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BCR-ABL MUTATIONS IN CHRONIC MYELOID LEUKEMIA PATIENTS

by

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Dedicated to my parents,

Lee & Donna

And my grandparents,

Ken, Mary, Sylvia & Louis

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LIST OF ABBREVIATIONS

Abl PP	Abl P223E/P230E mutant
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ARG	Abelson-related gene
BCR	breakpoint cluster region
CML	Chronic myeloid leukemia
CCR	complete cytogenetic response
Das	dasatinib
D-HPLC	denaturing high-performance liquid chromatography
Dox	doxycycline
FACS	fluorescence activated cell sorting
FISH	fluorescence in situ hybridization
FLT3	fms-related tyrosine kinase 3
GIST	gastrointestinal stromal tumor
HSC	hematopoietic stem cell
HES	hypereosinophilic syndrome
IM	imatinib
IRIS	International Randomized Study of Interferon and STI571
MNC	mononuclear cells
MTS	methanethiosulfonate
NTA	nitrilotriacetic acid
OOF	out of frame

qPCR	Quantitative RT-PCR
P-loop	ATP-binding loop
Ph	Philadelphia chromosome
PDGFRA	platelet-derived growth factor receptor A
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	streptavidin-coated membrane
SAXS	small angle x-ray diffraction
WT	wild type

ABSTRACT OF THE DISSERTATION

BCR-ABL MUTATIONS IN CHRONIC MYELOID LEUKEMIA PATIENTS

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The Bcr-Abl tyrosine kinase causes CML and is the target for therapy by imatinib, a clinically successful kinase inhibitor. More than 80% of newly diagnosed patients with chronic-phase chronic myeloid leukemia achieve a complete cytogenetic response (CCR) with imatinib treatment. However, patients who relapse during imatinib therapy often harbor mutations in the kinase domain of *BCR-ABL* that are imatinib-resistant. In this dissertation, three scerarios of acquired *BCR-ABL* mutations during imatinib therapy were studied. First, the presence of kinase domain mutations in the setting of minimal residual disease was investigated in patients with a stable CCR. Mutations were detected in a subset of these patients and were those commonly associated with drug resistance. However, detection of *BCR-ABL* kinase domain mutations in patients with a stable CCR did not consistently predict relapse. Second, mutations outside the kinase domain in the regulatory linker, SH2, SH3 and Cap domains were investigated. Imatinib-resistant mutations in these domains have been described in vitro, but not yet in patients. Mutations in the regulatory domains of Bcr-Abl were found in patients during imatinib treatment, but only a single mutation proved to be substantially imatinib-resistant. Third, deletion mutants of Bcr-Abl are described to occur in a subset of CML patients, apparently the result of missplicing. Most commonly these deletion mutants lack a

significant portion of the kinase domain that includes the P-loop. These mutants are demonstrated to be catalytically inactive. We hypothesized that coexpressed Bcr-Abl deletion mutants would have a dominant-negative effect on the native form through the oligomerization domain of Bcr. Although such an effect was not found on signaling activity or growth factor independence, cells coexpressing deletion mutants did have increased imatinib sensitivity compared to cells expressing only native Bcr-Abl. In summary, the Bcr-Abl oncogene is a frequent site for mutagenesis during imatinib treatment, and the consequences of the mutations are diverse.

CHAPTER 1

APPLYING THE DISCOVERY OF THE PHILADELPHIA CHROMOSOME

Applying the Discovery of the Philadelphia Chromosome

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1. 1 Abstract

The identification of the Philadelphia chromosome in cells from individuals with chronic myeloid leukemia (CML) led to the recognition that the Bcr-Abl tyrosine kinase causes CML. This, in turn, led to the development of imatinib, a clinically successful inhibitor of the Bcr-Abl kinase. Incorporating markers of Bcr-Abl kinase inhibition into clinical trials allowed for the realization that imatinib-resistant kinase domain mutations are the major cause of relapse during imatinib therapy and the subsequent development of new inhibitors to treat these patients. The development of imatinib validates an emerging paradigm in cancer where a tumor is defined by genetic abnormalities and effective therapies are developed that target events critical to the growth and survival of a specific tumor.

