BCR-ABL* kinase domain. Although the effect of insertions, deletions and duplications in the *BCR-ABL* kinase domain on patient prognosis is not fully understood, it has been suggested that these mutations can result in acquired resistance. Thus, for effective management of CML treatment, it is becoming increasingly important to detect not only single base mutations but also insertions, deletions and duplications in *BCR-ABL* in the region of the kinase domain.

While DNA sequencing is very effective in detecting mutations in *BCR-ABL* in the region of the kinase domain, the technique is costly and time consuming. Furthermore, unnecessary sequencing of samples with a suboptimal response due to non-compliance to treatment and not mutations in the BCR-ABL kinase domain increases the cost of mutational screening. Therefore, it would be ideal to screen for mutations in *BCR-ABL* in the region of kinase domain in a cost-and time-efficient manner, prior to sequencing, to exclude samples without mutations. Recently, high-resolution melting (HRM) has been shown by Poláková *et al.* (2008) and Doi *et al.* (2009) to screen *BCR-ABL* in the region of kinase domain for single base mutations, prior to sequencing. Based on current HRM literature, the assay has the potential to screen for not only the presence of single base mutations but insertions, deletions or duplications in *BCR-ABL* in the region of the kinase domain as well. Screening for mutations in *BCR-ABL* in the region of the kinase domain with HRM, may reduce the number of samples for sequencing as only samples with potential mutations will be sequenced to characterize the mutation.

CHAPTER TWO

MUTATIONAL SCREENING OF THE REGION OF *BCR-ABL* ENCODING FOR THE KINASE DOMAIN

2.1 Introduction

CML is a clonal myeloproliferative disorder that is characterized by the *BCR-ABL* fusion oncogene which encodes a constitutively active tyrosine kinase (Goldman and Melo, 2003). Tyrosine kinase inhibitors are currently considered the standard first line treatment for CML (Druker *et al.*, 2001; Sawyers *et al.*, 2002). Monitoring of CML patients being treated with a tyrosine kinase inhibitor is necessary in order to determine patient response to treatment and to identify when a suboptimal response is present. Monitoring of CML patients is therefore important so that the treatment regime with a tyrosine kinase inhibitor can be adjusted to achieve an optimal response (Hughes and Branford, 2009).

ELN has published guidelines for the assessment of patient response to treatment with a tyrosine kinase inhibitor (Baccarani *et al.*, 2006). These guidelines are based on haematological, cytogenetic and molecular response criteria (Baccarani *et al.*, 2006; Marin *et al.*, 2008; Hughes and Branford, 2009; Jabbour *et al.*, 2009). Unfortunately, some patients experience a suboptimal response and/or failure to treatment with a tyrosine kinase inhibitor (Jabbour *et al.*, 2009; Groves *et al.*, 2011; Jabbour *et al.*, 2011). A suboptimal response and/or failure to treatment with a tyrosine kinase inhibitor is suspected when the expected response is not achieved in the predetermined time period (Baccarani *et al.*, 2006). A lack of response could be a result of either non-compliance to treatment or acquired resistance due to mutations in the *BCR-ABL* kinase domain.

The development of acquired resistance to tyrosine kinase inhibitors is mainly due to single base mutations in the *BCR-ABL* kinase domain. Single base mutations in

the BCR-ABL kinase domain alter the conformation of the kinase domain by either preventing or reducing the binding of the tyrosine kinase inhibitor (Hochhaus *et al.*, 2002; Shah *et al.*, 2002; von Bubnoff *et al.*, 2002). Although single base mutations are considered the primary cause of resistance to a tyrosine kinase inhibitor, insertions, deletions and duplications associated with the BCR-ABL kinase domain are also being reported in literature (Melo and Chuah, 2007; Sherbenou *et al.*, 2008; Hanfstein *et al.*, 2011; Wienand *et al.*, 2011). However, the clinical impact of insertions, deletions and duplications in the kinase domain on patient response to a tyrosine kinase inhibitor is not well understood. Therefore, the detection and characterization of mutations in *BCR-ABL* in the region of the kinase domain is important for treatment management, since the type and position of the mutation may affect patient response to treatment with a tyrosine kinase inhibitor (Ernst *et al.*, 2008; Jabbour *et al.*, 2009). It is therefore recommended that mutational screening of the region of *BCR-ABL* encoding for the kinase domain needs to be performed in cases of a suboptimal response to a tyrosine kinase inhibitor (Baccarani *et al.*, 2006; Branford, 2007; Hughes and Branford, 2009). The aim of this study was to report on sequencing of the region of *BCR-ABL* encoding for the kinase domain of CML patients being treated with a tyrosine kinase inhibitor to detect single base mutations, insertions, deletions or duplications.

2.2 Materials and methods

CML patients for this study were selected from a group of approximately 100 individuals being treated for CML at the Haematology Clinic in Bloemfontein. The selected patients were being treated with tyrosine kinase inhibitors and had been requested for mutational sequencing analysis by the treating physician due to a lack of response to treatment and/or increasing levels of *BCR-ABL* mRNA. Informed consent was obtained under existing ethics approval (ETOVS 32/07) with Prof Viljoen as principal investigator and of which this project forms an addendum. After obtaining informed consent, peripheral blood was collected in EDTA tubes from the selected 40 CML patients for mutational screening of the kinase domain. Each blood sample received a unique number, which is specific to that patient and

acted as an identifier to anonymize the sample. Due to the patients being selected from the group of approximately 100 individuals, patient sample numbers do not necessarily occur in numerical order.

2.2.1 Trizol stabilization

Approximately 20 ml of peripheral blood was treated with 50 ml of lysis buffer (0.144 M NH_4Cl and 0.01 M NH_4HCO_3 , pH 7.4). Samples were incubated for approximately 10 minutes at room temperature followed by centrifugation at 3,500 rpm for 10 minutes. The supernatant was discarded and an additional 25 ml of lysis buffer was added, followed by incubation for 5 minutes at room temperature. The sample was then centrifuged at 3,500 rpm for 10 minutes. The supernatant was discarded and the remaining white cell pellet dissolved in 1.6 ml Trizol reagent (Invitrogen). Additional Trizol was added if necessary, up to a maximum of 5 ml to ensure that the cell pellet was fully dissolved. The white blood cells were mixed by pipetting until the solution was homogenous. The sample was subsequently stored at -70°C .

2.2.2 RNA extraction

RNA extraction was performed according to a modified method of Branford and Hughes (2006) in a dedicated area where all equipment was treated with DEPC and RNase-Zap to prevent RNase degradation. The method of Branford and Hughes (2006) was modified to ensure that maximum amount of quality RNA was extracted with minimal degradation under the conditions of our laboratory. For each extraction, a standard amount of 1.6 ml of Trizol homogenate was thawed at room temperature followed by the addition of 20 mg/ml proteinase K. The sample was then incubated at 65°C for 20 minutes with mixing by inversion every 5 minutes. Chloroform (350 μl) was added to the sample followed by vortexing for 15 seconds and incubation on ice for 3 minutes. The sample was subsequently centrifuged at 12,000 rpm for 15 minutes and approximately 1 ml of the aqueous upper phase was retained to which 1 ml cold isopropanol was added. The RNA was precipitated on ice for 30 minutes, followed by centrifugation at 12,000 rpm for

10 minutes. The supernatant was discarded and the remaining RNA pellet was washed with 1 ml 75% ethanol, followed by centrifugation at 10,000 rpm for 10 minutes. The wash step was repeated and the RNA pellet was air dried for approximately 15 minutes after which it was re-dissolved in 50 μ l DEPC treated water at 55°C for 15 minutes followed by incubation on ice for 1 minute. The concentration of RNA was determined using the Qubit Fluorometer (Invitrogen) and stored at -70°C.

2.2.3 cDNA synthesis

cDNA synthesis was performed according to a modified method of Branford and Hughes (2006) using the Fermentas Revert-Aid MMLV kit and a standard amount of 2 μ g RNA per reaction. The method of Branford and Hughes (2006) was modified in order to produce quality cDNA with minimal degradation under the conditions of our laboratory. The RNA/Primer mix was prepared containing 2 μ g RNA, 50 ng/ μ l random hexamers and DEPC treated water to a final volume of 11 μ l. The RNA/Primer mixture was incubated at 70°C for 5 minutes followed by incubation on ice for 1 minute. A cDNA synthesis cocktail was prepared containing 5 X RT-buffer, 10 mM MgCl₂, 10 mM dNTP mix, 0.1 M DTT, 40 U/ μ l Riboblock and 200 U/ μ l M-MuLV RT, of which 12 μ l was added to each RNA/Primer mix. The cDNA synthesis/RNA primer mix was incubated for 10 minutes at room temperature followed by 65 minutes at 42°C. The cDNA synthesis reaction was terminated by heating to 70°C for 10 minutes followed by cooling on ice for 2 minutes. Thereafter, 10 U/ μ l RNase-H was added to each sample and incubated at 30°C for 20 minutes. The cDNA was stored at 4°C.

2.2.4 DNA sequencing of the region of *BCR-ABL* encoding for the kinase domain

Semi-nested polymerase chain reaction (PCR) was performed to amplify the region of *BCR-ABL* encoding for the kinase domain, prior to sequencing. The first PCR was used to amplify a segment of DNA spanning *ABL* including the region of the kinase domain of *BCR-ABL*. The amplicon of the first PCR was then used as

template in a second semi-nested PCR reaction to amplify the region of *BCR-ABL* encoding for the kinase domain.

Table 2.1: Primers (Branford and Hughes, 2006) for semi-nested PCR to amplify the region of *BCR-ABL* encoding for the kinase domain.

Primer	Primer sequence (5' to 3')
Forward Primer 1 (1 st PCR)	TGACCAACTCGTGTGTGAAACTC
Forward Primer 2 (2 nd PCR)	CGCAACAAGCCCACTGTCT
Reverse Primer (1 st and 2 nd PCR)	AATCCAGTATCTCAGACGAAGTGGA

PCR reactions (final volume 25 μ l) contained: 10 X PCR Gold Buffer, 25 mM MgCl₂, 5 U/ μ l *AmpliTaq Gold*, 10 mM dNTPs, 10 pmol/ μ l forward primer 1 and reverse primer (Table 2.1), respectively, and cDNA (2 μ g RNA). The PCR cycling conditions were as follows: 94°C for 10 minutes followed by 40 cycles at 94°C for 10 seconds, 55°C for 1 minute and 68°C for 150 seconds. This was followed by a 10 minute extension at 72°C and cooling at 4°C. The semi-nested PCR was performed using the same reaction conditions as the first PCR using forward primer 2 and 1 μ l of the first PCR product as template. The semi-nested PCR product was resolved on a 2% agarose gel at 200 volts for 40 minutes. The gel was stained with ethidium bromide (0.5 μ g/ml) on the Gyrotory shaker Model G2 for 20 minutes at 50 rpm. The gel was visualized under UV light using the Gel Logic 200 Imaging System and documented with the Kodak 1D 3.6 Program.

Following visual confirmation of the correct fragment size, the semi-nested PCR product was purified with the GFX PCR DNA and the Gel band purification kit (GE Healthcare). The whole purified PCR product was sequenced using the ABI Prism Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction contained 2 μ l Terminator mix, 5 μ l template, 0.8 pmol/ μ l forward primer 2 and reverse primer (Table 2.1), respectively, and 1 μ l buffer to a final volume of 10 μ l. The cycling conditions were as follows: 96°C for 1 minute followed by 25 cycles at 96°C for 1 minute, 55°C for 5 seconds and 60°C for 4

minutes with a final holding temperature at 25°C. The cycle sequence product was purified using the ethanol/sodium acetate precipitation according to the manufacturer's instructions by the addition of 3 µl sodium acetate (3 M, pH 5.2), 62.5 µl 95% ethanol, 14.5 µl sterile water and 10 µl sequencing product. The mixture was vortexed for 30 seconds and centrifuged for 10 seconds followed by incubation at room temperature in the dark for 30 minutes. Thereafter, the precipitate was retained and centrifugation at 13,500 rpm for 30 minutes. The supernatant was carefully aspirated and the DNA pellet washed by the addition of 250 µl 70% ethanol, vortexing for 2 minutes and centrifugation for 10 minutes. The supernatant was aspirated and the DNA pellet air dried at 90°C for 1 minute. High dye formamide (25 µl) (Applied Biosystems) was added to the DNA pellet followed by vortexing for 1 minute and centrifugation at 13,500 rpm for 30 seconds. The sample was denatured at 95°C for 2 minutes followed by an incubation on ice for 5 minutes. The final product was vortexed for 10 seconds and centrifuged for an additional 30 seconds and stored in the dark until it was loaded on the sequencer. Sequencing samples were run on the ABI prism 3130 Genetic Analyser. The sequences were analysed using the Sequence Analysis Software 3.1 and edited using Chromas 2.31. The online program, LALIGN (Huang and Muller, 1991), was used to compare the alignment of the sequence data to a reference sequence M14752 from GenBank (M14752) (Branford and Hughes, 2006).

2.3 Results and discussion

In total, ten of the 40 patient samples sequenced were confirmed to contain mutations in *BCR-ABL* in the region of the kinase domain. The mutations found included four single base mutations, one previously described insertion and three novel deletion variants (Table 2.2 and 2.3).

Table 2.2: Summary of mutations in *BCR-ABL* in the region of the kinase domain, detected with sequencing.

Sample number	Mutation	Reference
11-3	F311L and L324Q	Roche-Leistienne <i>et al.</i> , (2002); Nicolini <i>et al.</i> , (2006)
48-6	S348L	Von Bubnoff <i>et al.</i> , (2004)
62-2	G250E	Branford <i>et al.</i> , (2002)
60-1	35 Ins	Laudadio <i>et al.</i> , (2008)
50-6 and 66-1	Δ 296 to 408	Not previously described in literature
19-3	Δ 296 to 446	Not previously described in literature
44-12, 55-16, 62-4 and 76-1	Δ 362 to 423	Not previously described in literature

Table 2.3: Summary of patient characteristics who have a mutation in *BCR-ABL* in the region of the kinase domain.

Patient number	Sex, Age, ethnicity ¹	Date of diagnosis	HIV status	Phase CML	Extramedullary disease ⁴	Sokal Score ³	Best Response ²	Cytogenetic finding at diagnosis	Survival from time of mutation detection
19	M, 23, B	September 2004	NEG	Chronic phase	Skin infiltrates, Spleen 10 cm	2	HR	67% + FISH	13 Months
44	M, 53, B	September 2007	NEG	Chronic phase	Skin infiltrates, Spleen 10 cm	0.9	Unknown	Monosomy 8 82% Ph ⁺ FISH	>15 months
50	F, 2, B	August 2008	NEG	Accelerated	Spleen 11.4 cm	1.5	Unknown	90% Ph ⁺ FISH	12 Months
76	F, 38, B	July 2010	NEG	Chronic phase	Spleen 14 cm	1.2	PCgR	62% Ph ⁺ FISH	>10 Months
55	M, 29, B	August 2008	NEG	Chronic phase	Spleen 20 cm	2.8	Unknown	78% Ph ⁺ FISH	>14 Months
62	F, 54, B	1997	NEG	Unknown	Unknown	Unknown	Unknown	No molecular cytogenetics	8 Months
66	M, 44, B	2003	NEG	Chronic phase	Spleen 13 cm	0.9	Unknown	XYY syndrome	>13 Months
2	F, 57, B	February 2001	NEG	Chronic phase	Spleen 14 cm	1.1	HR	92% Ph ⁺ FISH	5 Months
4	F, 51, B	June 2001	NEG	Chronic phase	Spleen 10 cm	1.1	Unknown	Unknown	26 Months
11	F, 76, B	January 2004	NEG	Chronic phase	Spleen 12 ml	2.7	Unknown	60% Ph ⁺ FISH	6 Months
48	F, 34, B	March 2008	NEG	Chronic phase	Spleen 20 cm	2.3	CHR	81% Ph ⁺ FISH	>19 Months
60	M, 57, B	January 2003	NEG	Chronic phase	Unknown	Unknown	Unknown	Unknown	>22 Months

¹Sex and ethnicity were indicated as M (male) or F (female) and B (black).

²The best response that the patient achieved while being treated with a tyrosine kinase inhibitor was indicated as HR (haematological response), PCgR (partial cytogenetic response) and CHR (complete haematological response).

³A Sokal score of <0.8 is considered low, between 0.8 to 1.2 intermediate and >1.2 high.

⁴Spleen size of <10 cm is considered normal and >11 cm up to 20 cm enlarged.

2.3.1 Single base mutations

Four single base mutations were detected and confirmed in *BCR-ABL* in the region of the kinase domain, in samples 11-3, 48-6 and 62-2 (Tables 2.2, 2.3, 2.5 and Figures 2.1 to 2.3). The importance of these different single base mutations in the *BCR-ABL* kinase domain is as follows:

- Single base mutations F311L and L324Q, in the gatekeeper domain, decrease sensitivity of the *BCR-ABL* kinase domain to imatinib and nilotinib resulting in partial resistance (Melo and Chuah, 2007; Bixby and Talpaz, 2009; Volpe *et al.*, 2009; Diamond and Melo, 2011). Studies have indicated that partial resistance as a result of F311L and L324Q can be overcome with a dose increase of imatinib or nilotinib as well as treatment with second generation tyrosine kinase inhibitor dasatinib (Corbin *et al.*, 2003; Melo and Chuah, 2007; Volpe *et al.*, 2009; Diamond and Melo, 2011). Reports in the literature have also shown that patients with F311L can achieve a haematological response (Roche-Lestienne *et al.*, 2002; Corbin *et al.*, 2003). Patient 11 was diagnosed in 2004 with 60% *BCR-ABL* Ph⁺ cells and having a Sokal score¹ of 2.7. Patient 11 was treated with 2 g/d Hydrea in 2004 and from November 2005 was treated with 400 mg/d imatinib. From 2007, the dosage of imatinib was increased to 800 mg/d due to lack of treatment response as well as disease progression to the accelerated phase and in 2008 mutational screening of the kinase domain detected single base mutations F311L and L324Q. Although F311L and L324Q result in partial resistance to imatinib, the combined effect of the two mutations in patient 11 could have been a negative contributing factor to the lack of response to treatment with imatinib.

¹ The Sokal score relates to the pathophysiology of the disease is used to predict response for survival of patients being treated with imatinib taking platelet count, age, spleen size and blast cell count into consideration. The Sokal score categorizes patients into groups with different survival rates such as a patient with a high Sokal score has a median survival of 2.5 years (Hasford *et al.*, 1998; Laneuville *et al.*, 2006; Guilhot and Guilhot, 2011).

- S348L is less frequently observed in patients and confers a decrease in sensitivity of the kinase domain to imatinib resulting in partial resistance (Melo and Chuah, 2007; Diamond and Melo, 2011). This mutation can be overcome with a dose increase of imatinib or the use of an alternative tyrosine kinase inhibitor such as nilotinib or dasatinib (Jones *et al.*, 2010). Patient 48 was diagnosed in 2008 in the chronic phase with 81% *BCR-ABL* Ph⁺ cells and having a Sokal score of 2.2. The bone marrow of patient 48 was hypercellular and displayed dysplasia in the granulocyte lineage with clumping in the megakaryocyte lineage. The patient was treated from 2008 with 800 mg/d imatinib, which was adjusted to 300 mg/d in 2009 after the patient repeatedly experienced toxicity and persistent thrombocytopenia. Patient 48 achieved a complete haematological response however this response was not maintained. In March 2010, patient 48 had still not regained the complete haematological response and mutational screening of the kinase domain detected the single base mutation S348L with no additional mutations. After the detection of S348L, dosage of imatinib remained between 200 mg/d and 300 mg/d due to persistent side effects of the treatment. Thus, the lack of maintaining the complete haematological response could possibly be due to the presence of S348L as well as the lower dosage of imatinib.
- G250E is one of the most frequently observed mutations in the *BCR-ABL* kinase domain, accounting for approximately 85% of all resistance associated with single base mutations to imatinib therapy (Gambacorti-Passerini *et al.*, 2003; Soverini *et al.*, 2006; Cowen-Jacob *et al.*, 2007). The mutation results in a conformational change of the kinase domain that reduces the binding efficacy for the tyrosine kinase inhibitor to *BCR-ABL* leading to an insensitivity to imatinib and nilotinib (Shah *et al.*, 2002; Gambacorti-Passerini *et al.*, 2003; Cowen-Jacob *et al.*, 2007; Melo and Chuah, 2007; Volpe *et al.*, 2009; Diamond and Melo, 2011). Patient 62 was diagnosed in 1997 with CML and from 2002 was treated with 300 mg/d imatinib which was increased to 600 mg/d in March 2006. From 2008, patient 62 was treated with the standard dosage of dasatinib but failed to

achieve an optimal response and from October 2009 treatment was changed to 400 mg nilotinib twice daily. Compliance to treatment was considered an issue since the patient was known to adjust the dosage of the tyrosine kinase inhibitor according to the side-effects experienced. In February 2010, patient 62 had a level of 146% *BCR-ABL* mRNA and mutational screening of the kinase domain detected single base mutation G250E. Although the high *BCR-ABL* mRNA level could be as the result of G250E and the insensitivity experienced to various tyrosine kinase inhibitors, compliance does remain a factor in the poor treatment response of this patient.

Table 2.3: Characteristics of the single base mutations in *BCR-ABL* in the region of the kinase domain, detected with sequencing.

Patient number	Single base mutation	Domain	Position from start codon	Nucleotide position (M14752)	Base change	Resistance ¹
11	F311L	Gatekeeper	931	1295	T to C	Partial (imatinib)
11	L324Q	Gatekeeper	971	1335	T to A	Partial (imatinib)
48	S348L	Gatekeeper	1046	1410	C to T	Partial (imatinib, dasatinib)
62	G250E	P-loop	749	1113	G to A	Partial (imatinib, nilotinib)

¹Resistance to the respective tyrosine kinase inhibitor is indicated in brackets.

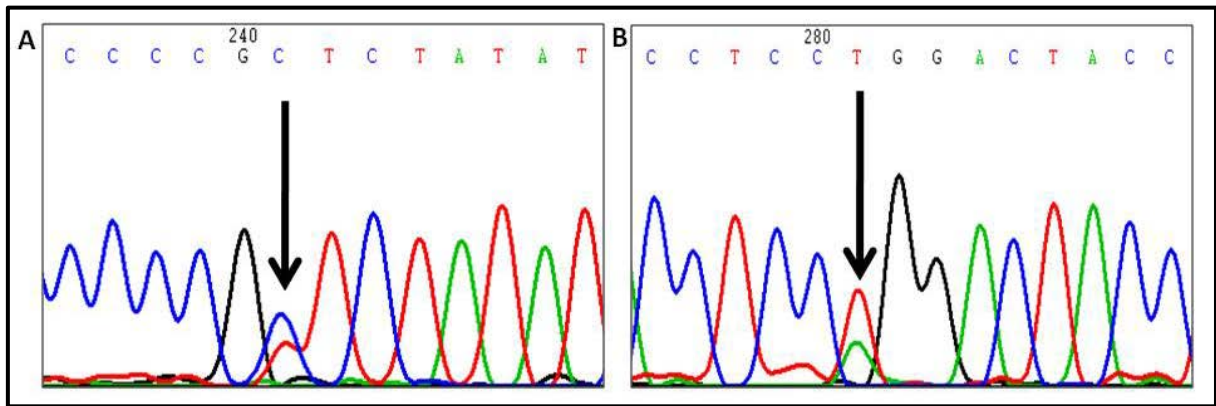


Figure 2.1: Sequencing chromatogram of single base mutations F311L and L324Q in sample 11-3. Single base mutations F311L (A) and L324Q (B) were observed at a level of approximately 70% and 50%, respectively, relative to the wild-type *BCR-ABL* sequence.