1.2 Introduction

The study of chronic myeloid leukemia (CML) affords a unique opportunity to understand the process of cancer development. This was the first human cancer in which a consistent genetic abnormality was demonstrated to cause the disease. For CML this is a shortened chromosome 22, which was first reported in 1960.[1] This chromosomal abnormality, referred to as the Philadelphia (Ph) chromosome, was later recognized to result from a reciprocal (9;22) translocation [2] ; that is, genetic material from the ends of chromosomes 9 and 22 is exchanged.

The molecular consequences of the (9;22) translocation are to fuse the *ABL* tyrosine kinase gene from chromosome 9 to the breakpoint cluster region (*BCR*) gene on chromosome 22.[3-5] The resulting fusion protein, Bcr-Abl, functions as an oncogenic tyrosine kinase that causes CML (Figure 1.1).[6,7] The oncogenic capabilities of Bcr-Abl have been demonstrated in mouse models in which Bcr-Abl expression is sufficient to cause leukemia.[8-10]

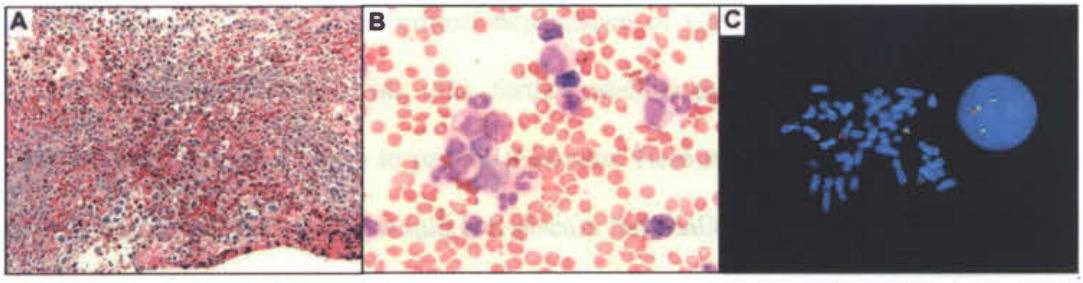

Figure 1.1. The phenotype and genotype of chronic myeloid leukemia. (**A**) A bone marrow biopsy from a patient with CML shows the typical hypercellularity with granulocytic and megakaryocytic hyperplasia (original magnification, 200x). (**B**) The peripheral blood is characterized by a full spectrum of myeloid cells, including immature myeloid cells with rare blasts. Basophilia is also observed (original magnification, 630x). Micrograph courtesy of Marc Loriaux. (**C**) Dual-color, dual-fusion fluorescence in situ hybridization (FISH) displaying BCR-ABL signals in bone marrow cells in metaphase (left) and interphase (right). The red fluorescent probe is specific for ABL, while the green probe is specific for BCR. Yellow signals the presence of BCR-ABL and ABL-BCR fusions. Micrograph courtesy of Susan Olson and Helen Lawce.

CML also functions as a dissectible model for other malignancies with clinically apparent phases coinciding with genetic progression of the disease. In the chronic phase of CML, the (9;22) translocation arises in a hematopoietic stem cell [11] and seems to be the sole genetic abnormality.[12] The chronic phase is characterized by a massive increase in cells of the myeloid lineage, but these cells mature and function normally. Historically, the chronic phase lasted an average of three to five years. Over time, the disease transforms into an invariably fatal acute leukemia (known as blast crisis) of either myeloid or lymphoid phenotype [13], often with a recognizable intermediate stage termed the accelerated phase. The accelerated phase and blast crisis are collectively referred to as advanced phase disease. Although it is clear that additional genetic abnormalities are responsible for blast crisis [14], the specific genetics lesions that cause disease progression are poorly characterized.

The clinical success of imatinib (Gleevec, formerly STI571), an inhibitor of the Bcr-Abl kinase, confirmed the critical dependence of CML cells on Bcr-Abl tyrosine kinase activity for their survival; thus, further establishing CML as a paradigm for the contributions of basic science to patient treatment. Here we will review the pre-clinical and clinical development of imatinib and discuss the challenges of treating patients with accelerated phase and blast crisis; the issue of imatinib resistance and the development of 2nd generation inhibitors of Bcr-Abl will be discussed; and finally, the application of the imatinib paradigm to other malignancies will be reviewed.

1.3 Beginning of the Imatinib Era

Imatinib is a small organic molecule synthesized for the purpose of protein kinase inhibition.[15,16] It has activity against all the Abl tyrosine kinases, including Bcr-Abl, c-ABL, v-ABL, and ARG (Abelson-related gene).[15-17] In addition to the Abl tyrosine kinases, other kinases inhibited by imatinib are platelet-derived growth factor receptor A (PDGFRA), PDGFRB, and Kit.[15,16,18] Given the critical role of tyrosine kinases in the regulation of cell growth and known activation in several cancers, such as Bcr-Abl in CML and Her2 overexpression in breast cancer, it was hypothesized that specific inhibitors of these protein kinases might be effective anticancer agents.[19]

Beginning in the late 1980's, scientists at Ciba Geigy (now Novartis) performed high-throughput screens of chemical libraries searching for compounds with kinase inhibitory activity. From this time-consuming approach, a lead compound was identified; its inhibitory activity against PDGFR was optimized by synthesizing a series of chemically related compounds and analyzing their relationship between structure and activity. The most potent molecules in the series were dual inhibitors of the PDGFR and Abl kinases.

Of the several compounds generated from this program, imatinib emerged as the lead compound for clinical development based on its superior in vitro selectivity against CML cells and its drug-like properties, including pharmacokinetic and formulation properties.[20]

In a critical set of preclinical experiments, imatinib specifically killed cell lines expressing Bcr-Abl and did not kill the parental cell lines from which they were derived.[16] Imatinib also inhibited tumor formation by cells expressing Bcr-Abl in vivo.[16] In colony-forming assays of peripheral blood or bone marrow from patients with CML, imatinib caused a 92–98% decrease in the number of colonies expressing Bcr-Abl that formed.[16] These experiments suggested that cells expressing Bcr-Abl become addicted to that oncogene and are dependent on it not only for proliferation, but also for survival. Thus, inhibition of the Bcr-Abl tyrosine kinase resulted in apoptosis instead of merely reverting the cells to normal. A mouse model with inducible Bcr-Abl expression has further extended this notion by demonstrating full dependence on oncogene expression in maintaining leukemia.[21]

In 1998, a Phase I clinical trial with imatinib was begun.[22] This initial phase I trial was a dose escalation trial that enrolled 83 patients with chronic-phase CML. Their clinical features were the following and were typical of the disease. Their ages ranged from 19–76 years and they had a median time from diagnosis of 3.8 years. At study entry, their white blood cell counts ranged from 9,400 to 199,000/mm^3 (normal is 4,000 to 11,000/mm^3) and the Ph chromosome was present in all leukemic cells in each patient. As this was a Phase clinical I trial, it was required that all patients had received prior therapy with the current standard of care, interferon-α which was either no longer controlling their disease (N=70) or was poorly tolerated (N=13). Interferon-α therapy had previously been

shown to prolong survival by a median of 20 months over the natural disease course of 3 to 5 years.[23] However, the mechanism of action of interferon-α is poorly understood and its side effects are significant. Therefore, the patients enrolled in the Phase I clinical trial of imatinib had late chronic-phase CML with no standard treatment options available. Although there was optimism at the beginning of the trial based on the promising preclinical experiments, there was also concern that blockade of the normal, ubiquitously expressed c-Abl, PDGFR, and/or Kit would lead to unforeseen toxicities. After 29 patients were enrolled, therapeutic doses of 300 mg per day or greater were reached and 53 of 54 patients achieved a complete hematologic response (CHR = normal blood counts with a white-cell count less than 10,000/mm^3 and platelet count less than 450,000/mm^3). Side effects of imatinib were relatively minimal.[22] Based upon the results of the Phase I trial, the use of imatinib was expanded to large international Phase II and Phase III clinical trials.