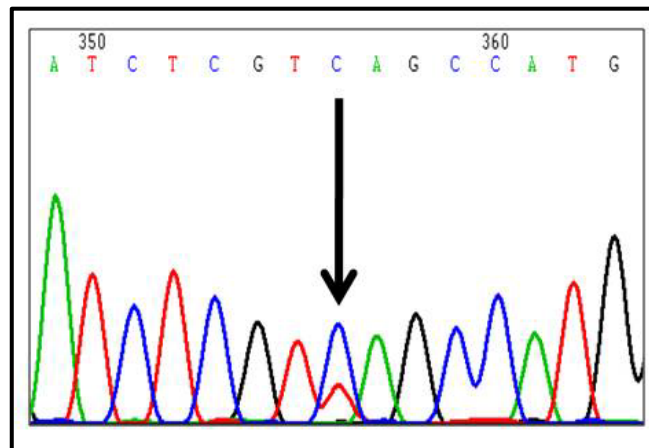


Figure 2.2: Sequencing chromatogram of single base mutation S348L in sample 48-6. Single base mutation S348L was observed at a level of approximately 40% relative to the wild-type *BCR-ABL* sequence.

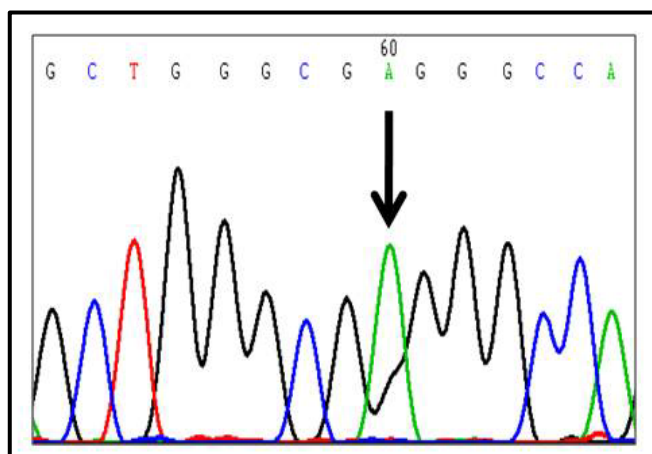


Figure 2.3: Sequencing chromatogram of single base mutation G250E in sample 62-2. Single base mutation G250E was observed at a level of approximately 30% relative to the wild-type *BCR-ABL* sequence.

2.3.2 35 bp insertion

A previously described 35 bp insertion was found to be present in patient sample 60-1 (Figures 2.4, 2.5 and Table 2.3). The 35 bp insertion was found at nucleotide position 1787 (GenBank M14752) and amino acid position 474 resulting in the addition of 10 amino acids after amino acid 474 followed by premature termination. Laudadio *et al.* (2008) and Ma *et al.* (2009) suggested that the 35 bp insertion confers absolute resistance to a tyrosine kinase inhibitor as conformational alterations of the kinase domain were similar to that in T315I. However, a recent report by O'Hare *et al.* (2011) suggested that *in vitro* the 35 bp insertion does not on its own confer resistance to tyrosine kinase inhibitors. Patient 60 was diagnosed with the 95% *BCR-ABL* Ph⁺ cells in 2003 and from 2008 was treated with 400 mg/d imatinib which was increased to 600 mg/d in 2009 due to lack of response. In January 2010, patient 60 was experiencing treatment failure and mutational screening of the kinase domain detected the 35 bp insertion with no additional mutations. After detection of the insertion, the dosage of imatinib was increased to 800 mg/d. Despite the dose increase of imatinib, the patient continued to experience a suboptimal response to treatment. Although recent literature suggests that the 35 bp insertion does not result in resistance to tyrosine kinase inhibitor, our findings suggest that the insertion may affect treatment response.

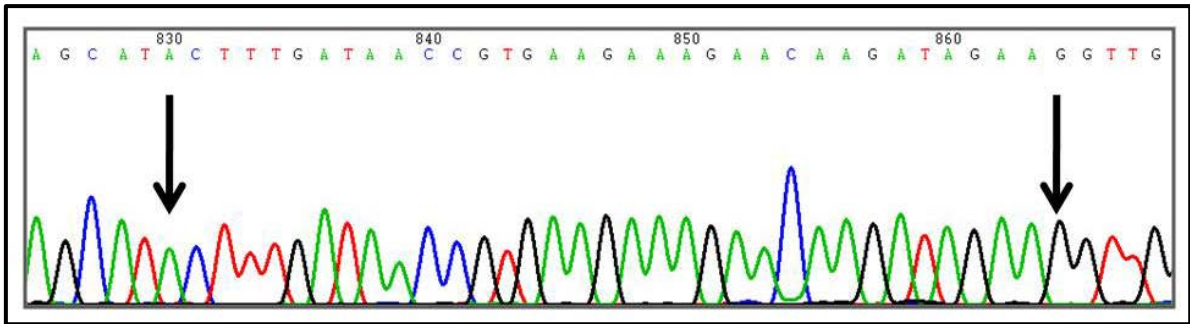


Figure 2.4: Sequencing chromatogram of the 35 bp insertion in sample 60-1. The insertion is at position 733 to position 768 on the chromatogram. The insertion was observed in the same percentage relative to the wild-type *BCR-ABL* sequence.

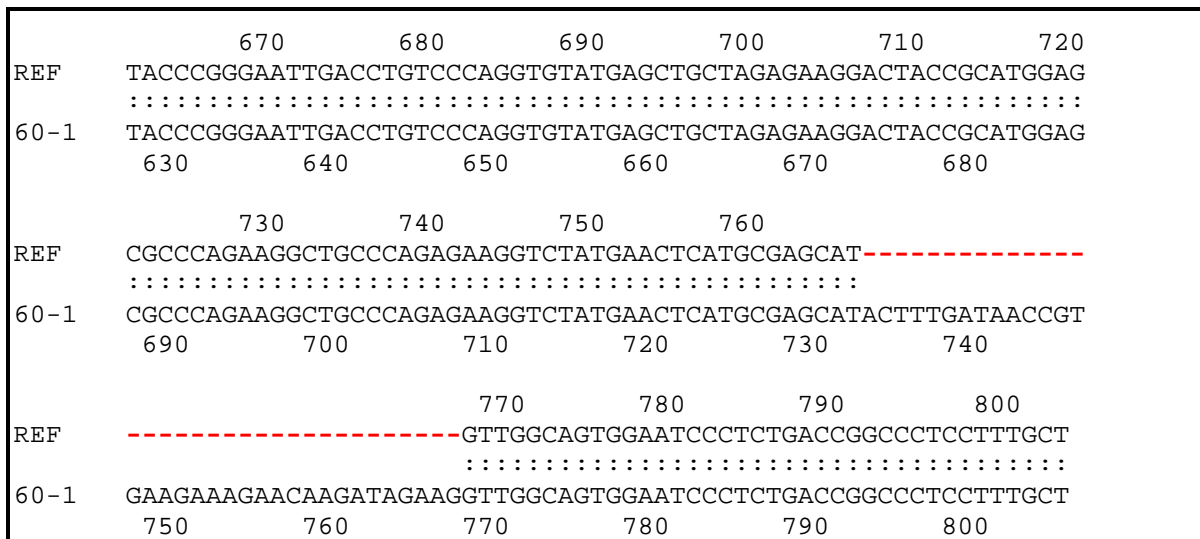


Figure 2.5: Multiple alignments of sample 60-1 and *BCR-ABL* (REF) (GenBank M14752) indicating the 35 bp insertion. The start of the 35 bp insertion was observed at position 767 and 734 in the reference and sample 60-1, respectively, resulting in a premature termination.

2.3.3 Deletion variants

Deletion variants, not previously described in the literature, were also identified and confirmed in seven patient samples (Figures 2.6 to 2.10 and Table 2.4). A second sequence was clearly present in the sequencing chromatogram that was not attributed to background. After subtracting the expected *BCR-ABL* sequence, the second sequence of the sample was aligned to normal *BCR-ABL* to determine its position (GenBank M14752). From this, it was possible to determine that the second sequence was the result of a deletion variant in *BCR-ABL*. The deletion variants included $\Delta 296$ to 446 (sample 19-3), $\Delta 296$ to 408 (samples 50-6 and 66-1) and $\Delta 362$ to 423 (samples 44-12, 55-16, 62-4 and 76-1) (Figures 2.6 and 2.7). By comparison of the deletion variants to normal *BCR-ABL* it was observed that the deletion variants resulted in a premature termination with the loss of the activation loop and catalytic domain (Figures 2.9 to 2.10, Table 2.4). It is suggested that the presence of an intact P-loop and loss of the activation loop could result in the kinase being continually in the active conformation. Therefore, the tyrosine kinase inhibitor is unable to bind to the kinase domain resulting in resistance to treatment (Sherbenou *et al.*, 2008; Gruber *et al.*, 2011).

Table 2.5: Characteristics of the deletion variants in *BCR-ABL*, detected with sequencing.

Sample number	Deletion (amino acid)	Number of nucleotides bases deleted	Nucleotide position (GenBank M14752)	Sections of kinase domain deleted
50-6 and 66-1	$\Delta 296$ to 408	337	1252 to 1588	Most of the C-helix, gate keeper domain, catalytic domain and activation loop
19-3	$\Delta 296$ to 446	452	1252 to 1703	Most of C-helix, gate keeper domain, catalytic domain and activation loop
44-12, 55-16, 62-4 and 76-1	$\Delta 362$ to 423	184	1449 to 1633	Most of the catalytic domain and activation loop

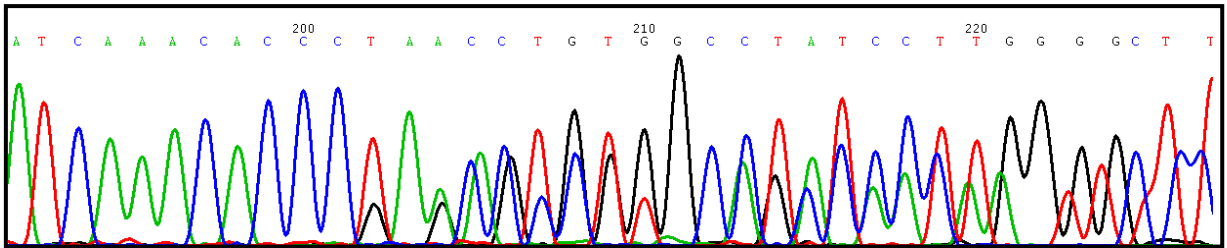


Figure 2.6: Sequencing chromatogram of novel deletion variants $\Delta 296$ to 408 and $\Delta 296$ to 446. Both deletion variants $\Delta 296$ to 408 and $\Delta 296$ to 446 were observed on the chromatogram from bp position 202 until either bp position 538 or 652 for $\Delta 296$ to 408 and $\Delta 296$ to 446, respectively.

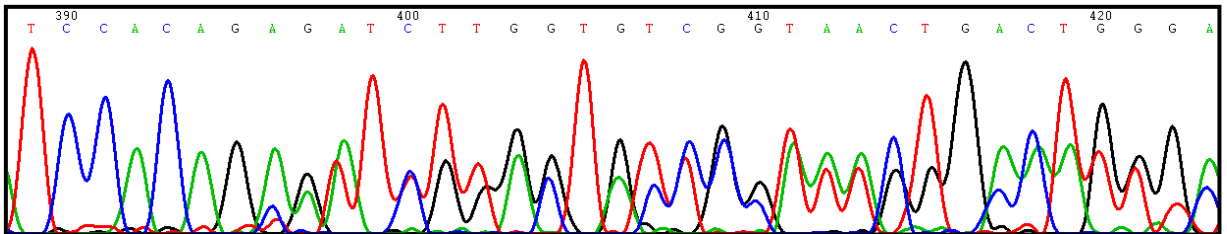


Figure 2.7: Sequencing chromatogram of novel deletion variant $\Delta 362$ to 423. Deletion variant $\Delta 362$ to 423 was observed from bp position 396 until bp position 579 on the chromatogram.

BCR-ABL	AAGAGATCAAACACCCCTAACCTGGTGCAGCTCCTTGGGGTCTGCACCCGGGAGCCCCCGT
Δ296 - 408	AAGAGATCAAACACCC-----
Δ296 - 446	AAGAGATCAAACACCC-----
Δ362 - 423	AAGAGATCAAACACCCCTAACCTGGTGCAGCTCCTTGGGGTCTGCACCCGGGAGCCCCCGT
BCR-ABL	TCTATATCATCACTGAGTTCATGACCTACGGGAACCTCCTGGACTACCTGAGGGAGTGCA
Δ296 - 408	-----
Δ296 - 446	-----
Δ362 - 423	TCTATATCATCACTGAGTTCATGACCTACGGGAACCTCCTGGACTACCTGAGGGAGTGCA
BCR-ABL	ACCGGCAGGAGGTGAACGCCGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCCA
Δ296 - 408	-----
Δ296 - 446	-----
Δ362 - 423	ACCGGCAGGAGGTGAACGCCGTGGTGCTGCTGTACATGGCCACTCAGATCTCGTCAGCCA
BCR-ABL	TGGAGTACCTGGAGAAGAAAACTTCATCCACAGAGATCTTGCTGCCCCGAAACTGCCTGG
Δ296 - 408	-----
Δ296 - 446	-----
Δ362 - 423	TGGAGTACCTGGAGAAGAAAACTTCATCCACA-----
BCR-ABL	TAGGGGAGAACCACTTGGTGAAGGTAGCTGATTTTGGCCTGAGCAGGTTGATGACAGGGG
Δ296 - 408	-----
Δ296 - 446	-----
Δ362 - 423	-----
BCR-ABL	ACACCTACACAGCCCATGCTGGAGCCAAGTTCCCCATCAAATGGACTGCACCCGAGAGCC
Δ296 - 408	-----GAGAGCC
Δ296 - 446	-----
Δ362 - 423	-----
BCR-ABL	TGGCCTACAACAAGTTCTCCATCAAGTCCGACGTCTGGGCATTTGGAGTATTGCTTTGGG
Δ296 - 408	TGGCCTACAACAAGTTCTCCATCAAGTCCGACGTCTGGGCATTTGGAGTATTGCTTTGGG
Δ296 - 446	-----
Δ362 - 423	-----GCATTTGGAGTATTGCTTTGGG
BCR-ABL	AAATTGCTACCTATGGCATGTCCCCTTACCCGGGAATTGACCTGTCCCAGGTGTATGAGC
Δ296 - 408	AAATTGCTACCTATGGCATGTCCCCTTACCCGGGAATTGACCTGTCCCAGGTGTATGAGC
Δ296 - 446	-----AGGTGTATGAGC
Δ362 - 423	AAATTGCTACCTATGGCATGTCCCCTTACCCGGGAATTGACCTGTCCCAGGTGTATGAGC

Figure 2.8: Multiple alignments of the deletion variants and *BCR-ABL* (GenBank M14752) indicating the starting and ending point of the deletion, respectively. The sections of the kinase domain that are deleted are indicated by a coloured hyphen for the deletion variants, respectively.

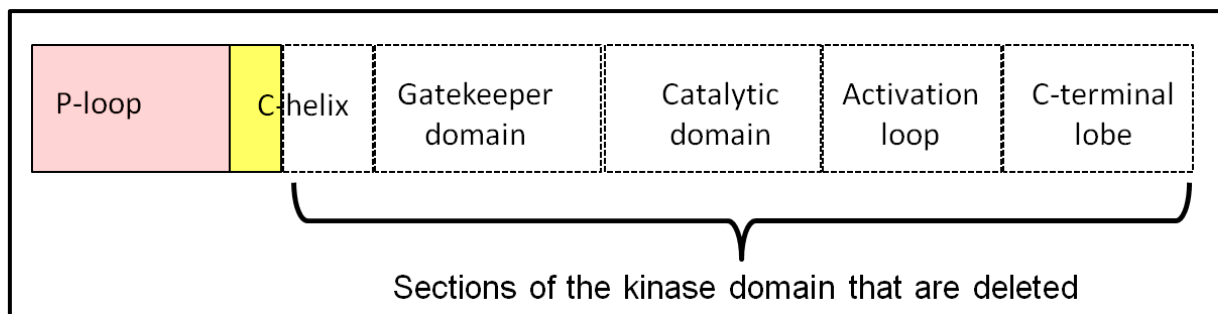


Figure 2.9: Schematic diagram representing the deletion variants $\Delta 296$ to 408 and $\Delta 296$ to 446. The deletion variants $\Delta 296$ to 408 or $\Delta 296$ to 446 had an intact P-loop as well as the first section of the C-helix. The remaining sections of the kinase domain were deleted resulting in a premature stop codon leading to protein truncation.

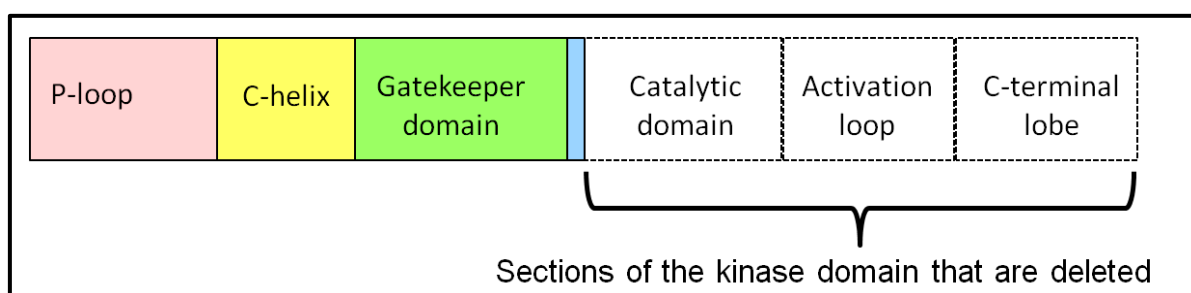


Figure 2.10: Schematic diagram representing the deletion variant $\Delta 362$ to 423. The deletion variant had an intact P-loop, C-helix, gatekeeper domain and the first few amino acids of the catalytic domain. The remaining sections of the kinase domain were deleted resulting in a premature stop codon and protein truncation.

Clinical data of some of the patients with deletion variants suggest that they were predisposed to the development of additional genetic abnormalities (Table 2.3). Baseline cytogenetics indicated the presence of XYY syndrome in patient 66 and monosomy 8 in patient 44. The presence of skin infiltrates² was observed in patients 44 and 19. Furthermore, patients with a deletion variant were diagnosed with Sokal scores ranging from 0.9 to 2.8. The importance of the cytogenetic findings, the presence of skin infiltrates and levels of *BCR-ABL* mRNA in the patients with deletion variants is as follows:

- In patient 66, conventional cytogenetics indicated the presence of the Ph chromosome as well as XYY syndrome. It has been described that the Ph chromosome can also occur coincidentally with sex chromosome linked diseases and in 1974, Ohyashiki *et al.* described the first incidence of CML and XYY syndrome. Since then the occurrence of CML and XYY syndrome has been observed in five different studies affecting six patients (Moore *et al.*, 1974; Potter *et al.*, 1975; Chaganti *et al.*, 1981; Alimena *et al.*, 1985; Oguma *et al.*, 1996). Patient 66 was diagnosed in 2003 with a hypercellular bone marrow with dysplasia in the erythroid and myeloid lineage with a normal megakaryocyte lineage as well as the presence of XYY syndrome. From 2009, patient 66 was treated with 400 mg/d imatinib and in May 2010 imatinib treatment failure was experienced. In addition to the imatinib treatment failure, XYY was observed in approximately 50% of the cells analysed following karyotyping of bone marrow of patient 66. Studies have indicated that chromosomal abnormalities in cancer patients have been associated with an increased risk of the development of additional abnormalities (Cerretini *et al.*, 2002; Welborn, 2004; Panani, 2009). Thus, we suggest that, even though the clinical implications of XYY syndrome on the disease progression and treatment with a tyrosine kinase inhibitor is uncertain, it may be indicative of genomic instability or an acquired problem that could result in deletions in *BCR-ABL*.

² Skin infiltrates is referred to as leukaemia cutis and is defined as the skin infiltration by a myeloid malignancy with a prevalence of 3% involvement in the cancer (Bénet *et al.*, 2011).

- In patient 44 a chromosomal abnormality, monosomy 8, was observed at diagnosis. Trisomy 8 is one of the most common additional chromosomal changes in the blast crisis, suggesting that chromosome 8 may be involved in CML progression (Tarkan-Arguden *et al.*, 2009). Patient 44 was diagnosed in the chronic phase with 82% *BCR-ABL* Ph⁺ cells as well as monosomy 8 at a level of approximately 16%. Bone marrow results displayed dysplasia in the granulocyte and megakaryocyte lineage as well as mild dysmaturation of the erythroid lineage. In addition to monosomy 8, patient 44 also presented with skin infiltrates at diagnosis, which are generally associated with an increased risk of blastic transformation (Bénet *et al.*, 2011). Patient 44 was treated with 600 mg/d to 800 mg/d imatinib from diagnosis, however failed to achieve an optimal response to treatment. In May 2010, patient 44 was in blast crisis, experiencing imatinib treatment failure. Mutational screening of the kinase domain detected the deletion variant in *BCR-ABL*. This suggested that monosomy 8 and the presence of skin infiltrates at diagnosis were indicative that patient 44 was predisposed to blastic transformation and genomic instability.
- The clinical background of patient 19 revealed the presence of skin infiltrates, similar to that of patient 44, at diagnosis. Skin infiltrates are not common and are only observed in approximately 2% of CML patients (Kaddu *et al.*, 1999; Nagarajarao *et al.*, 2009). In the majority of cases skin infiltrates are associated with disease progression and can proceed or accompany changes in blastic transformation in the bone marrow (Kaddu *et al.*, 1999; Bénet *et al.*, 2011). Patient 19 was diagnosed with 67% *BCR-ABL* Ph⁺ cells, a hypercellular bone marrow and skin infiltrates in the chronic phase. Diagnosis of skin infiltrates in the chronic phase may be an indication of later clinical progression of the disease and the possible development of genetic alterations. Although patient 19 was treated with imatinib (600 mg/d) from 2004 and later from 2008 with dasatinib (100 mg/d), levels of *BCR-ABL* mRNA remained above 500%. In May 2009, following mutational screening of kinase domain, a deletion variant was observed suggesting partial resistance to treatment.