1.4 Cumulative Experience with Imatinib

The experience with imatinib has yielded a wealth of information, including a randomized clinical trial with five years of follow-up (the IRIS (International Randomized Study of Interferon and STI571 (IRIS) study) [24], a crystal structure of the Abl kinase domain in complex with imatinib [25,26] and important insights into the mechanism of imatinib resistance. The IRIS trial was initiated in 2000 for patients newly diagnosed with CML in chronic-phase.[24] Initially designed as a comparison of imatinib to interferon-α plus cytarabine, the substantial superiority of imatinib resulted in study results being disclosed early and most patients being crossed over to the imatinib arm. Accordingly, this study is now a long-term follow-up study of patients who received imatinib as initial

therapy, with the median follow-up now being 5 years. The overall survival at 5 years is 89%.[24] Previous experience found 5-year survival rates for patients treated with interferon-α plus cytarabine to be 68-70%.[27,28] An estimated 93% of imatinib-treated patients remain free from disease progression to the accelerated phase or blast crisis.[24] An additional 6% of patients have some evidence of loss of response to imatinib, but their disease has not progressed to the accelerated phase or blast crisis.[24] Most of the side effects of imatinib are mild to moderate, with the most common being edema, muscle cramps, diarrhea, nausea, skin rashes, and myelosuppression. Thus, imatinib therapy has substantially increased survival for patients with CML while simultaneously greatly decreasing side effects.

Imatinib was the first small-molecule kinase inhibitor tested in patients, and its use has illuminated some fundamental characteristics of CML biology as well as establishing guiding principles for kinase inhibitor use in general. Most patients on imatinib therapy achieve a complete cytogenetic response [24], which is defined as the absence of the Ph chromosome in 20 bone marrow cells in metaphase. Despite the high frequency of complete cytogenetic responses, *BCR-ABL* transcripts are detectable in most patients by RT-PCR spanning the breakpoint in the fusion mRNA. Even patients in whom *BCR-ABL* is undetectable by this technique (classified as a complete molecular response) typically relapse if imatinib therapy is discontinued.[29,30] Therefore, a picture of disease response has emerged in which Bcr-Abl kinase inhibition triggers apoptosis in differentiated cells and halts progenitor cell proliferation, but does not eliminate hematopoietic stem cells that harbor the translocation.[31,32] The kinetics of these effects fit a biphasic mathematical model based on *BCR-ABL* transcript reduction in patients.[33] A rapid initial decline occurs

as differentiated cells undergo apoptosis, followed by a slower decline associated with progenitor cell turnover. Whether the hematopoietic stem cells expressing Bcr-Abl are resistant to imatinib or continue to be slowly eliminated over time is not clear. Of interest, another mathematical model has recently been reported also featuring a biphasic imatinib response, but instead raises the possibility of an additional slow decline in hematopoietic stem cells expressing Bcr-Abl when these cells enter the cell cycle.[34] Whether hematopoietic stem cells expressing Bcr-Abl are resistant to imatinib or sensitive only upon cell division is not clear. Insights into the mechanism that allows leukemic stem cells to persist during therapy would be an important contribution to the understanding of leukemogenesis and kinase inhibitor treatment.

1.5 Imatinib in Advanced Ph Chromosome Positive Leukemia

Soon after the first trial of imatinib for the treatment of chronic-phase CML was initiated, the efficacy of imatinib in treating two more challenging manifestations of Bcr-Abl-positive leukemia was investigated: blast-crisis CML and Ph chromosome–positive acute lymphoblastic leukemia (ALL).[35] Despite high response rates in these clinical trials, it quickly became apparent that most responses were short-lived.[35] Hematologic responses were seen in 55% (N=38) of patients with blast crisis CML of myeloid phenotype and 70% (N=20) of patients with lymphoid blast crisis CML or Ph chromosome positive ALL. Subsequent findings in larger Phase II clinical trials confirmed these observations.[36] Hematologic responses were observed in 52% of patients (N = 260), with a median response duration of 10 months.[36] Of note, 48% of patients in this trial had obvious clonal evolution as evident by cytogenetic abnormalities in addition to the Ph chromosome. The short duration of response highlighted the necessity for combination therapy to combat

resistance. Recent studies of patients with Ph chromosome–positive ALL treated with combinations of imatinib and a standard chemotherapy have shown high response rates and suggest improved durability of responses as compared to historical controls.[37-40] As specific genetic abnormalities are identified in the more aggressive manifestations of Ph chromosome–positive disease (blast crisis CML and Ph chromosome–positive ALL), these will also become candidates for targeted therapeutic agents.

The association between genomic instability and CML progression has been recognized for many years. In 1976, it was documented that the leukemic cells of approximately 80% of patients in accelerated phase or blast crisis harbored additional recurrent chromosomal abnormalities.[41] An interesting question is whether Bcr-Abl itself has a causal role in the generation of secondary genetic abnormalities. Bcr-Abl has been observed to translocate to the nucleus and disrupt signaling integral to checkpoint signaling from the ATR kinase following DNA damage, a checkpoint pathway that is essential for maintenance of genomic integrity.[42] It has also been reported that Bcr-Abl induces reactive oxygen species, which cause DNA double-strand breaks and point mutations.[43-45] By either mechanism, it can be postulated that Bcr-Abl is responsible, or at least contributes to the genomic instability that results in secondary abnormalities leading to blast crisis. This hypothesis would bode well for the Bcr-Abl-centric approach that dominates current clinical efforts, as it suggests that kinase inhibition in CML might prevent or delay the development of blast crisis. This hypothesis is consistent with the clinical data showing that few patients have progressed to the advanced phases of the disease with imatinib therapy.

The genetic complexity of blast-crisis CML implicates a multi-stage pathogenesis.[14] Bcr-Abl promotes the excess proliferation of myeloid cells during chronic-phase and as noted, may contribute to genetic instability that leads to additional mutations that drives disease progression. These additional mutations produce a block in cell differentiation or disable the key tumor suppressor pathways of p53 or Rb. Possible culprits that would block differentiation are transcription factors that are also commonly disrupted in acute myeloid leukemia (AML). For example, the (3;21) translocation (observed in approximately 2% of patients with blast crisis CML) fuses the Aml1 (Runx1) core binding factor to the Evi1 transcriptional repressor.[46] Aml1 is essential to the differentiation of hematopoietic cells and the Aml1-Evi1 fusion protein blocks this process.[47] In mice, co-expression of Bcr-Abl and Aml1-Evi1 results in AML.[48] Although the cases of CML patients with Aml1-Evi1 are few, it represents a general mechanism for disease progression. A recent study has examined the characteristics of the CML initiating cells in advanced disease.[49] By comparing cells from patients in blast crisis with those from individuals with chronic-phase CML it was found that granulocyte-macrophage progenitors from patients with blast-crisis, but not chronic-phase, displayed self-renewal capability.[49] These data suggest that cells that normally do not have the capacity for self-renewal can adopt stem cell–like properties with disease progression to blast crisis.