- Analysis of the clinical background of patients 62, 50, 55 and 76 indicated the possible development of resistance to treatment with a tyrosine kinase inhibitor. Patients 50, 55 and 76 were diagnosed with Sokal scores of 1.5, 2.8 and 1.2, respectively, as well as with 90%, 78% and 62% *BCR-ABL* Ph⁺ cells, correspondingly. Patients 50, 55 and 76 were treated with 400 mg/d imatinib and patient 62 with 400 mg nilotinib twice daily. Increases in the level of *BCR-ABL* mRNA, during routine monitoring, was observed in samples 50-6, 55-16, 62-4 and 76-1. For example, the level of *BCR-ABL* mRNA of sample 64-4 (April 2010) increased from 146% (sample 62-2, February 2010) to 168% with no treatment regime changes. Previous mutational screening of sample 62-2 had identified the single base mutation G250E. However, this mutation was no longer present when the deletion variant was detected. This suggests that the increase in *BCR-ABL* mRNA was possibly due to partial resistance as a result of the deletion variant. Although patients 62, 50, 55 and 76 did not present with skin infiltrates and chromosomal abnormalities at diagnosis, the various treatment regimes and increases in the level of *BCR-ABL* mRNA suggests the deletion variants could have resulted in partial resistance to treatment.

2.3.4 Problematic samples with sequencing

Two samples (2-2 and 4-6) proved problematic to sequence as it was not possible to amplify the region in *BCR-ABL* encoding for the kinase domain using semi-nested PCR, even after repeated attempts. These samples were not excluded from the study as their clinical background suggested that there was a possibility of resistance to tyrosine kinase inhibitor (Table 2.3). Both patients had been treated with one or more tyrosine kinase inhibitor but had failed to achieve an optimal response. In patient 2, additional chromosomal abnormalities including trisomy 1 (present at approximately 50%), isochromosome 17q10 (present at approximately 75%) and a duplication of the Ph chromosome were known to be present. Additional chromosomal abnormalities are known to occur in up to 80% of CML patients in blast crisis (Sawyers *et al.*, 2002; Calabretta and Perrotti, 2004; Ilaria, 2005). Isochromosome 17q10 and trisomy 1 have also been linked to loss

of gene function as well as the development of resistance towards treatment and rapid progression of the disease (Alimena *et al.*, 1980; Mamaeva *et al.*, 1983; Calabretta and Perrotti, 2004; Caramazzo *et al.*, 2009). Thus, the clinical background of patients 2 and 4 suggests that possible acquired resistance to treatment with tyrosine kinase inhibitors may be present and therefore they were included for mutational screening with HRM.

2.4 Conclusions

PCR and sequencing were successfully used to detect single base mutations, insertions and deletions in *BCR-ABL* in the region of the kinase domain. Three of the deletions have not been described previously in the literature. Although the prognostic impact of the deletions on treatment response is not fully understood, it has been suggested insertions, deletions and duplications may also result in resistance to treatment with a tyrosine kinase inhibitor. When taking the clinical background of the patients with mutations in the *BCR-ABL* kinase domain into consideration, it is possible to predict that these patients were at a higher risk of developing genetic alterations involving the *BCR-ABL* kinase domain. Six patients had a high Sokal score at diagnosis which is associated with an increased risk of molecular relapse or development of resistance to tyrosine kinase inhibitor (Hasford *et al.*, 1998; Laneuville *et al.*, 2006; Guilhot and Guilhot, 2011). Although, there is no definite reason for the development of mutations, the presence of additional chromosomal abnormalities are indicative of genomic instability.

Based on the results from this chapter, mutational analysis of the region of *BCR-ABL* encoding for the kinase domain in CML patients should not just focus on single base mutations, but also include insertions and deletions in the kinase domain. The deletion variants and the 35 bp insertion in the *BCR-ABL* kinase domain, may in the future emerge as a novel category of mutations that is associated with resistance to tyrosine kinase inhibitors. In conclusion, sequencing was useful to identify novel deletion variants in *BCR-ABL* in the region of the

kinase domain that are possibly associated with resistance to treatment with a tyrosine kinase inhibitor.

CHAPTER THREE

SCREENING FOR MUTATIONS IN *BCR-ABL* IN THE REGION OF THE KINASE DOMAIN WITH HIGH-RESOLUTION MELTING

3.1 Introduction

Mutations in the *BCR-ABL* kinase domain constitute one of the major mechanisms of resistance to tyrosine kinase inhibitors in patients with CML (Branford *et al.*, 2003; Goldman *et al.*, 2004; Melo and Chuah, 2007). A mutation in the *BCR-ABL* kinase domain is suspected when a patient experiences a suboptimal response and/or loss of response to treatment with a tyrosine kinase inhibitor (Branford, 2007; Jabbour *et al.*, 2009). Depending on the location of the mutation within the *BCR-ABL* kinase domain, binding of the tyrosine kinase inhibitor is reduced or prevented and results in either partial or absolute resistance (Branford *et al.*, 2003; Branford and Hughes, 2006). More recently, insertions, deletions and duplications associated with the *BCR-ABL* kinase domain have also been reported in the literature (Gruber *et al.*, 2011). Although the impact of insertions, deletions and duplications is unknown, it has been suggested that they may induce conformational changes in the *BCR-ABL* kinase domain resulting in resistance to tyrosine kinase inhibitor (Sagilo *et al.*, 2002; Ma *et al.*, 2009). Therefore, mutational screening of the kinase domain is important since early detection of mutations can guide therapeutic decisions for maximum treatment efficacy in CML patients (Branford and Hughes, 2006; Wang *et al.*, 2006; Ernst *et al.*, 2008).

DNA sequencing, DHPLC and ASO-PCR are the most widely used methods to detect mutations in *BCR-ABL* in the region of the kinase domain. However, DHPLC and ASO-PCR have limitations and are not routinely used. DNA sequencing is mostly used to detect single base mutations in *BCR-ABL* in the region of the kinase domain, but the assay is costly and time consuming.

Furthermore, a suboptimal response to tyrosine kinase inhibitor may also be due to non-compliance to treatment and not as a result of a mutation in the kinase domain. This makes mutation monitoring expensive since sequencing of the kinase domain may be performed unnecessarily (Branford *et al.*, 2003; Wang *et al.*, 2006). Therefore, it would be time-and cost-effective to screen the region of *BCR-ABL* encoding for the kinase domain for mutations prior to sequencing.

Recently, a method of HRM has proven effective in screening for mutations in *BCR-ABL* (Poláková *et al.*, 2008). HRM is based on the principle that mutational changes in a nucleic acid will alter the melting profile of the sequence in comparison to a sample without mutations (wild-type) (Ririe *et al.*, 1997; Taylor, 2009). In 2008, Poláková *et al.* published a method using four primer sets, denoted HRM 1 to 4, to screen the region of *BCR-ABL* encoding for the kinase domain for single base mutations using HRM. The application of this method to detect mutations in *BCR-ABL* in the region of the kinase domain was confirmed by Doi *et al.* (2009). However, to date HRM has not been used to screen for insertions, deletions or duplications in *BCR-ABL*.

Several studies have shown that HRM can be used to detect insertions, deletions or duplications in genes. A study by Vaughn and Elenitobe-Johnson (2004) demonstrated that HRM could be used to detect a tandem duplication of approximately 430 bp in *FLT3*, a gene that is important in growth and differentiation of haematopoietic progenitors. Rouleau *et al.* (2009) used HRM to detect sequence deletions in *MHL1*, responsible for the development of Lynch syndrome. Heideman *et al.* (2009) showed that sequence insertions that result in cancer could be detected using HRM in *k-ras*, a gene involved in signal transduction. Therefore, HRM has the potential for screening *BCR-ABL* in the region of the kinase domain for insertions, deletions or duplications. The aim of this part of the research was to determine whether HRM can be used as a screening method to detect insertions, deletions or duplications in *BCR-ABL* in the region of the kinase domain in CML patients.

3.2 Materials and methods

3.2.1 HRM of *BCR-ABL* in the region of the kinase domain

HRM was used to screen 40 CML patients' samples, of which 10 had known mutations in *BCR-ABL* in the region of the kinase domain detected by sequencing. High-resolution melting was performed on the ABI 7500 Fast using the MeltDoctor HRM Reagent Kit (Applied Biosystems). HRM reactions (final volume 20 μ l) contained 2 X master mix, 10 pmol/ μ l forward and reverse primer 10 pmol/ μ l (Table 3.1 and Figure 3.1), respectively, and cDNA (2 μ g RNA). cDNA was same as that prepared in section 2.2.3. The region of *BCR-ABL* encoding for the kinase domain was subdivided into four consecutive PCR segments referred to as HRM regions (1 to 4) (Figure 3.1). The HRM cycling conditions were as follows: 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 58°C for 1 minute. Thereafter, melting curve analysis was performed by heating to 95°C for 10 seconds and cooling to 60°C for 1 minute followed by an incremental increase in temperature to 95°C with a transition of 0.03°C per second and a final cooling to 60°C for 15 seconds.

3.2.2 Semi-nested PCR of the kinase domain

Semi-nested PCR of the region of *BCR-ABL* encoding for the kinase domain was used to determine at which dilution of HRM amplicon the cDNA was no longer amplifiable. Amplicon for region HRM 1 was tested at different dilutions including undiluted, 1:10, 1:100 and 1:1,000. Semi-nested PCR followed the same method described in chapter two (refer to section 2.2.4) using different dilutions of HRM 1 amplicon, as template.

3.2.3 Conventional PCR with HRM primers

Conventional PCR of *BCR-ABL* in the region of the kinase domain, designated as different HRM regions, was used to confirm the presence of a tandem duplication in two samples. Furthermore, conventional PCR was used to demonstrate that PCR amplification of the HRM region 1 was being extended into adjacent HRM

regions in samples that contained deletion variants in *BCR-ABL*. PCR reactions (final volume 50 μ l) contained 10 X PCR Gold Buffer, 25 mM $MgCl_2$, 5 U/ μ l *AmpliTaq Gold*, 10 mM dNTP's, 10 pmol/ μ l forward and reverse primer (Table 3.1), respectively, and cDNA (2 μ g RNA) or PCR amplicon. cDNA was prepared the same as that in section 2.2.3. The PCR cycling conditions were as follows: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute. This was followed by a 7 minute extension at 72°C and cooling to 4°C.

3.2.4 Real-time PCR to amplify HRM regions 2 to 4 using HRM 1 amplicon as template

Real-time PCR was used to confirm the PCR extension of HRM 1 into adjacent HRM regions in *BCR-ABL* in samples containing deletions. Real-time PCR was performed using region HRM 1 amplicon diluted 1:100. Real-time PCR reactions (final volume 20 μ l) contained MeltDoctor HRM Reagent Kit (Applied Biosystems), 10 pmol/ μ l HRM forward and reverse primer (Table 3.1), respectively, and 1:100 dilution of HRM 1 PCR amplicon. PCR reactions were performed on the ABI 7500 Fast with the following cycling conditions 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 58°C for 1 minute.

3.2.5 Real-time PCR to amplify HRM regions 2 to 4 using probes and cDNA as template

Real-time PCR with probes was used to further confirm PCR extension of HRM 1 into the adjacent HRM regions in *BCR-ABL* in samples containing deletion variants. Real-time PCR was performed using HRM region 1 primers and cDNA as template. cDNA was the same as that prepared in section 2.2.3. Real-time PCR reactions (final volume 20 μ l) contained Taqman Fast Advanced Master Mix (Applied Biosystems), 10 pmol/ μ l HRM 1 forward and reverse primer (Table 3.1), respectively, 5 pmol/ μ l each of the different probes (Table 3.2) and cDNA (2 μ g RNA). Probes were designed using Primer3Plus (Untergasser and Nijveen, 2007)

and Nucleic acid sequence massager (Horton, 2006). PCR reactions were performed on the ABI 7500 Fast with the following cycling conditions 95°C for 10 minutes followed by 60 cycles at 95°C for 15 seconds and 50°C for 1 minute.

3.2.6 Gel electrophoresis

Gel electrophoresis was used to resolve the PCR amplicon in order to determine the size of the amplified fragments. Conventional and Real-time PCR products were resolved on a 2% agarose gel at 200 volts for 40 minutes. The gel was stained with ethidium bromide (0.5 µg/ml) on the Gyrotory shaker Model G2 for 20 minutes at 50 rpm and visualised under UV light using the Gel Logic 200 Imaging System and documented with the Kodak 1D 3.6 Program.

3.2.7 DNA sequencing of the four HRM regions

Sequencing of HRM amplicon was used to confirm that the PCR extension of HRM 1 into adjacent HRM regions in *BCR-ABL* in samples containing deletions. Sequencing of HRM region 1 followed the same method described in chapter two (refer to section 2.2.4) with the following modified conditions: purified PCR amplicon (5 µl) from HRM region 1 was used as template for sequencing with 2 µl forward and reverse primer (0.8 pmol/µl) (Table 3.1), respectively, and cycle conditions were 96°C for 1 minute followed by 25 cycles at 96°C for 1 minute, 58°C for 5 seconds and 60°C for 4 minutes with a final holding temperature at 25°C. Cycle conditions were modified specific to HRM primers in order to prove the hypothesis of extended PCR amplification.

Table 3.1: Primers used to PCR amplify the different HRM regions of *BCR-ABL* encoding for the kinase domain.

HRM Region	Primer	Primer sequence (5' to 3')
HRM 1	Primer 1F	CTCATCACCACGCTCCATTA
	Primer 1R	TCTTCCACCTCCATGGTGTC
HRM 2	Primer 2F	ACACCATGGAGGTGGAAGAG
	Primer 2R	TGGCTGACGAGATCTGAGTG
HRM 3	Primer 3F	ATGGCCACTCAGATCTCGTC
	Primer 3R	ACGTCGGACTTGATGGAGAA
HRM 4	Primer 4F	CGTCTGGGCATTTGGAGTAT
	Primer 4R	ACTGGATTCTGGAACATTG

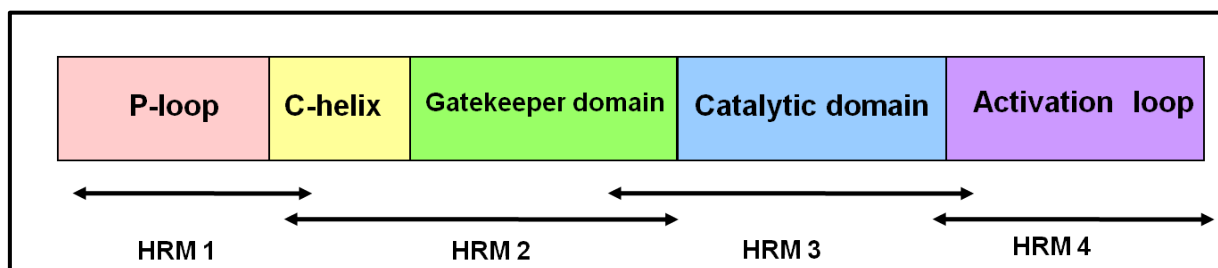


Figure 3.1: Schematic diagram of *BCR-ABL* corresponding to the kinase domain that was subdivided into four adjacent HRM amplification regions (1 to 4).

Table 3.2: Real-time probes used in the detection of the amplification of regions adjacent to HRM region 1.

Probe	Probe sequence (5' to 3')
Probe upstream from HRM 1 region	TTCATCATCATTCAACGGTG
Probe in HRM region 1	GACATCACCATGAAGCACAA
Probe in HRM region 2	AGCCCCGTTCTATATCATC
Probe in HRM region 4	GAGAAGGACTACCGCATGGA

3.3 Results and discussion

3.3.1 Summary

High-resolution melting (HRM) was successfully used to detect all mutations, including an insertion and novel deletion variants in *BCR-ABL* in the region of the kinase domain that had previously been identified using sequencing. In addition to single base mutations, an insertion and deletion variants detected using sequencing, HRM also identified a tandem duplication in *BCR-ABL* in the region of the kinase domain in two samples that was further confirmed using conventional PCR (Table 3.3). The efficiency of HRM compared to sequencing in the detection of previously confirmed single base mutations, a 35 bp insertion and deletion variants was 100%. Furthermore, a tandem duplication involving *BCR-ABL* in the region of the kinase domain, not previously identified by sequencing, was detected with HRM. The sensitivity of HRM and sequencing was similar since, with the exception of the tandem duplication, no other mutations were detected using HRM that were not identified with sequencing.

Table 3.3: Summary of results from the difference plots generated by HRM analysis of HRM regions 1 to 4 indicating the detection of different variants for patient samples containing mutations.

Sample number	Mutation	HRM region implicated by the mutation	HRM region			
			HRM 1	HRM 2	HRM 3	HRM 4
2-2 and 4-6	Tandem duplication	1 to 4	V	V	V	V
11-3	F311L and L324Q	2	Wt	V	Wt	Wt
48-6	S348L	2, 3	Wt	V ¹	V ¹	Wt
62-2	G250E	1	V	Wt	Wt	Wt
60-1	35 Ins	4	Wt	Wt	Wt	V
50-6 and 66-1	Δ296 to 408	2, 3	V	Wt	Wt	Wt
19-3	Δ296 to 446	2, 3, 4	V	Wt	Wt	Wt
44-12, 55-16, 62-4 and 76-1	Δ362 to 423	3, 4	V	V	Wt	Wt

Samples that were grouped as different variants to the wild-type on the difference plot are indicated as V, while samples that were grouped with the wild-type variant are indicated as Wt.

¹Mutation S348L was detected in HRM region 2 and 3 since it occurs in the overlapping area of the two adjacent HRM regions.

3.3.2 Temperature melting and difference plots

HRM was used to determine the melting temperature (T_m) of PCR fragments from the region of *BCR-ABL* encoding for the kinase domain (HRM 1 to 4) of CML patients. It was observed that the T_m for sequences with mutations did not differ considerably to that of the wild-type (Sample 1-7 confirmed to have no mutations by sequencing). Thus, the difference in T_m between samples with mutations compared to samples without mutations in *BCR-ABL* in the region of the kinase domain was not sufficient to be indicative of the presence of mutations. The T_m for regions HRM 1 to 4 containing mutations was found to be within the range of

normal variation compared to patients without mutations (Table 3.4). Thus, as has been found in other studies, differences in T_m were not found to be a good indicator of the presence of a mutation in the DNA sequence.

Table 3.4: A summary of T_m for the different HRM regions (1 to 4) of CML patients based on the mutation status of the region of *BCR-ABL* encoding for the kinase domain.

Mutation status of samples	HRM 1 T_m range (°C)	HRM 2 T_m range (°C)	HRM 3 T_m range (°C)	HRM 4 T_m range (°C)
Without mutation	85.56 to 86.04 (56)	84.37 to 86.49 (56)	84.91 to 85.31 (56)	84.83 to 85.21 (56)
Single base mutation	85.58 (2)	84.92 to 85.43 (4)	85.28 (2)	-
Deletion variant ¹	85.63 to 85.93 (14)	85.35 to 86.05 (14)	-	-
Insertion	-	-	-	85.05 (2)
Tandem duplication ²	85.62 to 85.98 (6)	85.26 to 85.94 (6)	85.04 to 85.14 (6)	85.01 to 85.07 (6)

The number of data points included in the T_m range is indicated in brackets. Samples were tested in duplicate or triplicate.

¹ T_m ranges for samples with a deletion variant were calculated for the HRM regions where the samples were grouped in a different variant to that of the wild-type, on the difference plot.

²Tandem duplications in the region of the kinase domain were only confirmed after further investigation of the HRM results.

Since there is normal variation in the T_m of a particular sequence, small changes in the T_m of that sequence is not necessarily indicative of the presence of a mutation. Of greater importance are the changes in the melting profile of a particular sequence. As a result, a difference plot is used to identify differences in melting profiles of a fragment compared to that of a known wild-type (samples

without mutations). However, it is first necessary to normalize differences in melting curves between samples. Normalization is achieved by eliminating fluorescence offsets amongst samples between a standardized pre-melt and post-melt range (determined from numerous HRM runs). Following normalization a difference plot for each HRM region was generated. Algorithms for defining a variant on the difference plot included software criteria that is designed to flag a sample with a mutation and/or when there are five or more difference units of a sample compared to a wild type. It was observed that samples without mutations were grouped with the wild-type, compared to samples with mutations that were grouped as different variants (refer to section 2.3.1 to 2.3.4 regarding the mutational status of samples) (Table 3.3).

3.3.3 Single base mutations

High resolution melting of the four HRM regions spanning the *BCR-ABL* kinase domain detected the presence of four single base mutations that were previously identified by sequencing. Samples with single base mutations G250E, F311L, L324Q and S348L were grouped as separate variants to the wild-type (Figures 3.2 to 3.4, Table 3.4). Interestingly, sample 48-6 containing the S348L mutation was identified as a variant in HRM region 2 and 3, due to the position of the mutation in the overlapping area of both adjacent segments (Figures 3.3 and 3.4). Samples 11-3 (containing mutations F311L and L324Q) and 48-6 (containing mutation S348L) were found in the same variant group on the difference plot for HRM region 2, indicating that the number of single base mutations in the sequence did not necessarily influence variant grouping (Figure 3.3). These results confirm the findings of Poláková *et al.* (2008) and Doi *et al.* (2009), that HRM can effectively be used to screen *BCR-ABL* in the region of the kinase domain for single base mutations. In this study, cDNA was directly used as template for PCR and HRM analysis compared to the studies of both Poláková *et al.* (2008) and Doi *et al.* (2009) that made use of PCR amplicon as template. The purpose of using PCR amplicon as template for HRM is to reduce the background noise generated by the presence of total cDNA. However, the direct use of cDNA as template for HRM did not appear to affect the detection of mutations in region of *BCR-ABL* encoding for the kinase domain and reduced the time needed for additional PCR reactions and dilution of amplicon.

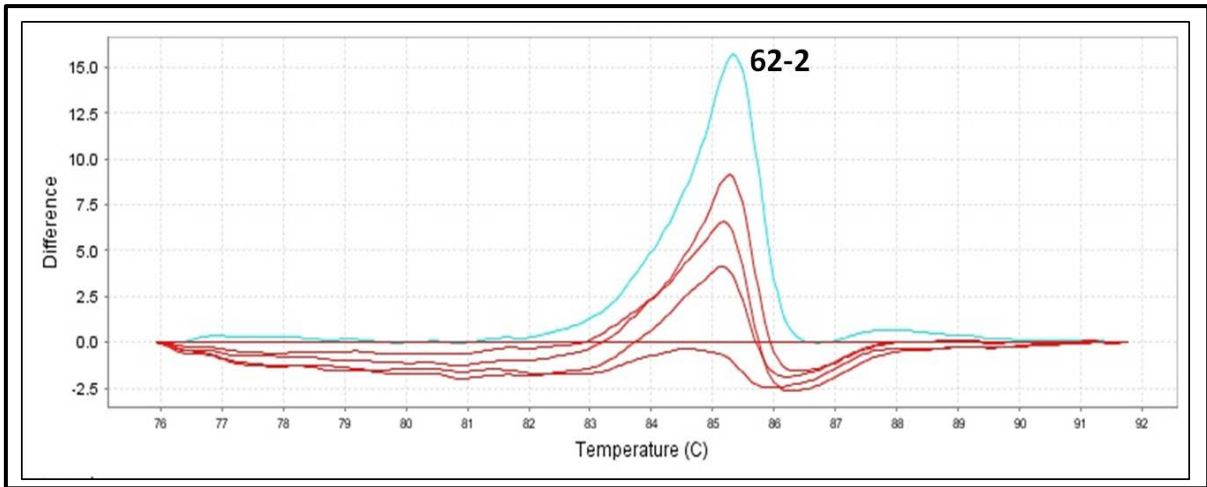


Figure 3.2: Difference plot following HRM analysis of HRM region 1 indicating one sample (62-2) that grouped as a different variant, compared to the wild-type. Sample 62-2 contained G250E.