1.6 The Challenge to Optimizing Therapy: Imatinib Resistance

Although most patients with chronic-phase CML treated with imatinib have well-controlled disease, some patients have relapsed and/or progressed to accelerated phase or blast crisis.[24] Furthermore, most patients with advanced phases of CML respond to imatinib, but subsequently relapse.[35,36] Therefore, the mechanisms of relapse emerged as a

major question. The first insights into relapse mechanisms came from assays that evaluated Bcr-Abl kinase inhibition. Bcr-Abl is a constitutively active tyrosine kinase in which the N-terminal domains of the Bcr signaling protein are joined to nearly the entire c-Abl kinase.[50,51] The functional consequence is the dysregulation of c-Abl, by two intramolecular effects (Figure 1.2). The first is a loss-of-function effect in which an N-terminal region of c-Abl, referred to as the Cap domain (see Figure 1.2), is partially lost from the Bcr-Abl fusion gene. This N-terminal Cap region is an important component of the intramolecular apparatus that inhibits the kinase activity of c-Abl.[52,53] The second effect is a gain-of-function resulting from the presence of a coiled-coil domain in Bcr that functions to dimerize or tetramerize Bcr-Abl.[54] This domain is essential for the transforming properties of Bcr-Abl.[55-57] Collectively, these two effects yield a kinase that is constitutively phosphorylated.[52,56] The result is the alteration of the tightly regulated c-Abl kinase into a predominantly active kinase that phosphorylates numerous intracellular proteins.[50] These phosphorylated proteins activate signaling pathways that control cell proliferation, including the PI3K, Ras, and Stat5 pathways.[58-62] Bcr-Abl also protects cells from apoptosis by upregulating Bcl_{XL} and by phosphorylation and inactivation of the pro-apoptotic molecule Bad.[63,64] One of the most heavily tyrosine phosphorylated proteins in CML patient samples is the SH3-SH2 domain–containing adaptor protein Crkl [65], and the level of Crkl phosphorylation was used in the clinical trials of imatinib to monitor the activity of the Bcr-Abl kinase.[22]

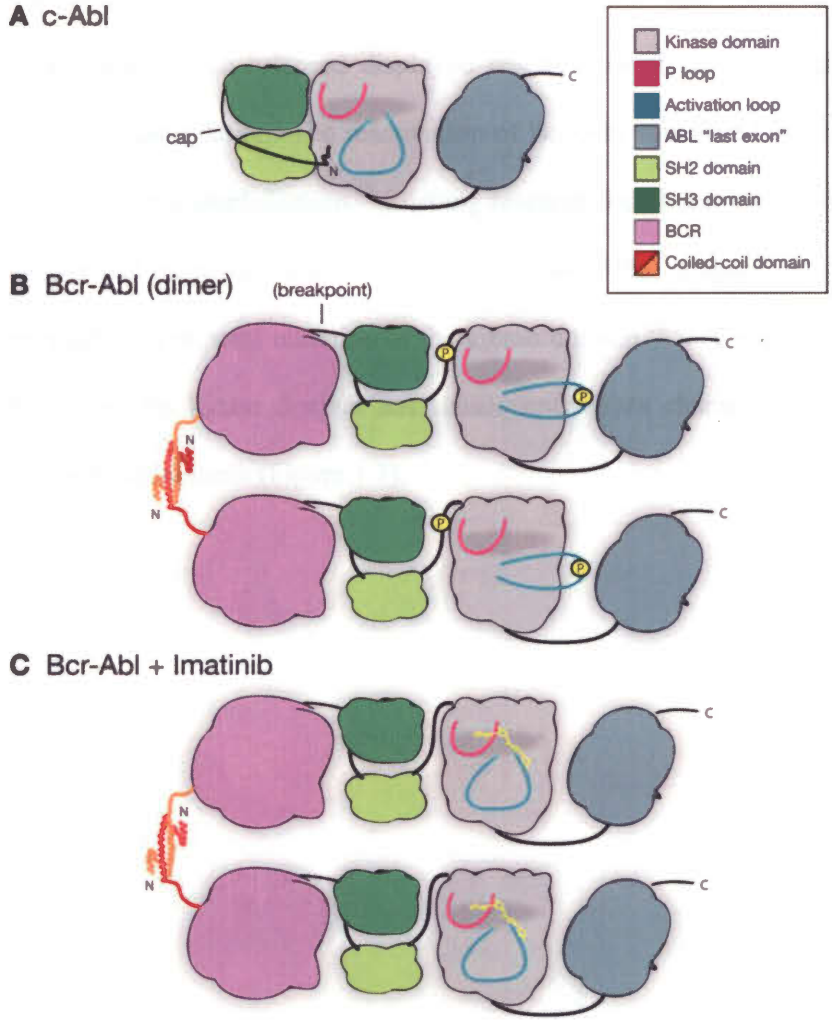

Figure 1.2. A model of the domains of the c-Abl and Bcr-Abl proteins displays the functional consequences of the translocation. (**A**) In c-Abl, the P-loop and the activation loop surround the active site for substrate phosphorylation (shaded). Under normal conditions c-Abl is inactive, with the SH3 and SH2 domains bound to the kinase domain, restricting its kinase activity.[53] This conformation is stabilized by the cap domain, which is anchored to the kinase domain by a myristoyl group (jagged line).[52,53] (**B**) In Bcr-Abl, the myristoyl group of the cap domain is lost and is replaced by Bcr, shown for simplicity as a single globular domain. This is presumed to destabilize binding of SH2 and SH3 to the kinase domain.[56] The N-terminus of Bcr, the coiled-coil domain, forms a helical dimer (that also tetramerizes) and tethers individual Bcr-Abl proteins together[54]. This allows trans-phosphorylation of the kinase (yellow circles), which promotes the kinase to adopt an active conformation with a significant outward twist of the activation loop. (C) Imatinib binds to the active site of the kinase domain, freezing it in the inactive conformation, preventing Bcr-Abl activation.[26]

In evaluating mechanisms of relapse, a major breakthrough occurred with the observation that patients who relapsed displayed reactivation of Bcr-Abl signaling, as assessed by Crkl phosphorylation. The reactivation of Bcr-Abl was found to occur either by point mutation or gene amplification.[66] Ensuing research found that the most common mechanism of Bcr-Abl reactivation, occurring in 76 of 144 (53%) relapsed patients in the initial cohorts studied, was point mutation of the kinase domain.[66-71] A spectrum of point mutations throughout the kinase domain has subsequently been characterized to confer decreased sensitivity to imatinib (Figure 1.3).