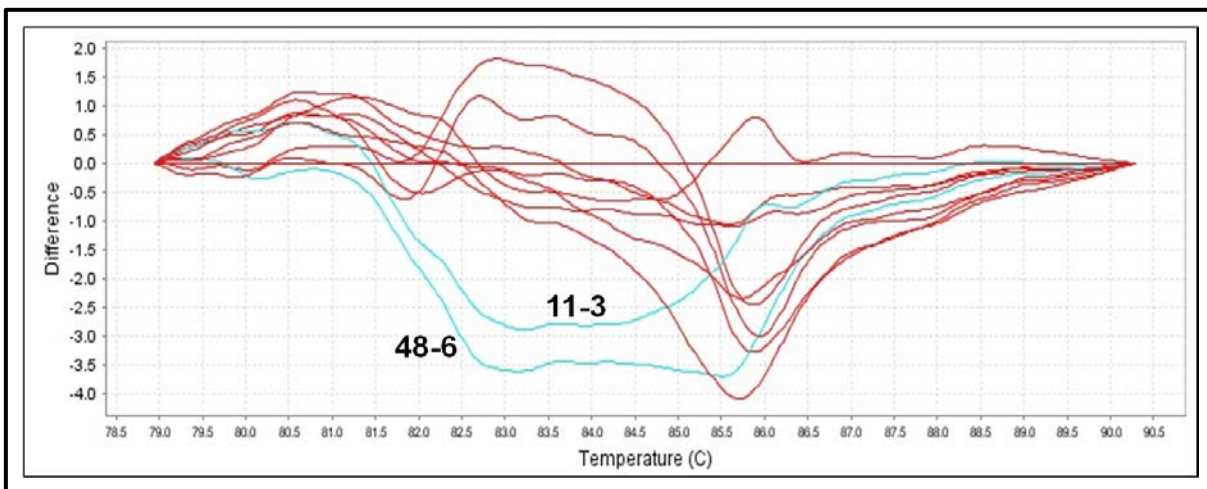


Figure 3.3: Difference plot following HRM analysis of HRM region 2 indicating two samples (11-3 and 48-6) that grouped as the same variant, compared to the wild-type. Sample 11-3 contained mutations F311L and L324Q, while sample 48-6 contained S348L.

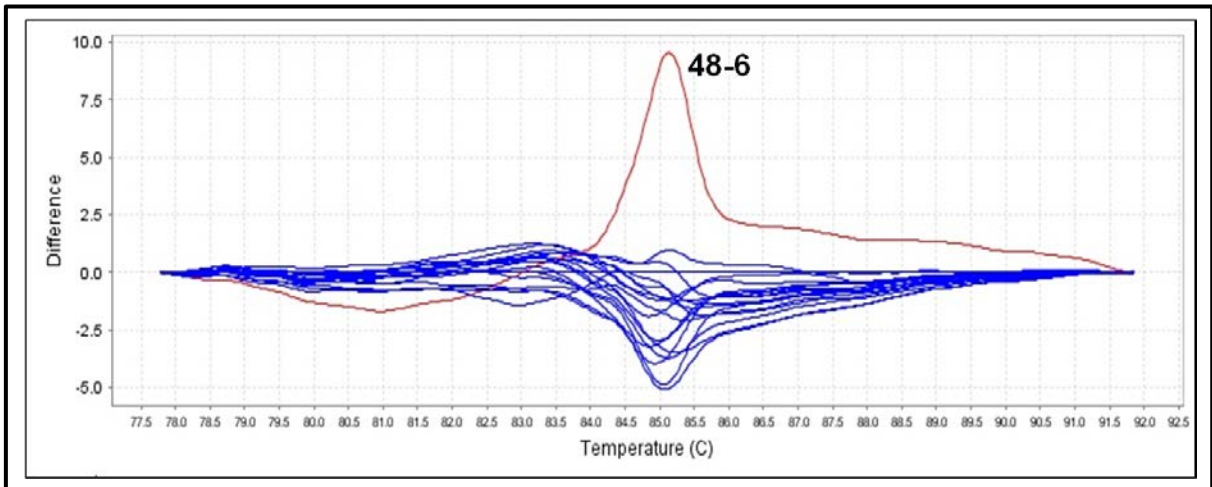


Figure 3.4: Difference plot following HRM analysis of HRM region 3 indicating one sample (48-6) that grouped as a different variant, compared to the wild-type. Sample 48-6 contained S348L.

3.3.4 35 bp insertion

High resolution melting of HRM region 4 detected the presence of a 35 bp insertion in the region of *BCR-ABL* encoding for the kinase domain in sample 60-1 (Table 3.4). Sample 60-1 was observed as a separate variant group to that of the wild-type on the difference plot for HRM region 4 (Figure 3.5). This is the first report of using HRM to screen for insertions in the region of *BCR-ABL* encoding for the kinase domain.

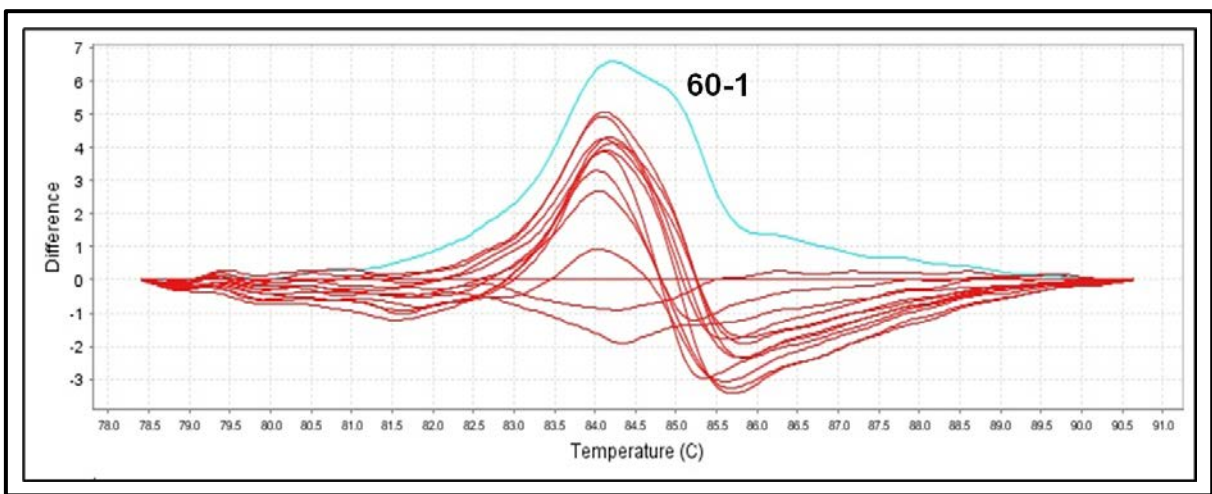


Figure 3.5: Difference plot following HRM analysis of HRM region 4 indicating one sample (60-1) that grouped as a different variant, compared to the wild-type. Sample contained a 35 bp insertion based on previous sequence analysis.

3.3.5 Deletion variants

Sequencing, as described in chapter two, the entire region of *BCR-ABL* encoding for the kinase domain identified several patient samples with deletion variants (19-3, 44-12, 50-6, 55-16, 62-4, 66-1 and 76-1). A comparison of the T_m of the samples with a deletion variant to wild-type was not indicative of the presence of a mutation. Samples with deletions in *BCR-ABL* were identified as variants, compared to the wild-type, on the difference plots. Of interest, was the observation that samples with a deletion in *BCR-ABL* were identified as variants on the difference plot preceding the region affected by the deletion. For example, samples 50-6 and 66-1 had a deletion affecting HRM region 2 and 3 while HRM analysis detected a variant on the difference plot in HRM region 1. Similarly, samples 44-12, 55-16, 62-4 and 76-1 had a deletion affecting HRM region 3 and 4 while HRM analysis detected a variant on the difference plot in HRM region 1 and 2 (Figures 3.6 and 3.7, Table 3.4). In other studies that have used HRM to identify the presence of deletion variants, the deletion was detected within the PCR fragment being studied (Takano *et al.*, 2008; Rouleau *et al.*, 2009). Compared to this, in the current study, the variant on the difference plot indicating the presence of a deletion in *BCR-ABL* was detected in the HRM region preceding the area affected by the deletion. Repeated experiments yielded the same result. Since there did not appear to be any logical explanation for this, it was decided to investigate this phenomenon further.

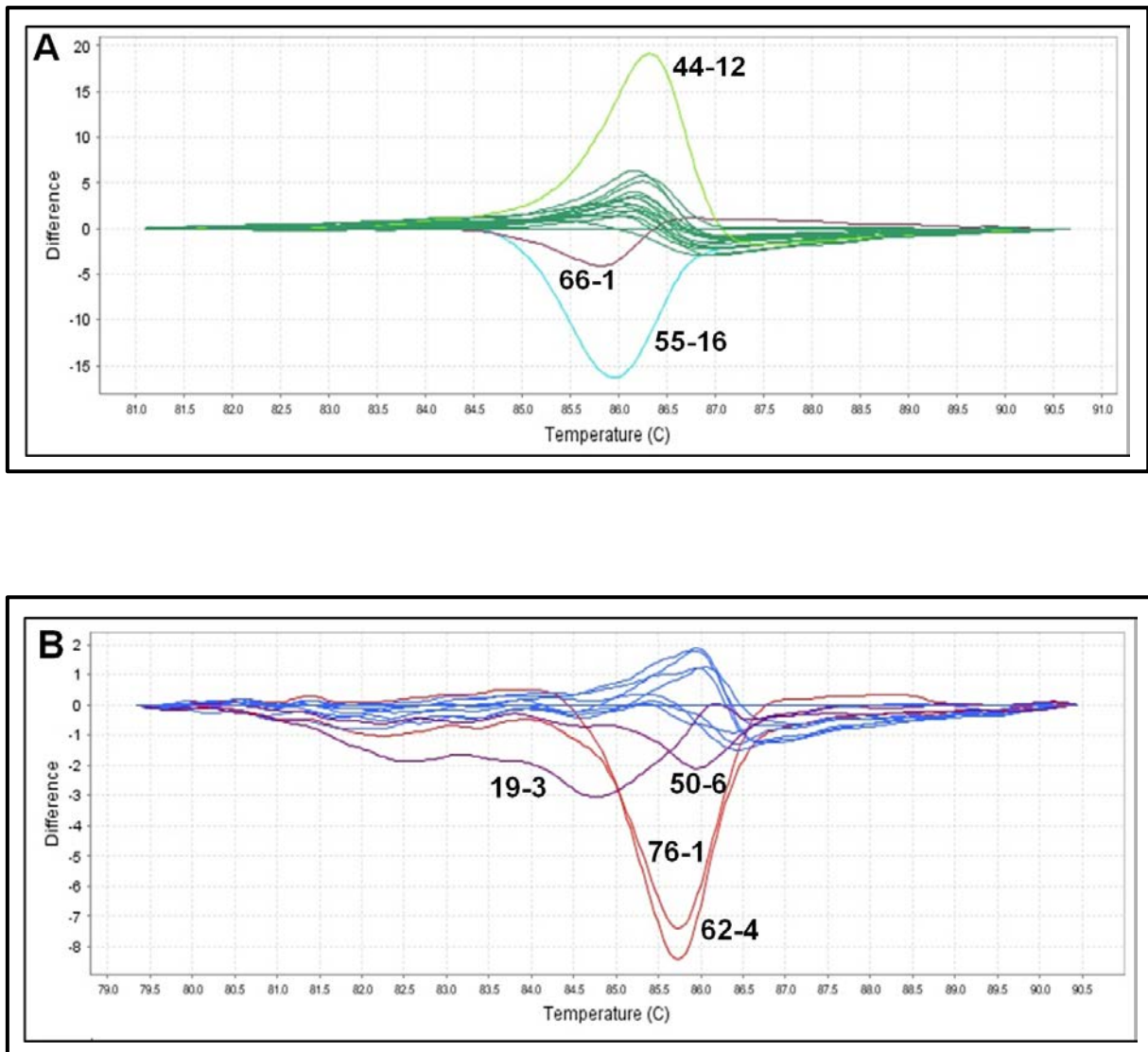


Figure 3.6: Difference plots A and B following HRM analysis of HRM region 1 indicating seven samples (19-3, 44-12, 50-6, 55-16, 62-4, 66-1 and 76-1) that were grouped as variants, compared to the wild-type. Samples 44-12, 55-16, 62-4 and 76-1 contained $\Delta 362$ to 423; samples 50-6 and 66-1 contained $\Delta 296$ to 408 and sample 19-3 with $\Delta 296$ to 446.

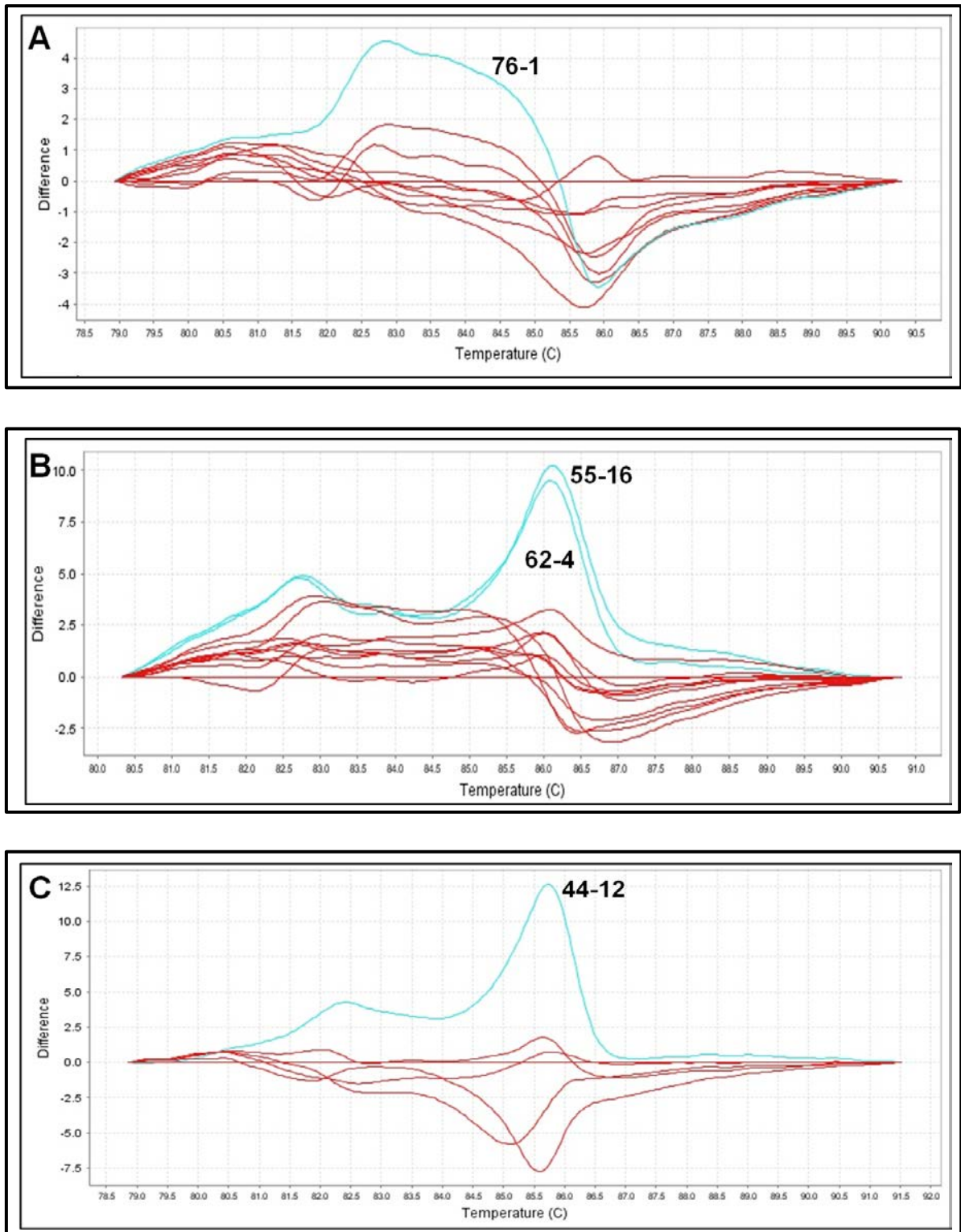


Figure 3.7: Difference plots A, B and C following HRM analysis of HRM region 2 indicating four samples (44-12, 55-16, 62-4 and 76-1) that were grouped as variants, compared to the wild-type. Samples 44-12, 55-16, 62-4 and 76-1 contained $\Delta 362$ to 423.

In order to understand why the deletion was being detected on the difference plot in the HRM region preceding the area of the deletion, PCR fragments from HRM analysis were resolved using gel electrophoresis to confirm the presence of the expected HRM 1 amplicon size for deletion variants. All samples with deletion variants were found to have the expected HRM 1 fragment size of 221 bp. However, it also appeared that there was linear amplification (Figure 3.8). It was hypothesized that the linear amplification may be as a result of the HRM 1 amplicon being extended by *Taq polymerase* into the HRM area of the deletion.

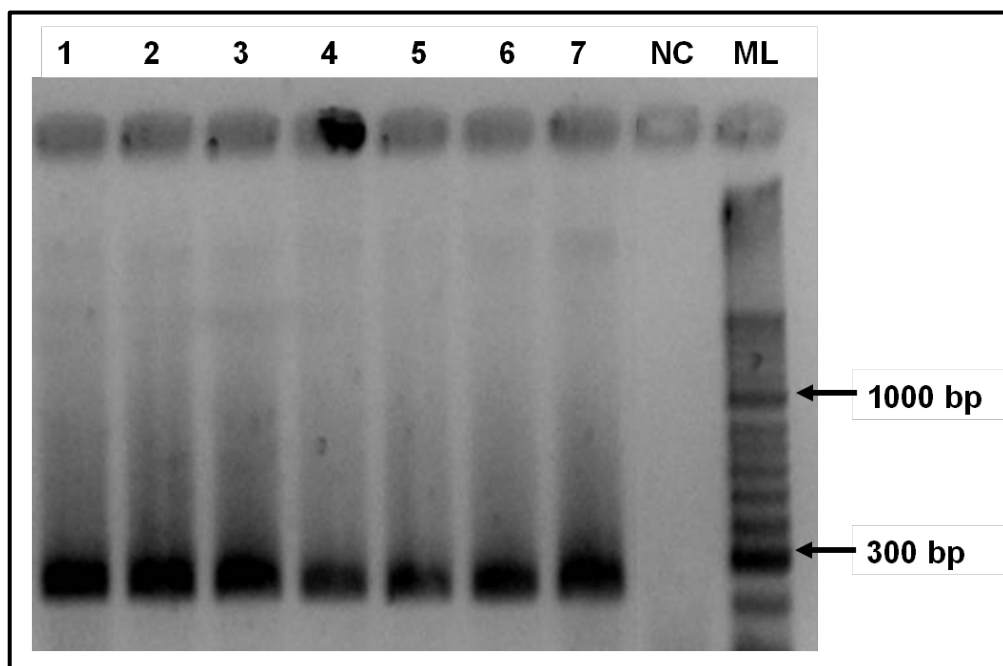
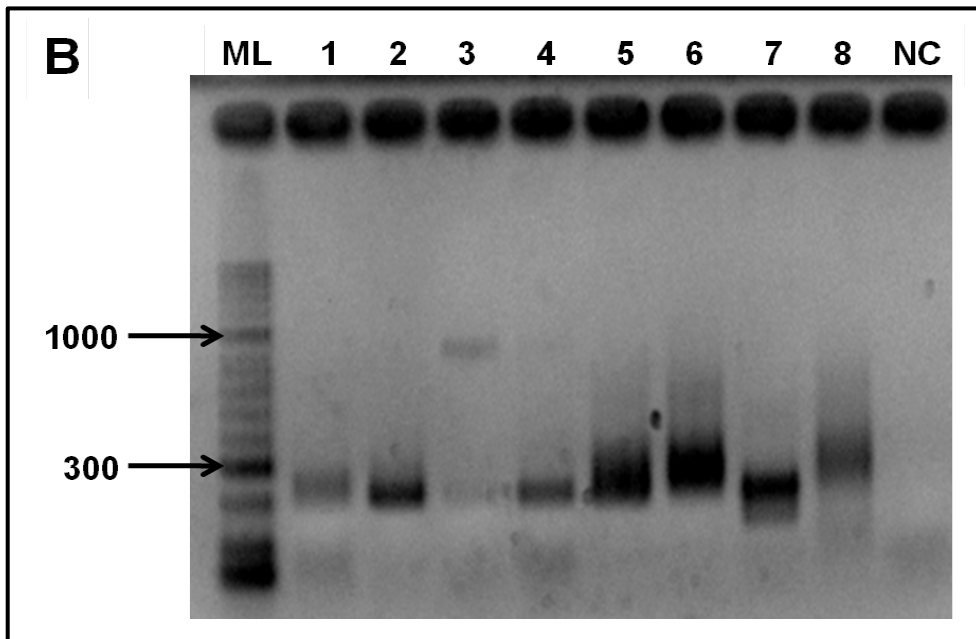
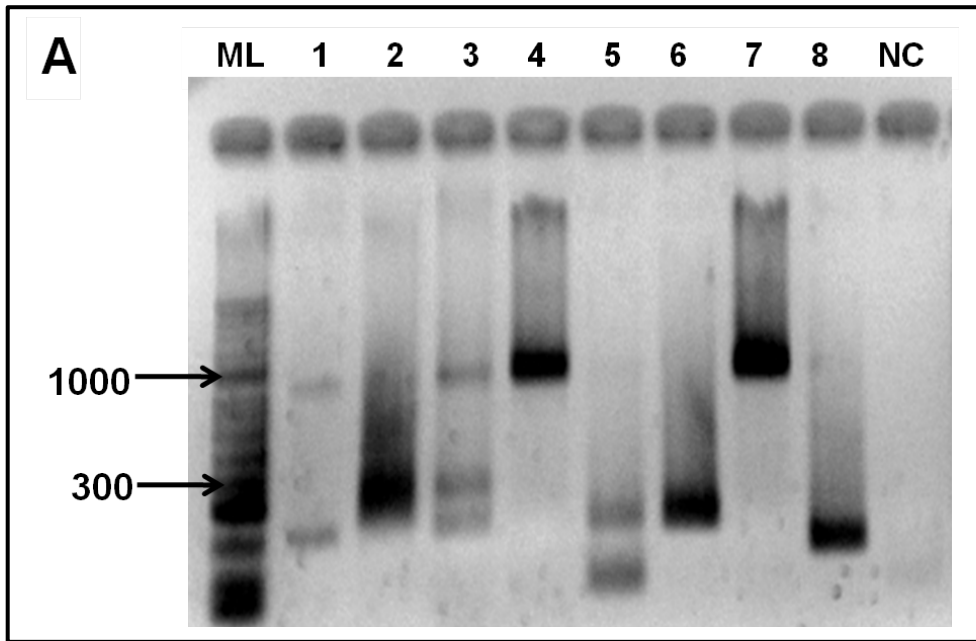


Figure 3.8: Negative inverted gel image of HRM 1 amplicon of samples with a deletion variant. HRM 1 amplicon of samples with a deletion variant was resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The expected fragment of 221 bp was observed as well as non-specific amplification. Lane 1 – sample 19-3; lane 2 – sample 50-6; lane 3 – sample 66-1; lane 4 – sample 62-4; lane 5 – sample 76-1; lane 6 – sample 55-16; lane 7 – sample 44-12; lane NC – negative control and lane ML – molecular ladder (Hyperladder II).

To test the theory that the HRM 1 amplicon is being extended by *Taq polymerase* into the HRM area of the deletion, the HRM 1 amplicon was used as template to PCR amplify (in house method) HRM region 1 and 2 using primers 1F and 2R (Section 3.2.3). However, in order to exclude PCR amplification of HRM region 1 and 2 from the residual cDNA as template instead of HRM 1 amplicon, a set of dilutions of HRM 1 amplicon was tested using PCR primers (Branford and Hughes, 2006) spanning the entire region of *BCR-ABL* encoding for the kinase domain (Section 3.2.2, Figure 3.9). At a dilution 1:100 of the HRM 1 amplicon no amplification of cDNA occurred and only the expected HRM 1 amplicon is observed after the product of amplification was resolved using gel electrophoresis (Figure 3.9 and 3.10). Furthermore, amplification using primers for the different HRM regions in combination was tested to confirm that the use of primers 1F and 2R would result in an amplification product (Section 3.2.3, Figure 3.11). Surprisingly, the PCR amplification of HRM region 1 and 2 using primers 1F and 2R was observed when using HRM 1 amplicon, of samples with a deletion variant, as template (Sections 3.2.3 and 3.2.4, Figure 3.12).



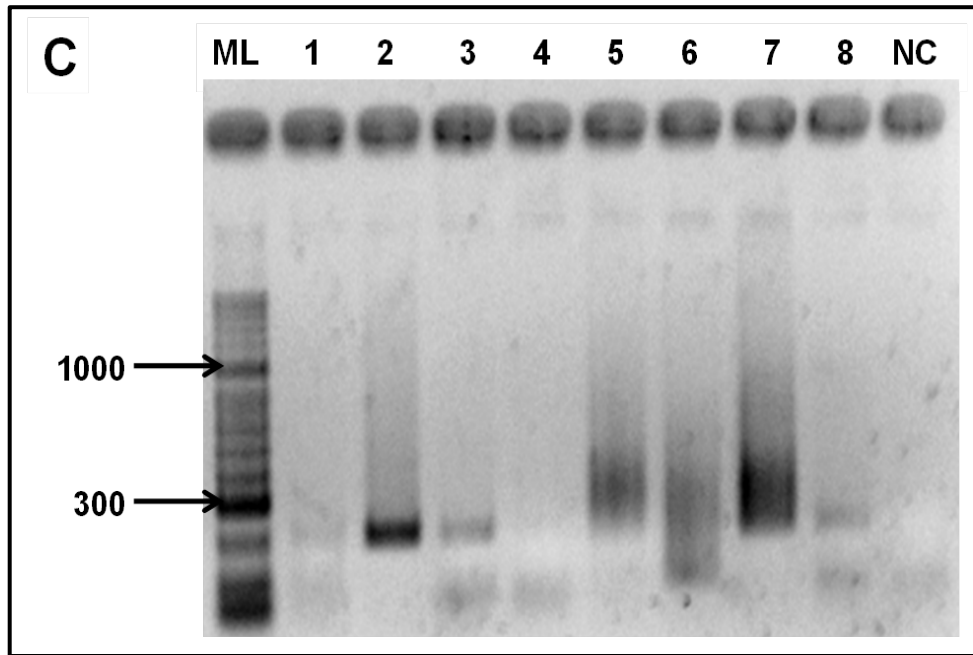


Figure 3.9: Negative inverted gel images of the semi-nested PCR of the kinase domain using different dilutions of HRM 1 amplicon as template. The semi-nested PCR products of the kinase domain were resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The expected fragment of 863 bp was observed when using undiluted HRM 1 amplicon (Gel A) as well as when using the 1:10 dilution of HRM 1 amplicon (Gel B). The kinase domain failed to amplify when using the 1:100 dilution of HRM 1 amplicon (Gel C) indicating cDNA was sufficiently diluted out as not to act as template in the semi-nested PCR reaction. The non-specific amplification observed is likely due to linear amplification (Figure 3.9). Gel images A to C: Lane ML – molecular ladder (Hyperladder II); lane 1 – wild-type; lane 2 – sample 19-3; lane 3 – sample 50-6; lane 4 – sample 66-1; lane 5 – sample 62-4; lane 6 – sample 76-1; lane 7 – sample 55-16; lane 8 – sample 44-12 and lane NC – negative control.

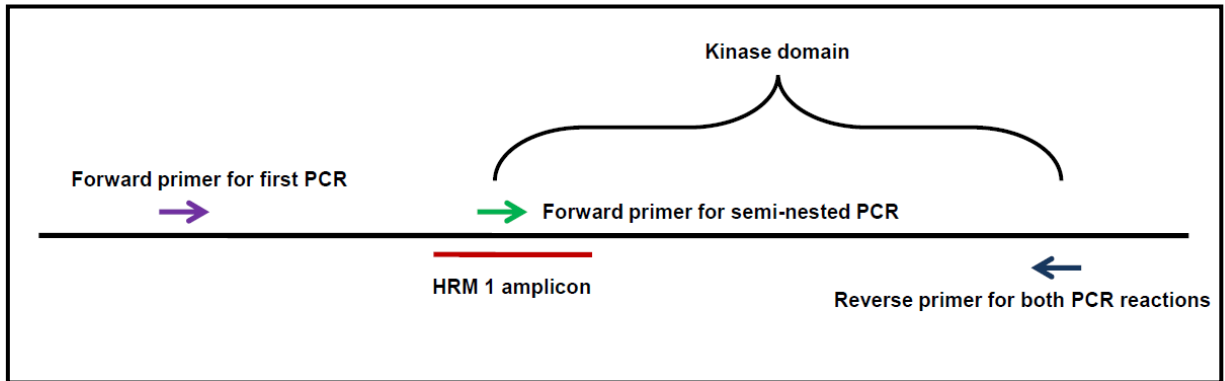


Figure 3.10: Schematic diagram representing the different primer binding sites in the semi-nested PCR reaction amplifying the kinase domain. The forward primer (green) for the semi-nested PCR binds 33 bases from the start of the 5' end of the HRM 1 amplicon resulting in linear amplification of an amplicon of 188 bp.

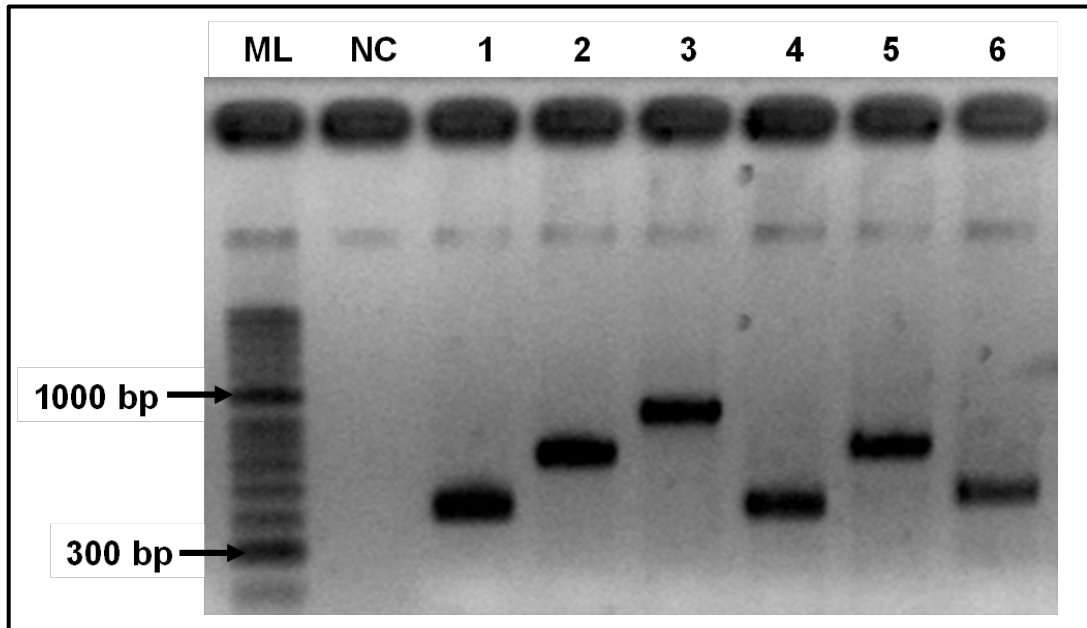


Figure 3.11: Negative inverted gel image of conventional PCR products using wild-type cDNA and different HRM primer combinations, respectively. PCR products were resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The observed amplicon sizes correlated with the expected sizes for the different HRM primer combinations. Lane ML – molecular ladder (Hyperladder II); lane NC – negative control; lane 1 – primers 1F and 2R (446 bp); lane 2 – primers 1F and 3R (685 bp); lane 3 – primers 1F and 4R (926 bp); lane 4 – primers 2F and 3R (454 bp); lane 5 – primers 2F and 4R (705 bp) and lane 6 – primers 3F and 4R (480 bp).

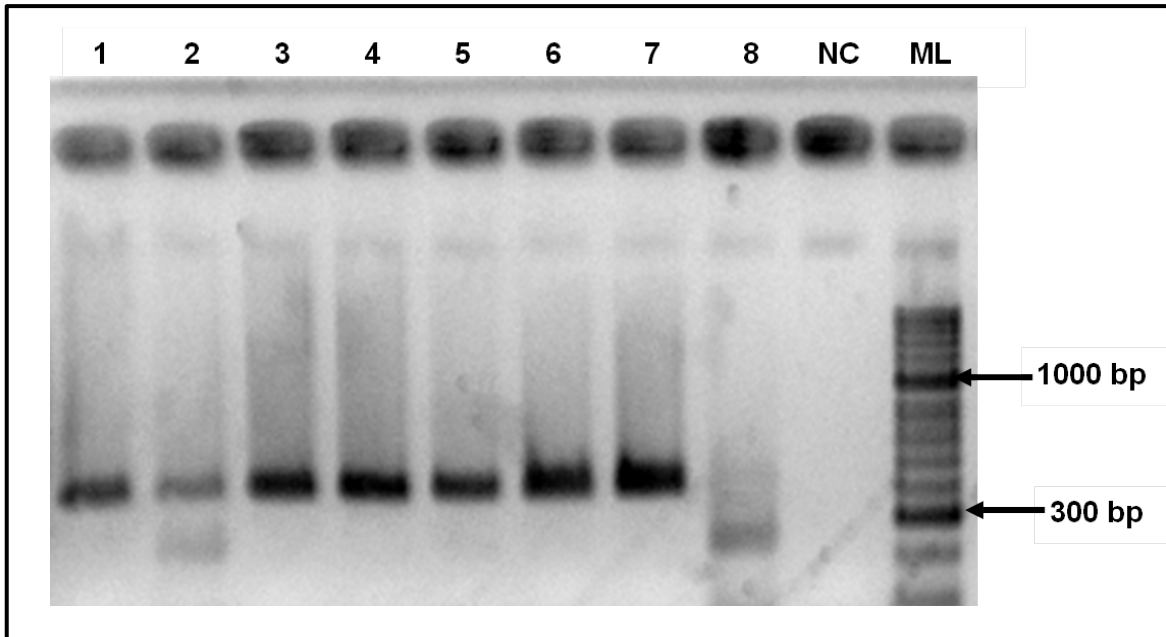


Figure 3.12: Negative inverted gel image of PCR product of HRM regions 1 and 2 (primers 1F and 2R) using 1:100 of dilution of HRM 1 amplicon as template. PCR products were resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The observed amplicon of approximately 450 bp in lanes 1 to 8 correlated to expected size fragment of 446 bp, indicating that it was possible to amplify the combination of regions HRM 1 and 2 from the targeted HRM 1 region. Lane 1 – wild-type; lane 2 – sample 19-3; lane 3 – sample 50-6; lane 4 – sample 66-1; lane 5 – sample 62-4; lane 6 – sample 76-1; lane 7 – sample 55-16; lane 8 – sample 44-12; lane NC – negative control and Lane ML – molecular ladder (Hyperladder II).

The observation that the HRM 1 region was being extended into HRM region 2 was further tested by using HRM 2 amplicon as template to amplify the HRM region spanning the area of the deletion using primers 2F and 4R in samples with deletion variants (Section 3.2.3). Similar to previous results, it was possible to PCR amplify the HRM region spanning the deletion using primers 2F and 4R (Figure 3.13). Finally, the extended amplification of the HRM region 1 was confirmed with sequencing of HRM region 1 (Section 3.2.7, Figure 3.14) as well as

Real-time PCR using probes for the different regions preceding and post HRM region 1 (Section 3.2.5, Figure 3.15). Thus, it appears that the hypothesis that the extended amplification of HRM 1 and/or 2, by *Taq polymerase*, was allowing the detection of the deletion variants in HRM regions 2 to 4 was correct.

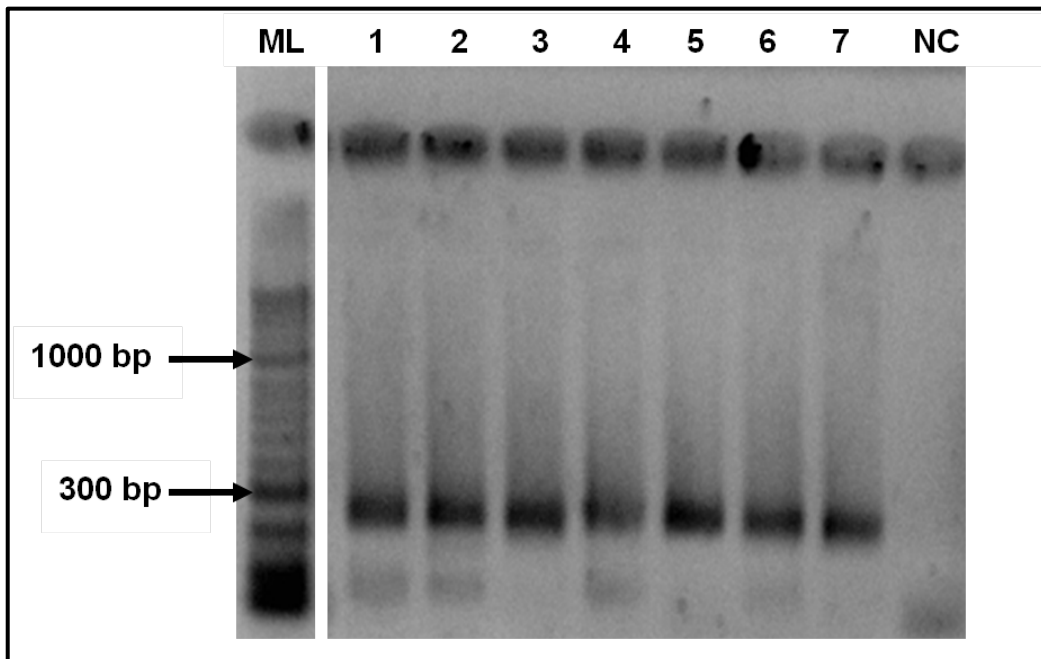


Figure 3.13: Negative inverted gel image of PCR product of the combination of regions HRM 2 to 4 (primers 2F and 4R) using HRM 2 amplicon. PCR products were resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The observed amplicon of approximately 250 bp in deletion variants indicated that amplification of HRM region 2 had been extended into the area of the deletion. Lane ML – molecular ladder (Hyperladder II); lane 1 – sample 19-3; lane 2 – sample 50-6; lane 3 – sample 66-1; lane 4 – sample 62-4; lane 5 – sample 76-1; lane 6 – sample 55-16; lane 7 – sample 44-12 and lane NC – negative control. The molecular ladder is from the same gel image as unnecessary data has been excluded.

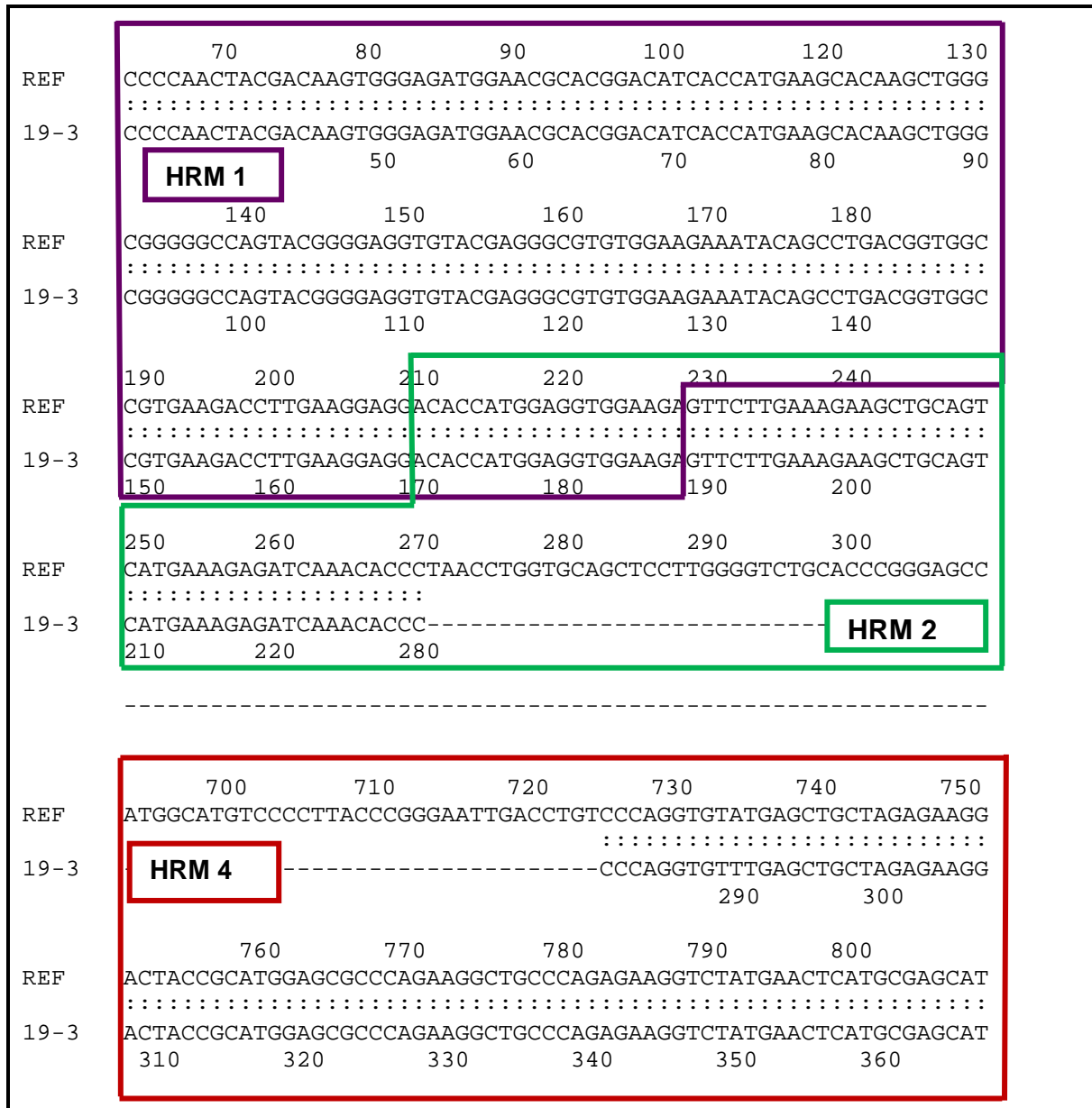


Figure 3.14: Alignment of sequencing results of HRM region 1 for sample 19-3 to *BCR-ABL* (REF) (GenBank M14752) using primer 1F. The alignment of sequencing results for HRM region 1 for sample 19-3 went beyond region the reverse primer binding site into HRM regions 2 and 4. The latter section of HRM region 1 is indicated in purple, the portion of HRM region 2 before the deletion in green and the remaining section of HRM region 4 after the deletion in red. The end point of HRM region 2 is at position 270 and 280 for the reference and sample 19-3, respectively, while the remaining section of HRM region 4 is indicated from position 281 and 725 for sample 19-3 and the reference, accordingly.

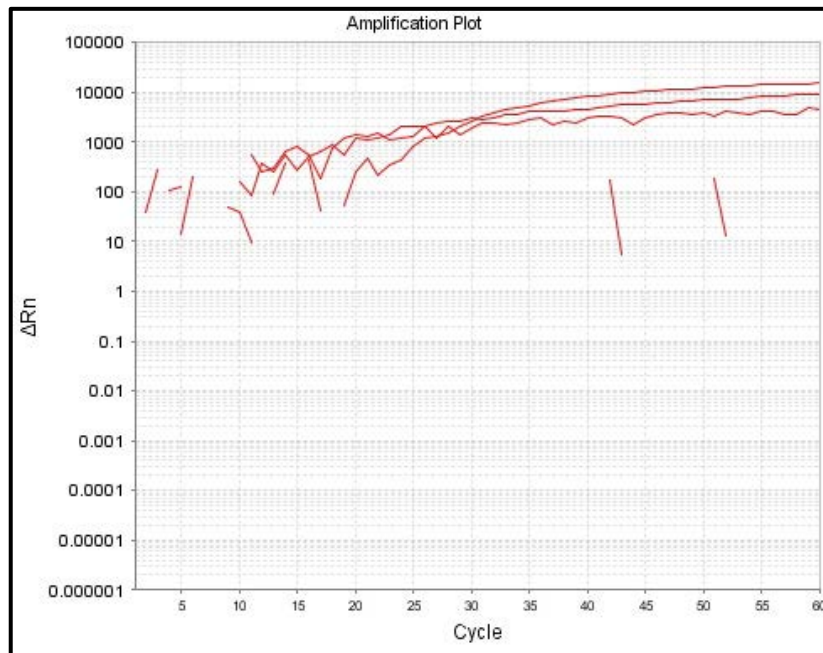


Figure 3.15: Real-time amplification of a sample with $\Delta 296$ to 446 using probes and HRM 1 primers. Linear amplification with three of the four probes was observed, indicating that extended amplification of HRM region 1 had occurred into the area of the deletion variant. The remaining section of HRM region 2 and start of HRM region 4 was lost due to the deletion. Therefore, the probes had been designed complementary to the remaining sections of HRM regions 2 and 4. The extended amplification of the HRM 1 amplicon beyond the reverse primer binding site for HRM region 1 was confirmed by the amplification of probes within HRM regions 2 and 4. This confirmed the extended amplification of HRM 1 amplicon was allowing for the detection of the deletion variants in HRM regions 2 to 4.

3.3.6 Tandem duplication of the kinase domain

Two samples (2-2 and 4-6) had proven previously impossible to sequence since the region of *BCR-ABL* encoding for the kinase domain could not be PCR amplified successfully using semi-nested PCR. However, when screened with HRM, variants in HRM regions 1 to 4 were identified for these samples compared to the wild-type on the difference plot (Figures 3.16 to 3.19). The HRM amplification product of these samples was resolved using gel electrophoresis and approximately 1,150 bp fragment was observed in addition to the expected fragment in samples 2-2 and 4-6 (Figure 3.20). It was suspected that the larger fragment of 1,150 bp may represent a tandem duplication in *BCR-ABL* in the region of the kinase domain. To test this hypothesis, the larger fragment was band stabbed from the gel, placed in sterile water and left over night at 4°C. The band stab product was used to PCR amplify HRM regions 1 to 4 confirming that the larger fragment in samples 2-2 and 4-6 represented a tandem duplication (Figure 3.21 and 3.22). Thus, HRM was used to detect a tandem duplication in *BCR-ABL* in the region of the kinase domain not identified by sequencing. Although the impact that the tandem duplication has on treatment with a tyrosine kinase inhibitor is not yet known, the literature suggests that duplications involving *BCR-ABL* can result in resistance to imatinib (Bixby and Talpaz, 2009).

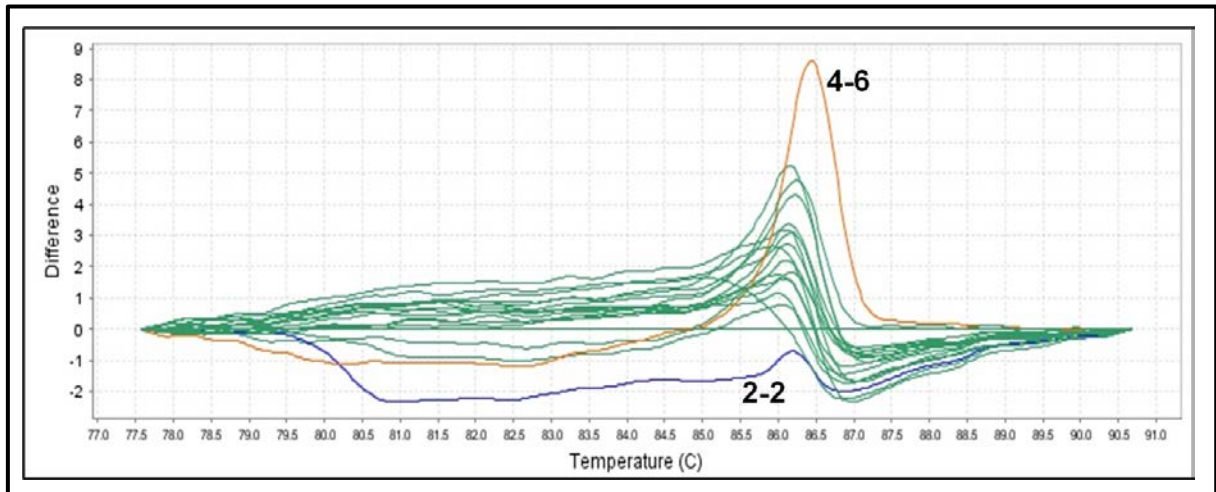


Figure 3.16: Difference plot following HRM analysis of HRM region 1 indicating two samples (2-2 and 4-6) that were grouped as variants, compared to the wild-type. Samples 2-2 and 4-6 contained a tandem duplication of the kinase domain.

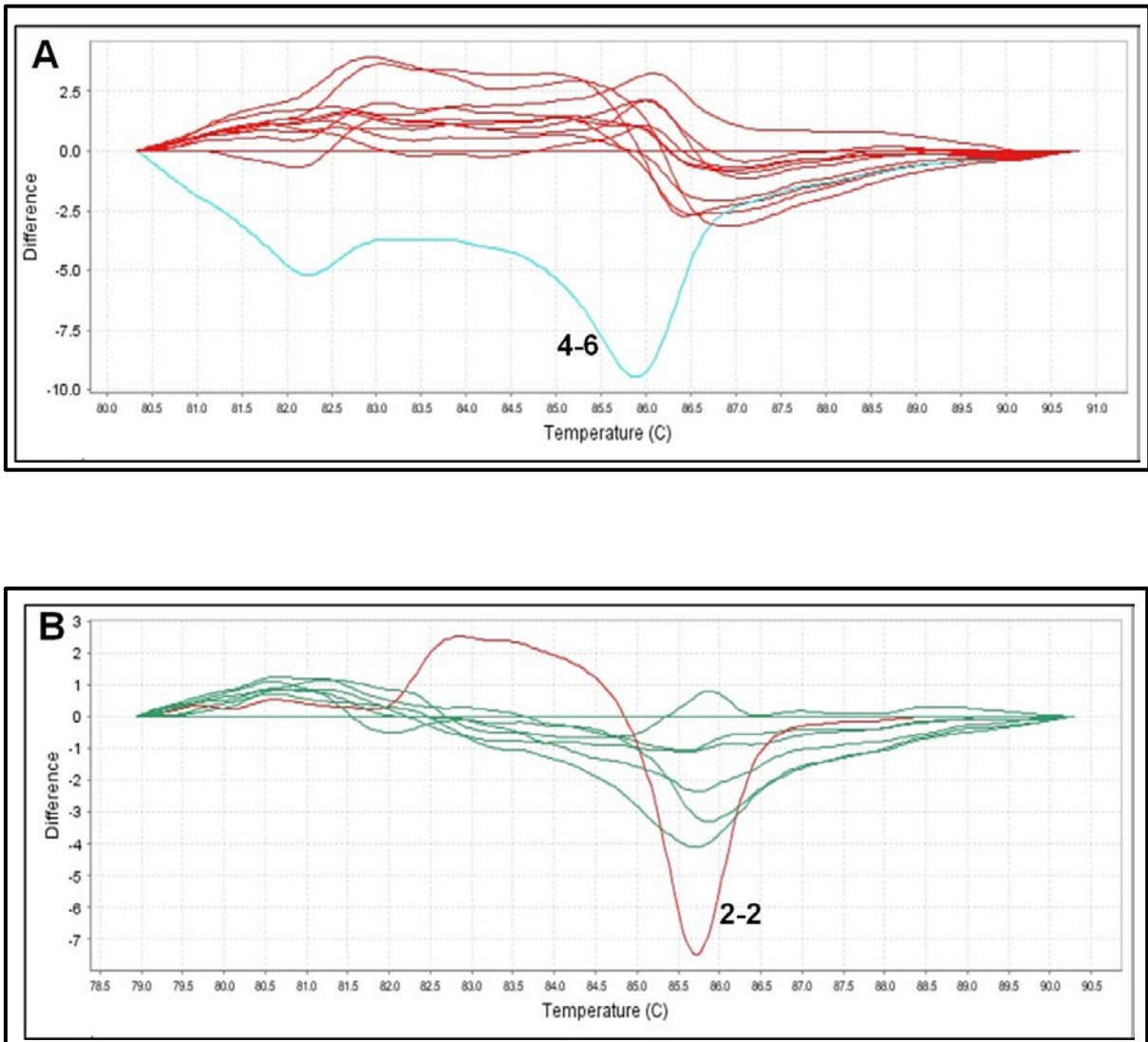


Figure 3.17: Difference plots A and B following HRM analysis of HRM region 2 indicating two samples (2-2 and 4-6) that were grouped as variants, compared to the wild-type. Samples 2-2 and 4-6 contained a tandem duplication of the kinase domain.

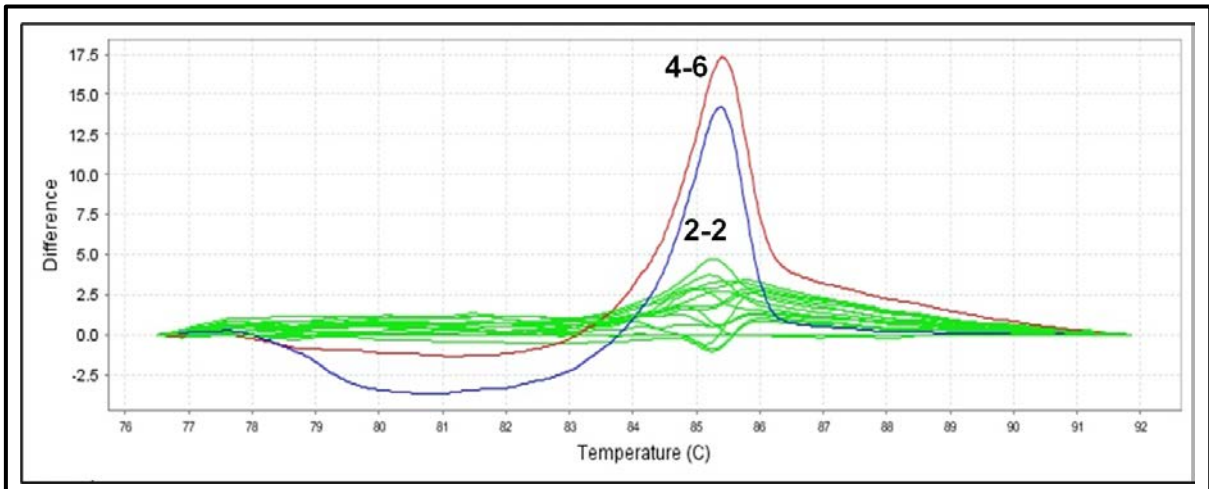


Figure 3.18: Difference plot following HRM analysis of HRM region 3 indicating two samples (2-2 and 4-6) that were grouped as variants, compared to the wild-type. Samples 2-2 and 4-6 contained a tandem duplication of the kinase domain.

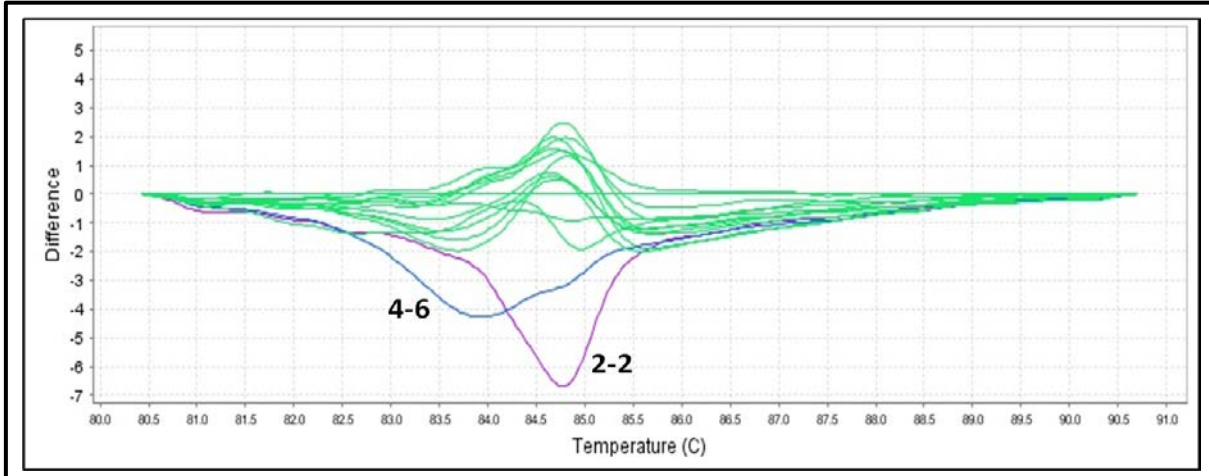


Figure 3.19: Difference plot following HRM analysis of HRM region 4 indicating two samples (2-2 and 4-6) that were grouped as variants, compared to the wild-type. Samples 2-2 and 4-6 contained a tandem duplication of the kinase domain.

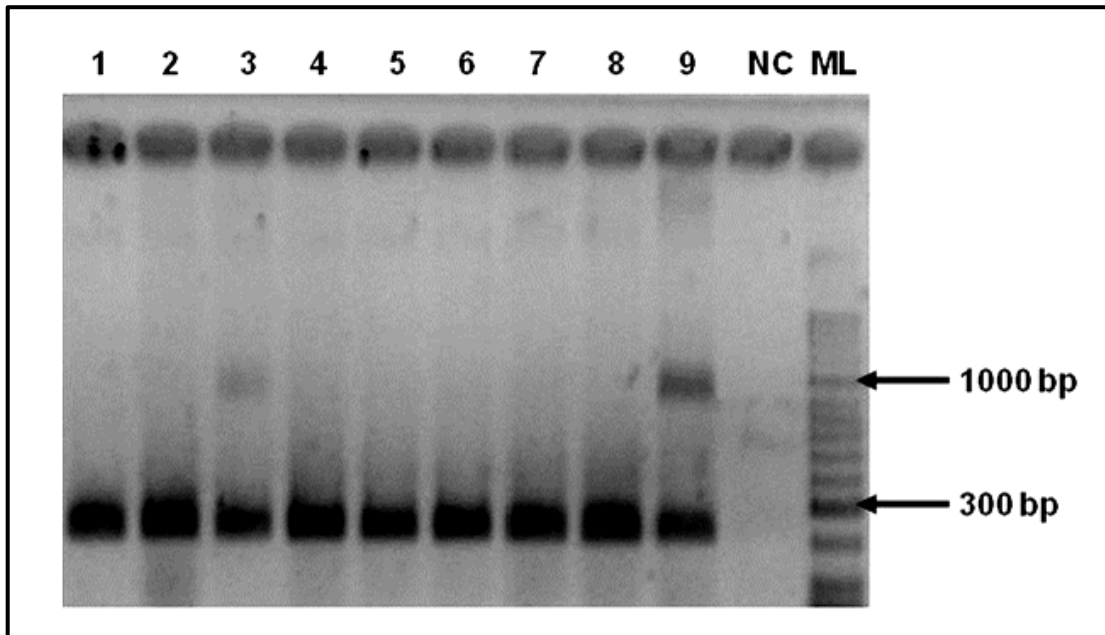


Figure 3.20: Negative inverted gel image of HRM 2 amplicon. HRM 2 amplicon for samples with mutations was resolved on a 2% agarose gel in 1X TAE buffer. Thereafter the gel was stained with ethidium bromide and visualised under UV light. The expected fragment of 225 bp was observed in lanes 1 to 9. An additional larger fragment of approximately 1,150 bp was faintly observed in lane 3 while in lane 9 it was more defined. The additional fragment was confirmed to be a tandem duplication of the kinase domain. Lane 1 – sample 19-3; lane 2 – sample 11-3; lane 3 – sample 2-2; lane 4 – sample 50-6; lane 5 – sample 48-6; lane 6 – sample 76-1; lane 7 – sample 44-12; lane 8 – sample 55-16; lane 9 – sample 4-6; lane NC – negative control and lane ML – molecular ladder (Hyperladder II).

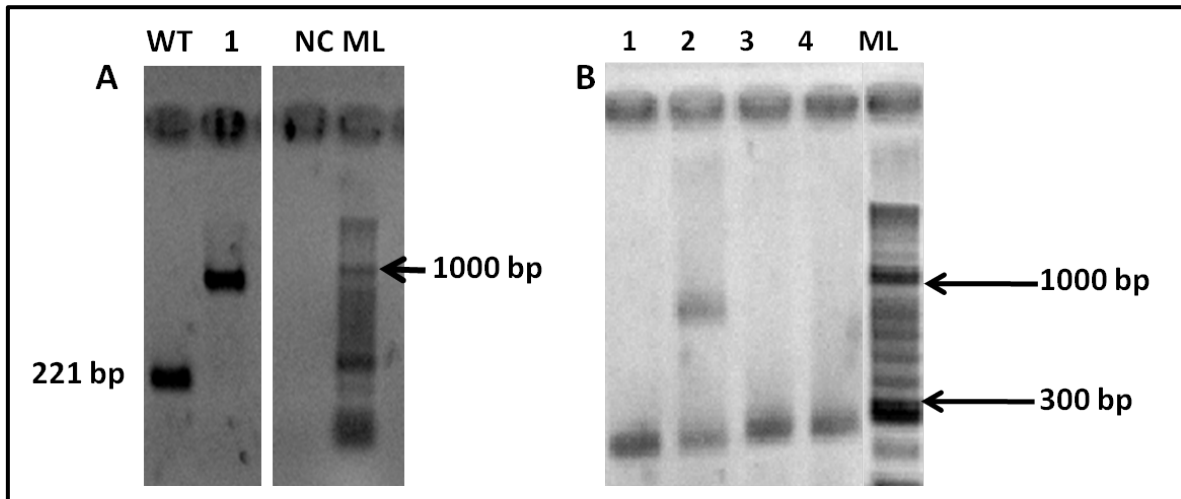


Figure 3.21: Negative inverted gel image of conventional PCR products of a sample with a tandem duplication of the kinase domain. Gel images of conventional PCR products that have been resolved on a 2% agarose gel in 1X TAE buffer after staining with ethidium bromide and visualized under UV light. **A:** Conventional PCR results of sample 4-6 with a tandem duplication of the kinase domain, indicated by the presence of a larger amplicon of approximately 1,150 bp. Lane WT – wild-type; lane 1 - sample 4-6; lane NC – negative control and lane ML – molecular ladder (Hyperladder II). **B:** Results of the conventional PCR using the larger amplicon (excised from gel A) as template and regions HRM 1 to 4 primers. The observed amplicon was the expected fragment size for HRM regions 1 to 4, respectively. Lane 1 – amplicon of HRM region 1 (221 bp); lane 2 – amplicon of HRM region 2 (225 bp); lane 3 – amplicon of HRM region 3 (239 bp); lane 4 – amplicon of HRM region 4 (240 bp) and lane ML – molecular ladder (Hyperladder II). The negative control and molecular ladder in gel A is from the same gel image as unnecessary data have been excluded. The same principle applies for gel B.

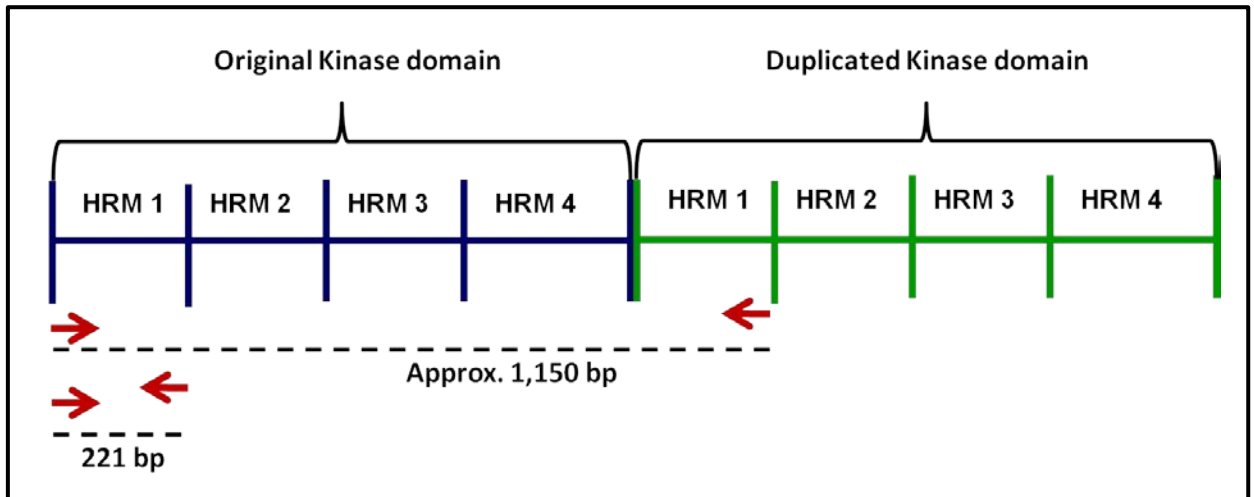


Figure 3.22: Schematic diagram representing of the tandem duplication of the region of *BCR-ABL* encoding for the kinase domain. The original kinase domain is represented in blue, while the tandem duplication of the kinase domain is in green. PCR amplification using HRM primers produced either the amplicon with expected fragment size of 221 bp or an amplicon with approximate size of 1,150 bp.

3.4 Conclusions

High-resolution melting was successfully used to screen for mutations including single base changes, insertions, deletions and duplications in *BCR-ABL* in the region of the kinase domain. While differences observed in T_m between samples with and without mutations were insufficient to indicate the presence of the mutation, the difference plot identified variants with mutations compared to the wild-type.

This is the first report of using HRM to identify sequence variants with insertions and duplications in *BCR-ABL* in the region of the kinase domain. Furthermore, HRM proved successful to detect deletion variants in HRM regions outside the area affected by the deletion. Although surprising, these results were repeated consistently during the duration of this study. In terms of its application in CML diagnostics, HRM has shown to be as efficient and sensitive as sequencing but with a time saving consideration as only samples with possible mutations would need to be sequenced.

One possible limitation in this study was the coverage of the region of *BCR-ABL* encoding for the kinase domain by HRM region primers. For example, primers 3F and 4R used in the amplification of HRM region 3 and 4, only had a two base overlap. Although no described single base mutations occur in the overlapping region of HRM 3 and 4, novel mutations could be missed to due to the lack of overlap. It is also important to note that if a mutation disrupts primer binding, no amplification of that HRM region would occur. Therefore, a future study could include designing primers for better coverage of the region of *BCR-ABL* encoding for the kinase domain including the use of probes to identify deleted HRM regions.

In this study, 40 patient samples were successfully screened for mutations using HRM. Using HRM as a pre-sequencing screen would have reduced the sequencing reactions by more than half (40% of current sequence) in addition to

the time saved (3 hrs vs 25 hrs). Furthermore, HRM identified a tandem duplication in *BCR-ABL* in the region of the kinase domain that was not detectable using sequencing. In conclusion, high-resolution melting is a suitable, rapid and cost-effective method to screen the region of *BCR-ABL* encoding for the kinase domain for mutations including single base mutations, insertions, deletions and duplications.

SUMMARY

CML is a haematological malignancy that is characterized by the *BCR-ABL* fusion oncogene that encodes a constitutively active tyrosine kinase. The treatment of choice for CML is a tyrosine kinase inhibitor and molecular monitoring of patients forms an integral part of disease management. When the expected response to tyrosine kinase inhibitor is not achieved within internationally accepted time frames, acquired resistance to tyrosine kinase inhibitors is suspected.

Acquired resistance to tyrosine kinase inhibitors is primarily due to mutations in the *BCR-ABL* kinase domain. Types of mutations include single base mutations, insertions, deletions as well as duplications. Characterization of these mutations is important for treatment, since the type and position of the mutation may have an effect on how the patient responds to treatment. Although several methods have been described for detecting mutations, DNA sequencing is mostly used. Sequencing is currently the only technique that can simultaneously detect single base mutations, insertions and deletions in the *BCR-ABL* kinase domain. However, sequencing is costly as some patient samples do not have mutations and the lack of response to treatment is due to non-compliance. Thus, a screening method to exclude samples without mutations would make mutational analysis more cost-effective.

High resolution melting (HRM) is a relatively new technique that is being used to screen for mutations, prior to sequencing. HRM has recently been used to screen the region of *BCR-ABL* encoding for the kinase domain for single base mutations. However, it was unknown whether HRM could be used to identify insertions, deletions or duplications in the kinase domain. This study has shown that HRM can be used to screen for mutations including insertions, deletions and duplications the region of *BCR-ABL* encoding for the kinase domain, prior to sequencing.

HRM was performed on 40 patient samples, 10 of which had confirmed mutations in *BCR-ABL* in the region of the kinase domain. Of the 10 samples with mutations, three had single base mutations, one with a previously described insertion, seven had novel deletion variants. Furthermore, HRM detected a tandem duplication of the kinase domain in two patient samples that was not previously been possible with sequencing. There was 100% congruency between the detection of mutations using HRM and sequencing results, indicating similar sensitivity. HRM proved successful to indicate the presence of deletion variants. However, the deletion variants were detected in the HRM region preceding the area affected by the deletion. It was confirmed that the detection of the deletion variants was due to the PCR extension of HRM 1 amplicon into the HRM area of the deletion.

It has been suggested that the insertion, deletions and duplications detected in this study may result in acquired resistance to tyrosine kinase inhibitor. In conclusion, this was the first study to use high-resolution melting to detect insertions, deletions and duplications in the region of *BCR-ABL* encoding for the kinase domain, indicating the suitability of the assay for screening for mutations prior to sequencing.

KEY WORDS:

Chronic myeloid leukaemia

BCR-ABL kinase domain

High-resolution melting

Insertion, deletions, tandem duplication

OPSOMMING

CML is 'n hematologiese maligniteit gekenmerk deur die BCR-ABL-fusie-onkogene wat 'n wesenlik aktiewe tirosienkinase kodeer. Die voorkeurbehandeling van CML is tirosienkinase-inhibeerders met molekulêre monitering van pasiënte as integrale deel daarvan. Wanneer die verwagte respons op behandeling nie binne bepaalde tydsraamwerke bereik word nie, word verworwe bestandheid teen tirosienkinase-inhibeerders vermoed.

Verworwe bestandheid teen tirosienkinase-inhibeerders is hoofsaaklik die gevolg van mutasies in die BCR-ABL-kinasedomein. Mutasies sluit enkelbasisveranderinge, invoegings, delesies en duplikasies in. Karakterisering van die mutasies is belangrik aangesien die tipe en posisie van die mutasie die verwagte respons op behandeling kan beïnvloed. Alhoewel verskillende metodes al vir die opsporing van mutasies beskryf is, word DNS-volgordebepaling meestal gebruik. DNS-volgordebepaling is tans die enigste metode wat gelyktydig enkelbasismutasies, invoegings, en delesies in die ABL-BCR-kinasedomein kan aantoon. Volgordebepaling is egter duur en nie alle pasiënte het mutasies nie want verworwe bestandheid is dikwels die gevolg van nienakoming van behandeling. Mutasie-analise sal meer koste-effektief wees met 'n siftingsmetode wat pasiënte sonder mutasies kan uitsluit.

Hoë-resolusie-smeltkromme-analise (HRM) is 'n relatiewe nuwe siftingsmetode wat gebruik word om mutasies te identifiseer voordat volgordebepaling gedoen word. HRM is onlangs gebruik om die kinasedomein van BCR-ABL vir enkelbasisveranderinge te ondersoek. Dit is egter nie bekend of HRM gebruik kan word om invoegings, delesies of duplikasies in die kinasedomein te identifiseer nie. Hierdie studie het getoon dat HRM wel vir hierdie doel aangewend kan word voordat volgordebepaling gedoen word.

HRM is op 40 CML-pasiënte, 10 met bevestigde mutasies in die BCR-ABL-kinasedomein, uitgevoer. Van die 10 pasiënte met mutasies, het drie

enkelbasisveranderinge gehad, een 'n voorheen beskryfde invoeging, en sewe 'n verskeidenheid van delesievariante. Nogtans, het HRM 'n duplikasie van die kinasedomein in twee pasiënte aangetoon wat nie moontlik was met volgordebepaling nie. Daar was 100% ooreenstemming tussen die resultate van HRM en volgordebepaling, wat dui op gelyksoortige sensitiwiteit. HRM was suksesvol om die teenwoordigheid van delesies aan te toon. Die delesie-variante is egter bespeur in die HRM-streek voor die gebied van die kinasedomein wat deur die delesie geraak word. Dit bevestig dat die opsporing van die delesie-variante die gevolg was van PKR (polimerasekettingreaksie-uitbreiding van HRM-1-amplikon in die HRM-gebied waar die delesie voorkom.

Dit wil voorkom asof die invoeging, delesies en duplikasies wat in hierdie studie aangetoon is, tot verworwe bestandheid teen tirosienkinase kan lei. Ten slotte, is hierdie die eerste studie waar hoë-resolusie-smeltekromme-analise gebruik is om die kinasedomein van BCR-ABL te ondersoek vir invoegings, delesies en duplikasies, en resultate dui op die geskiktheid daarvan as sitingstoets vir mutasies voordat volgordebepaling gedoen word.

REFERENCES

- Agis H, Sperr W, Herndhofer S, Semper H, Pirc-Danoewinata H, Haas O, Mannhalter C, Esterbauer H, Geissler K, Sillaber C, Jager U and Valent P. 2007. Clinical and prognostic significance of histamine monitoring in patients with CML during treatment with Imatinib (STI571). *Annals of Oncology* 343: 1 - 8.
- Alimena G, Dallapiccola B, Mitelman F and Montuoro A. 1980. Aberrations of chromosome no 1 in blastic phase of chronic myeloid leukemia. *Hereditas* 92: 59 - 63.
- Alimena G, Billstrom R, Casalone R, Gallo E, Mitelman F and Pasquali F. 1985. Cytogenetic pattern in leukemic cells of patients with constitutional chromosomal abnormalities. *Cancer Genetics and Cytogenetics* 16: 207 - 218.
- Baccarani M, Sagilo G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, Apperley J, Cervantes F, Cortes J, Deininger M, Gratwohl A, Guilhot F, Horowitz M, Hughes T, Kantarjian H, Larson R, Niederwieser D, Siver R and Hehlmann R. 2006. Evolving concepts in the management of chronic myeloid leukaemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* 108 (6): 1809 - 1820.
- Baccarani M, Cortes J, Pane F, Niederwieser D, Sagilo G, Apperley J, Cervantes F, Deininger M, Gratwohl A, Guilhot F, Hochhaus A, Horowitz M, Hughes T, Kantarjian H, Larson R, Radich J, Simonsson B, Silver R, Goldman J and Hehlmann R. 2009. Chronic myeloid leukaemia: An update of concepts and management recommendations of the European LeukemiaNet. *Journal of Clinical Oncology* 27 (35): 6041 - 6051.
- Bénet C, Gomez A, Aguilar C, Delattre C, Vergier B, Beylot-Barry M, Fraitag S, Carlotti A, Dechelotte P, Hospital V, d'Incan M, Costes V, Dereure O, Ortonne N, Bagot M, Laroche L, Blom A, Dalac S and Petrella T. 2011. Histologic and immunohistologic characterization of skin localization of myeloid disorders. *American Journal of Clinical Pathology* 135: 278 - 290.

- Bennett J. 1845. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. *Edinburgh Medical and Surgical Journal* 64: 413 - 423.
- Bikker J, Brooijmans N, Wissnwe A and Mansour T. 2008. Kinase domain mutations in Cancer: Implications for small molecular drug design strategies. *Journal of medical chemistry* 52 (6): 1 - 16.
- Bixby D and Talpaz M. 2009. Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance. *Hematology* 1: 461 - 475.
- Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch K and Hughes T. 2002. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with Chronic Myeloid Leukemia or Ph-positive Acute Lymphoblastic Leukemia who develop Imatinib (ST1571) resistance. *Blood* 99 (9): 3472 - 3475.
- Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Seer J, Taylor K, Herrmann R, Seymour J, Arthur C, Joske D, Lynch K and Hughes T. 2003. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 102 (1): 276 - 283.
- Branford S and Hughes T. 2006. Detection of BCR-ABL mutations and resistance to imatinib mesylate. *Methods in molecular medicine* 125: 93 - 106.
- Branford S. 2007. Chronic myeloid leukaemia: Molecular monitoring in clinical practice. *Haematology* 1: 376 - 383.
- Calabretta B and Perrotti D. 2004. The biology of the blast crisis. *Blood* 103 (11): 4010 - 4022.
- Caramazzo D, Hussein K, Siragusa S, Pardanani A, Knudson R, Ketterling R, Tefferi A. 2009. Chromosome 1 abnormalities in myeloid malignancies: a

- literature survey and karyotype – phenotype associations. *European Journal of Haematology* 84: 191 - 200.
- Cerretini R, Acevedo S, Chena C, Belli C, Larripa I and Slavutsky I. 2002. Evaluation of constitutional chromosomal aberrations in hematologic disorders. *Cancer Genetics and Cytogenetics* 134: 133 - 137.
- Chaganti R, Jhanwar S, Arlin Z and Clarkson B. 1981. Chronic myelogenous leukemia in an XYY male. *Cancer Genetics and Cytogenetics* 5 (3): 223 - 226.
- Chomel J, Sorel N, Bonnet M, Bertrand A, Brizard F, Saulnier P, Roy L, Guilhot F and Turhan A. 2009. Quantitative monitoring of the T315I mutation in patients with chronic myeloid leukaemia (CML). *Leukemia research* 33: 551 - 555.
- Corbin A, La Rosee P, Stoffregen E, Druker B and Deininger M. 2003. Several Bcr-Abl kinase domain mutants associated with Imatinib mesylate resistance remain sensitive to Imatinib. *Blood* DOI: 10.1182/blood-2002-12-3659.
- Cowen-Jacob S, Fendrich G, Floersheimer A, Furet P, Liebetanz J, Rummel G, Rheinberger P, Centeleghe M, Fabbro D and Manley P. 2006. Structural biology contributions to the discovery of drugs to treat Chronic Myelogenous Leukemia. *Biological Crystallography* 63: 80 - 93.
- Craigie D. 1845. Case of disease of the spleen in which death took place from suppuration of the blood. *Edinburgh Medical and Surgical Journal* 64: 400 - 412.
- Deininger M, Goldman J and Melo J. 2000. The molecular biology of Chronic Myeloid Leukemia. *Blood* 96(10): 3343 - 3356.
- Diamond J and Melo J. 2011. Mechanisms of resistance to BCR-ABL kinase inhibitors. *Leukemia and Lymphoma* 52 (S1): 12 - 22.
- Doi Y, Sasaki D, Terada C, Mori S, Tsuruda K, Matsuo E, Miyasaki Y, Nagai K, Hasegawa H, Yanagihara K, Yamada Y and Kamihira S. 2009. High-

- resolution melting analysis for a reliable and two-step scanning of mutations in the tyrosine kinase domain of the chimerical *bcr-abl* gene. *International journal of Haematology* 90 (1): 37 - 43.
- Druker B, Talpaz M, Resta D, Peng B, Buchdunger E, Ford J, Lydon N, Kantarjian H, Capdeville R, Ohne-Jones S and Sawyers C. 2001. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in Chronic Myeloid Leukemia. *The New England Journal of Medicine* 334 (14): 1031 - 1037.
- Druker B, Guilhot F, O'Brien S, Gathmann I, Kantarjian H, Gattermann N, Deininger M, Silver R, Goldman J, Stone R, Cervantes F, Hochhaus A, Powell B, Gabrilove J, Rousselot P, Reiffers J, Cornelissen J, Hughes T, Agis H, Fischer T, Verhoef G, Shepherd J, Saglio G, Gratwohl A, Nielsen J, Radich J, Simonsson B, Taylor K, Baccarani M, Letvak L and Larson R. 2006. Five –year follow up of patients receiving Imatinib for chronic myeloid leukaemia. *New England Journal of Medicine* 355 (23): 2408 - 2417.
- Erali M, Voelkerding K and Wittwer C. 2008. High resolution melting applications for clinical laboratory medicine. *Experimental and Molecular Pathology* 85: 50 - 58.
- Ernst T, Hoffmann J, Erben P, Hanfstein B, Leitner A, Hehlmann R, Hochhaus A and Müller M. 2008. ABL single nucleotide polymorphisms may masquerade as BCR-ABL mutations associated with resistance to tyrosine kinase inhibitors in patients with chronic myeloid leukaemia. *Haematologica* 93 (9): 1389 - 1393.
- Ernst T, Erben P, Müller M, Paschka P, Schenk T, Hoffmann J, Kreil S, La Rosée P, Hehlmann R and Hochhaus A. 2008. Dynamics of BCR-ABL mutated clones prior to hematologic or cytogenetic resistance to Imatinib. *Haematologica* 93 (2): 187 - 192.
- Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R and Kantarjian H. 1999. The Biology of Chronic Myeloid Leukemia. *The New England Journal of Medicine* 341 (3): 164 - 172.

- Fialkow P, Jacobson R and Papayannopoulou T. 1977. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *American Journal of Medicine* 63: 125 - 130.
- Foroni L, Wilson G, Gerrard G, Mason J, Grimwade D, White H, Gonzalez de Castro D, Austin S, Awan A, Burt E, Clench T, Farruggia J, Hancock J, Irvine A, Kizilors A, Langabeer S, Milner B, Nickless G, Schuh A, Sproul A, Wang L, Wickham C and Cross N. 2011. Guidelines for the measurement of BCR-ABL1 transcripts in chronic myeloid leukaemia. *British Journal of Haematology* 153: 179 - 190.
- Frazer R, Irvine A and McMullin M. 2007. Chronic Myeloid Leukemia in the 21st Century. *The Ulster Medical Society* 76 (1): 8 - 17.
- Gamabacorti-Passerini C, Barni R, le Coutre P, Zucchetti M, Cabrita L, Cleris F, Rossi E, Gianazza E, Brueggen J, Cozens P, Pioltelli P, Pogliani E, Corneo G, Formelli F and D'Incalci M. 2000. Role of α 1 acid glycoprotein in the *in vivo* resistance of human BCR-ABL (+) leukemic cells to the Abl inhibitor STI571. *Journal of the National Cancer Institute* 92: 1641 - 1650.
- Gamabacorti-Passerini C, Gunby R, Piazza R, Galiotta A, Rostagno R and Scapozza L. 2003. Molecular mechanisms of resistance to Imatinib in Philadelphia-chromosome-positive leukemiaeas. *The Lancet* 4: 75 - 85.
- Goldman J and Melo J. 2001. Targeting the BCR-ABL tyrosine kinase in Chronic Myeloid Leukemia. *The New England Journal of Medicine* 334 (14): 1084 - 1086.
- Goldman J and Melo J. 2003. Chronic Myeloid Leukemia – Advances in Biology and New approaches to Treatment. *The New England Journal of Medicine* 349 (15): 1451 - 1464.
- Goldman J. 2004. Chronic Myeloid Leukemia – still a few questions. *Experimental Haematology* 32: 2 - 10.

- Goldman J. 2007. How I treat chronic myeloid leukaemia in the imatinib era. *Blood* 110: 2828 - 2837.
- Gorre M, Mohammed M, Ellwood K, Hsu N, Raquette R, Rao P and Sawyers S. 2001. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 293: 876 - 880.
- Groffen J, Stephenson J, Heisterkamp N, de Klein A, Bartarm C and Grosveld G. 1984. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 36: 93 - 99.
- Gruber F, Lundan T, Goll R, Silye A, Mikkola I, Rekvig O, Knuutila S, Remes K, Gebbe-Dahl T, Porkka K and Hjorth-Hansen H. 2011. BCR-ABL isoforms associated with intrinsic or acquired resistance to imatinib: more heterogeneous than just ABL kinase domain point mutations. *Medical Oncology* epub: DOI 10.1007/s12032-010-9781-z.
- Guilhot F and Guilhot J. 2011. Predicting response in CML. *Blood* 117 (6): 173.
- Guitérrez M, Timson G, Siraji A, Barbhaya S, Banavali S and Bhatia K. 2005. Single monochrome Real-time RT-PCR assay for identification, quantification and breakpoint cluster region determination of t(9;22) transcripts. *Journal of Molecular diagnostics* 7 (1): 40 - 47.
- Gundry C, Vandersteen J, Reed G, Pryor R, Chen J and Wittwer C. 2003. Amplicon melting analysis with labelled primers: A closed-tube method for differentiating homozygotes and heterozygotes. *Clinical Chemistry* 49 (3): 396 - 406.
- Gundry C, Dobrowolski S, Martin Y, Robbins T, Nay L, Boyd N, Coyne T, Wall M, Wittwer C and Teng D. 2008. Base-pair neutral homozygous can be discriminated by calibrated high-resolution melting of small amplicons. *Nucleic acid research* 36 (10): 3401 - 3408.
- Hanfstein B, Muller M, Kreil S, Ernst T, Schenk T, Lorentz C, Schwindel U, Leitner A, Hehlmann R and Hochhaus A. 2011. Dynamics of mutant *BCR-ABL*

- positive clones after cessation of tyrosine kinase inhibitor therapy. *Hematologica* 96 (3): 361 - 366.
- Hasford J, Pfirrmann M, Hehlmann R, Allan N, Baccarani M, Kluin-Nelemans J, Steegmann G and Ansari H. 1998. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. *Journal of the National Cancer Institute* 90 (11): 1 - 9.
- Heaney N and Holyoake T. 2007. Educational article – What is new in Chronic Myeloid Leukemia? *Scottish Medical Journal* 52 (1): 36 - 41.
- Heideman D, Thunnisen F, Doeleman M, Kramer D, Verheul H, Smit E, Postmus P, Meijer C, Meijer G and Snijders P. 2009. A panel of high resolution melting (HRM) technology-based assays with direct sequencing possibility for effective mutation screening of EGFR and K-ras genes. *Clinical Oncology* 31: 329 - 333.
- Hehlmann R, Berger U and Hochhaus A. 2005. Chronic myeloid leukaemia: a model for oncology. *Annals of Hematology* 84: 487 - 497.
- Hehlmann R, Hochhaus A and Baccarani M. 2007. Chronic myeloid leukaemia. *The Lancet* 370: 342 - 350.
- Hehlmann R, Jung-Munkwitz S and Saußebe S. 2011. Treatment of chronic myeloid leukaemia when imatinib fails. *Expert Opinions of Pharmacotherapy* 12 (2): 269 - 283.
- Herrmann M, Durtschi J, Bromley L, Wittwer C and Voelkerding K. 2006. Amplicon DNA melting analysis for mutation scanning and genotyping: Cross-Platform comparison of instruments and dyes. *Clinical Chemistry* 52 (3): 494 - 503.
- Hochhaus A, Kreil S, Corbin A, La Rosée P, Müller M, Lahaye T, Hanfstein B, Schoch C, Cross N, Berger U, Gschaidmeier H, Druker B and Helmann R. 2002. Molecular and chromosomal mechanisms of resistance to Imatinib (ST1571) therapy. *Leukemia* 16: 2190 - 2195.

- Hochhaus A. 2006. Treatment with tyrosine kinase inhibitors. *Hematologic Malignancies: Myeloproliferative disorders 1*: 109 - 114.
- Horton R. 2006. Nucleic acid sequence massager [online] Available at <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi> [Accessed 30 September 2011].
- Huang and Muller. 1991. LALIGN [online] Available at http://www.ch.embnet.org/software/LALIGN_form.html [Accessed 2 February 2010].
- Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, Baccarani M, Cortes J, Cross N, Druker B, Gabet J, Grimwade D, Hehlmann R, Kamel-Reid S, Lipton J, Longtine J, Martinelli G, Saglio G, Soverini S, Stock W and Goldman J. 2006. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: A review and recommendation for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* 108 (1): 28 - 37.
- Hughes T and Branford S. 2009. Monitoring disease response to tyrosine kinase inhibitor therapy in CML. *Hematology* 1: 447 - 487.
- Ilaria R. 2005. Pathobiology of lymphoid and myeloid blast crisis and management issues. *American Society of Hematology* 1: 188 - 194.
- Iqbal Z, Siddiqui R, Qureshi J and Khalid A. 2004. Case study of primary Imatinib resistance and correlation of BCR-ABL multiple mutations in Chronic Myeloid Leukemia. *Therapy* 2: 249 - 254.
- Jabbour E, Cortes J, Kantarjian H. 2009. Suboptimal response to or failure of Imatinib treatment for chronic myeloid leukaemia: What is the optimal strategy? *Mayo Clin Proc* 84 (2): 161 - 169.
- Jabbour E, Kantarjian H, O'Brien S, Shah J, Garcia-Manero G, Wierda W, Ravandi F, Borthakur G, Rios M and Cortes J. 2011. Predictive factors for outcome and response in patients treated with second-generation tyrosine kinase

- inhibitors for chronic myeloid leukaemia in chronic phase after imatinib failure. *Blood* 117 (6): 1822 - 1827.
- Jeong S, Hahn Y, Rong Q and Pfeifer K. 2007. Accurate quantification of allele-specific expression by analysis of DNA melting. *Genome Research* 17: 1093 - 1100.
- Jones D, Kamel-Reid S, Bahler D, Dong H, Elenitoba-Johnson K, Press R, Quigley N, Rothberg P, Sabath D, Viswanatha D, Weck K and Zehnder J. 2009. Laboratory practice guidelines for detecting and reporting BCR-ABL drug resistance mutations in chronic myelogenous leukemia and acute lymphoblastic leukemia. *Journal of Molecular Diagnostics* 11 (1): 4 - 11.
- Kaddu S, Zenahlik P, Beham-Schmid C, Kerl H and Cerroni L. 1999. Specific cutaneous infiltrates in patients with myelogenous leukaemia: A clinicopathologic study of 26 patients with assessment of diagnostic criteria. *Journal of American Academy of Dermatology* 40 (6): 966 - 978.
- Kaddu S, Beham-Schmid C, Zenahlik P and Cerroni L. 1999. CD56⁺ blastic transformation of chronic myeloid leukaemia involving the skin. *Journal of cutaneous pathology* 26: 497 - 503.
- Kang H-Y, Hwang J-Y, Kim S-H, Goh H, Kim M and Kim D. 2006. Comparison of allele specific oligonucleotide-polymerase chain reaction and direct sequencing for high throughput screening of ABL kinase domain mutations in chronic myeloid leukaemia resistant to Imatinib. *Haematologica/The haematology journal* 91 (5): 659 - 662.
- Kantarjian H, O'Brien S, Smith T, Rios M, Cortes J, Beran M, Koller C, Giles F, Andreeff M, Kornblau S, Giralt S, Keating M and Talpaz M. 1999. Treatment of Philadelphia chromosome-positive early chronic phase chronic myelogenous leukaemia with daily doses of Interferon Alpha and low-dose Cytarabine. *Journal of Clinical Oncology* 17 (1): 284 - 292.
- Kantarjian H, Melo J, Tura S, Giralt S and Talpaz M. 2000. Chronic myelogenous leukemia: Disease biology and current and future therapeutic strategies. *Hematology* (1): 90 - 109.

- KAPABIOSYSTEMS version 1.10. 2011. Introduction to high-resolution melting curve analysis. 1 - 17.
- Khorashad J, Thelwell N, Milojkovic D, Marin D, Watson J, Goldman J, Apperley J, Foroni L and Reid A. 2008. A new rapid and sensitive assay for detection the T315I *BCR-ABL* kinase domain mutation in chronic myeloid leukaemia. *Journal of Clinical Pathology* 61: 863 - 865.
- Krause D and Van Etten R. 2005. Tyrosine kinases as targets for cancer therapy. *New England Journal of Medicine* 353: 172 - 187.
- Krypuy M, Newnham G, Thomas D, Conron M and Dobrovic A. 2006. High resolution melting analysis for the rapid and sensitive detection of mutations in clinical sample: *KRAS* codon 12 and 13 mutations in non-small cell lung cancer. *Biomedical Central Cancer* 6 (295): 1 - 12.
- Kurzrock R, Gutterman J and Talpaz M. 1988. The molecular genetics of Philadelphia chromosome positive leukemias. *New England Journal of Medicine* 319: 990 - 998.
- Kwong. 2007. Chronic myeloid leukaemia. *The Hong Kong medical diary* 12 (4): 13 - 15.
- Laneuville P, Barnett M, Bélanger R, Couban S, Forrest D, Roy D and Lipton J. 2006. Recommendations of the Canadian Consensus Group on the management of Chronic Myeloid Leukaemia. *Current Oncology* 13 (6): 201 - 221.
- Laudadio J, Deininger M, Mauro M, Druker B and Press R. 2008. An intron-derived insertion/truncation mutation in the *BCR-ABL* kinase domain in chronic myeloid leukaemia patients undergoing kinase inhibitor therapy. *Journal of Molecular Diagnostics* 10 (2): 177 - 180.
- Le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, Cabrita G, Marchesi E, Supino R and Gambacorti-Passerini C. 2000. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* 1 (95): 1758 - 1766.

- Le Coutre P. 2007. Nilotinib in chronic-phase chronic myeloid leukaemia. *Leukemias and Lymphomas*: 58 - 60.
- Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E and Wittwer C. 2004. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clinical Chemistry* 50 (7): 1156 - 1164.
- Linnet M. 1985. The leukaemias: epidemiologic aspects. *Lancet* 349: 344 - 349.
- Litz C, McClure J, Copenhaver C and Brunning R. 1993. Duplication of small segments within the major breakpoint cluster region in chronic myelogenous leukemia. *Blood* 8 (5): 1567 - 1572.
- Louw V, Dreosti L, Ruff P, Jogerssar V, Moodley D, Novitzky N, Patel A, Schmidt A and Willem P. 2011. Recommendations for the management of adult chronic myeloid leukaemia in South Africa. *South African Medical Journal* 101 (11): doi: samj.org.za/index.php/samj/article
- Lui W, Smith D, Rehtzigel K, Thibodeau S and James C. 1998. Denaturing high performance liquid chromatography (DHLPC) used in the detection of germline and somatic mutations. *Nucleic acid research* 26 (6): 1396 - 1400.
- Lui W and Markrigiorgos M. 2003. Sensitive and quantitative detection of mutations associated with clinical resistance to STI-571. *Leukemia Research* 27: 979 - 982.
- Lui Z and Cordes J. 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* 238: 1 - 37.
- Ma W, Kantarjian H, Yeh C, Zhang Z, Cortes J and Albitar M. 2009. BCR-ABL truncation due to premature translation termination as a mechanism of resistance to kinase inhibitors. *Acta Haematologica* 121: 27 - 31.
- Mahon F, Deininger M, Schultheis B, Chabrol J, Reiffers J, Goldman J and Melo J. 2000. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood* 96: 1070 - 1079.

- Mahon F, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F, Legros L, Charbonnier A, Guerci A, Varet B, Etienne G, Reiffers J and Rousset P. 2010. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncology* 11; 1029 – 1035.
- Mamaeva S, Mamaev N, Jartseva N, Belyaeva L and Scherbakova E. 1983. Complete or partial trisomy for the long arm of chromosome 1 in patients with various haematological malignancies. *Human Genetics* 63: 107 - 112.
- Marin D, Milojkovic D, Olavarria E, Khorashad J, de Lavallade H, Reid A, Foroni L, Rexvani K, Bua M, Dazzi F, Pavlu J, Klammer M, Kaeda J, Goldman J and Apperley J. 2008. European LeukemiaNet criteria for the failure or suboptimal response reliably identify patients with CML in early chronic phase treated with imatinib whose eventual outcome is poor. *Blood* 112 (12): 4437 - 4444.
- Marktel S, Marin D, Foot N, Szydlo R, Bua M, Karadimitris A, De Melo V, Kotzampaliris P, Dazzi F, Rahemtulla A, Olavarria E, Apperley J and Goldman J. 2003. Chronic myeloid leukemia in chronic phase responding to imatinib: the occurrence of additional cytogenetic abnormalities predicts disease progression. *Haematologica/journal of hematology* 88 (3): 260 - 267.
- Mathisen M, Kantarjian H, Cortes J and Jabbour E. 2011. Mutant *BCR-ABL* clones in chronic myeloid leukaemia. *Haematologica* 96 (3): 347 - 349.
- Melo J, Hughes T and Apperley J. 2003. Chronic Myeloid Leukemia. *Hematology* 1: 132 - 152.
- Melo J and Chuah C. 2007. Resistance to Imatinib mesylate in chronic myeloid leukaemia. *Cancer letters* 249: 121 - 132.
- Milojkovic D, Nicholson E, Apperley J, Holyoake T, Shepherd P, Drummond M, Szydlo R, Bua M, Foroni L, Reid A, Khorashad J, Lavallade H, Rezvani K, Paliompeis C, Goldman J and Marin D. 2010. Early prediction of success

- or failure of treatment with second-generation tyrosine kinase inhibitors in patients with chronic myeloid leukemia. *Hematologica* 95 (2): 224 - 231.
- Montgomery J, Wittwer C, Palais R and Zhou L. 2007. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. *Nature Protocols* 2 (1): 59 - 66.
- Moore M, Ekert H, Fitzgerald M and Carmichael A. 1974. Evidence of the clonal origin of chronic myeloid leukaemia from a sex chromosome mosaic: clinical, cytogenetic and marrow culture studies. *Blood* 43 (1): 15 - 22.
- Nagarajarao H, Akahtar I, Heard I and Baliga M. 2009. Unusual presentation of chronic myelogenous leukaemia as multiple skin chloromas. *Acta Cytologic* 53 (2): 235 - 238.
- Nardi V, Azam M and Daley G. 2003. Mechanisms and implications of Imatinib resistance mutations in BCR-ABL. *Current Opinions in Haematology* 11: 35 - 43.
- Nicolini F, Corm S, Le Q-H, Sorel N, Bories D, Leguay T, Roy L, Giraudier S, Tulliez M, Facon T, Mahon F, Cayuela J, Rousselot R, Michallet M, Preudhomme C, Guilhot F and Roche-Lestienne C. 2006. Mutation status and clinical outcome of 89 imatinib meylate-resistant chronic myelogenous leukaemia patients: a retrospective analysis from the French intergroup of CML. *Leukaemia* 20: 1061 - 1066.
- Nowell P and Hungerford D. 1960. A minute chromosome in human chronic granulocytic leukemia. *Science* 132: 1497.
- O'Brien S, Guilhot F, Larson R, Gathmann I, Baccarani M, Cervantes F, Cornelissen J, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen J, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwol A, Goldman J, Kantarjian H, Taylor K, Verhoef G, Bolton A, Capdeville R and Druker B. 2003. Imatinib compared with Interferon and low-dose Cytarabine for newly diagnosed Chronic-phase Chronic Myeloid Leukemia. *The New England Journal of Medicine* 348 (11): 994 - 1004.

- Oguma N, Shigeta C and Kamada N. 1996. XYY male and hematologic malignancy. *Cancer Genetics and Cytogenetics* 90: 179 - 181.
- O'Hare T, Zabriskie M, Eide C, Agarwal A, Adrian L, You H, Corbin A, Yang F, Press R, Rivera V, Toplin J, Wong S, Deininger M and Druker B. 2011. The BCR-ABL 35INS insertion/truncation mutant is kinase-inactive and does not contribute to tyrosine kinase inhibitor resistance in chronic myeloid leukemia. *Blood* DOI: 10.1182/blood-2011-05-349191.
- Ohyashiki K, Oshimura M, Toyoda T, Sakai N, Ito H, Yamamoto K and Tonomura A. 1984. Chronic myelogenous leukemia with a complex Ph1 translocation in an XYY male. *Cancer Genetics and Cytogenetics* 11 (2): 215 - 219.
- Panani A. 2009. Is there an association with constitutional structural chromosomal abnormalities and hematologic neoplastic process? A short review. *Annals of Hematology* 88: 293 - 299.
- Peggs K. 2004. Imatinib mesylate – gold standards and silver linings. *Clinical and Experimental Medicine* 4: 1 - 9.
- Pillar G. 1997. John Hughes Bennett: His life and the identification of leukaemia. *Proceedings of the Royal College of Physicians of Edinburgh* 27: 1 - 11.
- Poláková K, Lopotová T, Klamová H and Moravcová J. 2008. High-resolution melt curve analysis: Initial screening for mutations in BCR-ABL kinase domain. *Leukemia Research* 23: 1236 - 1243.
- Potter A, Sharp J, Brown M and Sokol R. 1975. Structural rearrangements associated with the Ph' chromosome in chronic granulocytic leukaemia. *Humangenetik* 29: 223 - 228.
- Quigley N, Henley D and Hubbard R. 2008. ABL kinase domain pseudoexon insertion is not uncommon in BCR-ABL transcripts. *Journal of Molecular Diagnostics* 10 (5): 475 - 476.
- Quintas-Cardama A and Cortes J. 2008. Molecular Biology of BCR-ABL1-positive chronic myeloid leukemia. *Blood*: DOI:10.1182/blood-2008-03-144790.

- Reed G and Wittwer C. 2004. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clinical Chemistry* 50 (10): 1748 - 1754.
- Reed G, O Kent J and Wittwer C. 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* 8 (6): 597 - 608.
- Ren R. 2005. Mechanisms of BCR-ABL in pathogenesis of Chronic Myelogenous Leukemia. *Nature Reviews* 5: 172 - 183.
- Ririe K, Rasmussen R and Wittwer C. 1997. Product differentiation by analysis of DNA Melting Curves during Polymerase Chain Reaction. *Analytical Biochemistry* 245: 154 - 160.
- Rix U, Hantelschel O, Dürnberger G, Rensing L, Rix R, Planyavsky M, Fernbach N, Kaupe I, Bennett K, Valent P, Colinge J, Köcher T and Superti-Furga G. 2007. Chemical proteomic profiles of the BCR-ABL inhibitors Imatinib, Nilotinib and Dasatinib reveal novel kinase and nonkinase targets. *Blood* 110 (12): 4055 - 4063.
- Roche-Lestienne C, Soenon-Cornu V, Gardel-Duflos N, Lai J.L, Philippe N, Facon T, Fenaux P and Preudhomme C. 2002. Several types of mutations in the Abl gene can be found in chronic myeloid leukaemia patients resistant to STI571, and they can pre-exist to the onset of treatment. *Blood* 100 (3): 1014 - 1018.
- Rouleau E, Lefol C, Bourdon V, Coulet F, Noguchi T, Soubrier F, Bieche I, Olschwang S, Sobol H and Lidereau R. 2009. Quantitative PCR High-Resolution Melting (qPCR-HRM) Curve Analysis, a new approach to simultaneously screen point mutations and large rearrangements: Application to *MLH1* germline mutations in lynch syndrome. *Human Mutation* 30 (6): 1 - 9.
- Roumiantsev S, Shah N, Gorre M, Nicoll J, Brasher B, Sawyers C and Van Etten R. 2002. Clinical resistance to the kinase inhibitor STI-571 in Chronic

- Myeloid Leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. *Proceedings of the National Academy of Sciences* 99 (16): 10700 - 10705.
- Rowley J. 1973. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 243: 290 - 293.
- Sagilo G, Storlazzi C, Giugliano E, Surace C, Anelli L, Rege-Cambrin G, Zagaria A, Velasco A, Heiniger A, Scaravaglio P, Gomez A, Gomez J, Archidiacono N, Banfi S and Rocchi M. 2002. A 76-kb duplication maps close to the BCR gene on chromosome 22 and the ABL gene on chromosome 9: Possible involvement in the genesis of the Philadelphia chromosome translocation. *Proceedings of the National Academy of Sciences* 99: 9882 - 9887.
- Sattler M, Golam Mohi M, Pride Y, Quinnan L, Malouf N, Podar K, Gesbert F, Iwasaki H, Li S, van Etten R, Gu H, Griffin J and Neel B. 2002. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell* 1: 479 - 492.
- Savage D and Antman K. 2002. Imatinib Mesylate – A new oral targeted therapy. *The New England Journal of Medicine* 346 (9): 683 - 693.
- Sawyers C. 1999. Chronic Myeloid Leukemia. *The New England Journal of Medicine* 340 (17): 1130 - 1340.
- Sawyers C, Hochhaus A, Feldman E, Goldman J, Miller C, Ottmann O, Schiffer C, Talpaz M, Guilhot F, Deininger M, Fischer T, O'Brien S, Stone R, Gambacorti-Passerini C, Russell N, Reiffers J, Shea T, Chapuis B, Coute S, Tura S, Morra E, Larson R, Saven A, Peschel C, Gratwohl A, Mandelli F, Ben-Am M, Gathmann I, Capdeville R, Paquette R and Druker B. 2002. Imatinib induces haematological and cytogenetic responses in patients with Chronic Myelogenous Leukemia in myeloid blast crisis: Results of a phase II study. *Blood* 99 (10): 3530 - 3539.
- Schindler T, Bornmann W, Pellicena P, Miller W, Clarkson B and Kuriyan J. 2000. Structural mechanism for STI-571 inhibition of Abelson Tyrosine Kinase. *Science* 289: 1938 - 1942.

- Seipp M, Durtschi J, Liew M, Williams J, Damjanovich K, Pont-Kingdon G, Lyon E, Voelkerding K and Wittwer C. 2007. Unlabeled oligonucleotides as internal temperature controls for genotyping by amplicon melting. *Journal of Molecular Diagnostics* 9 (3): 284 - 289.
- Shah N, Nicoll J, Nagar B, Gorre M, Paquette R, Kuriyan J and Sawyers C. 2002. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor Imatinib (ST1571) in chronic phase and blast crisis Chronic Myeloid Leukemia. *Cancer cell* 2: 117 - 125.
- Shah N, Lee F, Luo R, Jiang Y, Donker M and Akin C. 2006. Dasatinib (BMS-354825) inhibits KITD816V, an Imatinib-resistant activating mutation that triggers neoplastic growth in most patients with systematic mastocytosis. *Blood* 108: 286 - 291.
- Sherbenou D, Hantschel O, Turaga L, Kaupe L, Willis S, Bumm T, Press R, Superti-Furga G, Druker B and Deininger M. 2008. Characterization of BCR-ABL deletion mutants from patients with chronic myeloid leukaemia. *Leukemia* 22: 1184 - 1190.
- Shtivelman E, Lifshitz B, Gale R, Canaani E. 1985. Fused transcript of abl and bcr genes in chronic myeloid leukaemia. *Nature* 315: 550 - 554.
- Sivakumaran T, Kucheria K and Oefner P. 2003. Denaturing high performance liquid chromatography in the molecular diagnosis of genetic disorders. *Current Science* 84 (3): 291 - 296.
- Sokal J, Baccarani M, Russo D and Tura S. 1988. Staging and prognosis in chronic myelogenous leukaemia. *Semin Haematology* 25: 49 - 61.
- Soverini S, Colarossi S, Gnani A, Rosti G, Castagnetti F, Poerio A, Lacobucci I, Amabile M, Abruzzese E, Orlandi E, Radaelli F, Ciccone F, Tiribelli M, di Lorenzo R, Caracciolo C, Izzo B, Pane F, Saligo G, Baccarani M and Martinelli G. 2006. Contribution of ABL Kinase Domain Mutations to Imatinib Resistance in Different subsets of Philadelphia-Positive patients: By the GIMEMA working party on Chronic Myeloid Leukemia. *Cancer Therapy* 12 (24): 7374 - 7379.

- Soverini S, Hochhaus A, Nicolini F, Gruber F, Lange T, Sagilo G, Pane F, Muller M, Ernst T, Rosti G, Porkka K, Baccarani M, Cross N and Martinelli G. 2011. BCR-ABL kinase domain mutations analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. *Blood* DOI: 10.1182/blood-2010-12-326405.
- Stewart B and Kleihues P. 2003. World health organisation, International agency for research on cancer: www.scribd.com/doc/2350813/World-Cancer-Report-2003-Stuart-e-Kleihues-WHO-e-IARC
- Tajiri-Utagawa E, Hara M, Takahashi K, Watanabe M and Wakita T. 2009. Development of a Rapid High-Throughput Method for High-Resolution Melting Analysis for Routine Detection and Genotyping of Noroviruses. *Journal of Clinical Microbiology* 47 (2): 435 - 440.
- Takano E, Mitchell G, Fox S and Dobrovic A. 2008. Rapid detection of carriers with *BRCA1* and *BRCA2* mutations using high resolution melting analysis. *Biomedical Central Cancer* 8 (59): 1 - 7.
- Talpaz M, Shah N, Kantarjian H, Donato N, Nicoll J, Paquette R, Cortes J, O'Brien S, Nicaise C, Bleickardt E, Blackwood-Chirchir A, Iyer V, Chen T, Huang F, Decillis A and Sawyers C. 2006. Dasatinib in Imatinib-resistant Philadelphia chromosome-positive leukemias. *The New England Journal of Medicine* 354 (24): 2531 - 2541.
- Tan A, Westerman D, Carney D, Seymour J, Juneja S and Dobrovic A. 2008. Detection of *NPM1* exon 12 mutations and *FLT3* – internal tandem duplication by high resolution melting analysis in normal karyotype acute myeloid leukemia. *Journal of Haematology and Oncology* 1(10): DOI:10.1186/1756-8722-1-10
- Tarkan-Arguden Y, MCem M, Yilmaz S, Ongoren S, Kuru D, Ure U, Cirakoglu A, Eskazan A, Guven G, Cetin G, Purisa S, Baslar Z, Deviren A, Aydın Y, Hachanefi oglu Y, Ferhanoglu B, Tuzuner N, Ulku B, Soysal. 2009.

- Cytogenetic clonal evolution in patients with chronic myeloid leukemia. *Medical Biotechnology* (23): 1515 - 1520.
- Tauchi T and Ohyashiki K. 2004. Molecular mechanisms of resistance of Leukemia to Imatinib Mesylate. *Leukemia Research* 10: 39 - 45.
- Taylor C. 2009. Mutation screening using high-resolution melting. *Advances in Nucleic acid detection and quantification* 37: 433 - 437.
- Thomas J, Wang L, Clark R and Pirmohamed M. 2004. Active transport of imatinib into and out of cells: implications for drug resistance. *Blood* 104: 3739 - 3745.
- Twyman R. 2005. Single nucleotide polymorphisms (SNP) genotyping techniques – An overview. *Encyclopedia of diagnostics and proteomics*: DOI: 10.1081/E-EDGP 120020761.
- Untergasser A and Nijveen H. 2007. Primer3Plus [online] Available at <http://www.attotron.com/cybertory/analysis/seqMassager.htm> [Accessed on 30 September 2011].
- Van Etten R. 2011. Clinical manifestations and diagnosis of chronic myeloid leukemia. DOI: <http://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-chronic-myeloid-leukemia>.
- Vaughn C and Elenitoba-Johnson K. 2004. High-resolution melting analysis for detection of internal tandem duplications. *Journal of Molecular Diagnostics* 6 (3): 211 - 216.
- Volpe G, Panuzzo C, Ulisciani S and Cilloni D. 2009. Imatinib resistance in CML. *Cancer letters* 274: 1 - 9.
- von Bubnoff N, Peschel C and Duyster J. 2002. Resistance of Philadelphia-chromosome positive Leukemia towards the kinase inhibitor Imatinib (ST1571): a targeted oncoprotein strikes back. *Leukemia* 17: 829 - 838.
- Von Bubnoff N, Veach D, van der Kuip H, Aulitzky W, Sanger J, Seipel P, Bornmann W, Peschel C, Clarkson B and Duyster J. 2004. A cell-based screen for resistance of BCR-ABL positive leukemia identifies the mutation

- pattern for PD166326, an alternative Abl kinase inhibitor. *Blood*: DOI: 10.1183/blood-2004-06-2445.
- Wang L, Knight K, Lucas C, Clark R. 2006. The role of serial BCR-ABL transcripts monitoring in predicting the emergence of BCR-ABL kinase mutations in Imatinib-treated patients with Chronic Myeloid Leukemia. *The Haematology Journal* 91 (2): 235 - 239.
- Wei Y, Hardling M, Olsson B, Hezaveh R, Ricksten A, Stockelberg D and Wadenvik H. 2006. Not all Imatinib resistance in CML are BCR-ABL kinase domain mutations. *Annals of Haematology* 85: 841 - 847.
- Weisberg E and Griffin J. 2001. Mechanisms of resistance Imatinib (ST1571) in preclinical models and in Leukemia patients. *Drug Resistance Updates* 4: 22 - 28.
- Welbron J. 2004. Constitutional chromosome aberrations as pathogenetic events in hematologic malignancies *Cancer Genetics and Cytogenetics* 149: 137 - 153.
- Wienand K, Viljoen C, Sreenivasan S, Louw V. 2011. High-resolution melting curve analysis for screening of mutations in the BCR-ABL kinase domain of CML patients. *Haematologica* 96 (2): 607.
- Wittwer C, Reed G, Gundry C, Vandersteen J and Pryor R. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry* 49 (6): 853 - 860.
- Willis S, Lange T, Demehri S, Otto S, Crossman L, Niederwieser D, Stoffregen E, McWeeney S, Kovacs I, Park B, Druker B and Deininger M. 2005. High-sensitivity detection of BCR-ABL kinase domain mutations in Imatinib-naïve patients: Correlation with clonal cytogenetic evolution but not response to therapy. *Blood* 106 (6): 2128 - 2137.
- Yamamoto M, Kurosu T, Kakihana K, Mizuchi D and Miura O. 2004. The two major resistance mutations E255K and T315I enhance the activity of

- BCR/ABL fusion kinase. *Biochemical and Biophysical Research Communications* 319: 1272 - 1275.
- Yokota A and Kimura S. 2008. Novel agents to override Imatinib resistance mechanisms. *Drug development research* 69: 398 - 406.
- Zeeb H and Blettner M. 1998. Adult leukaemia: what is the role of currently known risk factors? *Radiation and Environmental Biophysics* 36: 217 - 228.
- Zhou L, Wang L, Palais R, Pryor R and Wittwer C. 2005. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. *Clinical Chemistry* 51 (10): 1770 - 1777.