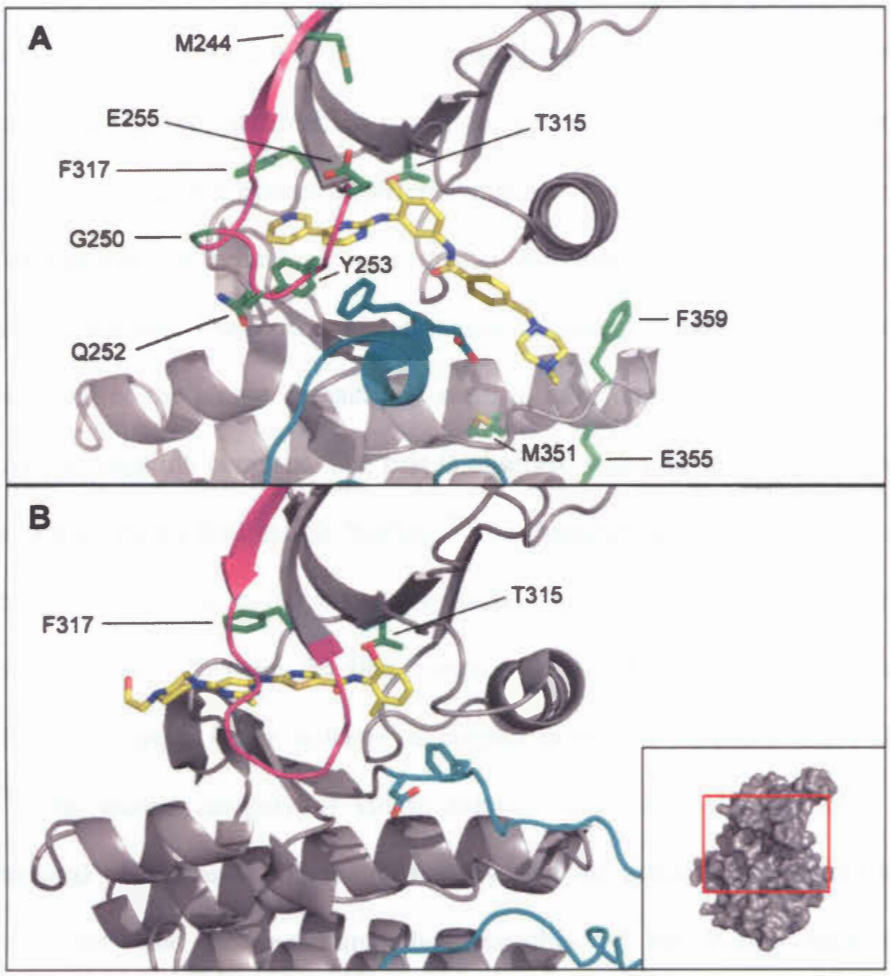

Figure 3. Magnified views into the active sites of the Abl kinase domain in complex with imatinib (**A**) and dasatinib (**B**). The two compounds have very different binding modes to the kinase, with dasatinib more confined to the ATP-binding pocket than imatinib. In addition, imatinib binds the inactive and dasatinib the active conformations, with opposite orientations of the catalytic DFG amino acid residues (shown in cyan stick format). In the dasatinib structure, the glutamic acid of the DFG motif that coordinates a Mg^{2+} ion during catalysis is oriented properly for catalysis, whereas in the imatinib structure this residue points away from the active site. Both inhibitors reside in close proximity to the T315 residue. The side chain atoms of the residues susceptible to resistant mutations for each inhibitor are shown in green and inhibitor atoms shown in yellow stick format (Nitrogen=blue, Oxygen=red, Sulfur=orange, Chlorine=hot pink). The P loop (residues 244-255) is shown in magenta and the activation loop (residues 381-402) is shown in cyan. (**A**) PDB entry 1IEP.[25] (**B**) PDB entry 2GQG.[72] The figure was created using PyMol.[73]

The understanding of relapses on imatinib therapy has been further aided by the solution of the crystal structure of the Abl kinase domain in complex with imatinib. This crystal structure showed that imatinib bound the kinase in its inactive conformation.[25,26] Although the active conformations of different kinases share many common features, their respective inactive conformations have greater differences.[26] This helps explain the specificity of imatinib. The most common imatinib-resistant mutations cluster around the active site cleft of the kinase domain and seem to have one of two effects on the Abl kinase domain–imatinib complex. The first is observed when the amino acid substitution occurs at contact points for imatinib binding.[70] The characteristic mutation of this category is the change of threonine to the more bulky isoleucine at residue 315 in the heart of the imatinib-binding site. This was the first mutation described [66] and has proven to be the most difficult to treat. Other mutants at contact points for imatinib are F317L and F359V.[70] The second category of kinase domain mutations is hypothesized to have conformational effects on the kinase, either favoring the active conformation to which imatinib cannot bind or decreasing the flexibility of the P-loop such that the conformational changes required for imatinib to bind this region cannot be adopted.[70] These mutations include those in the P-loop that bridges the ATP-binding pocket of the kinase domain (M244V, G250E, Q252H, Y253F/H and E255K/V) and the activation loop (H396R/P). These mutants, along with M351T and E355G, are the most common in patients who relapse.[74]

An issue that emerges from the study of patients who relapse on imatinib therapy is whether imatinib induces Bcr-Abl kinase domain mutations or whether the mutations pre-exist. Thus far, the clinical data is consistent with mutations pre-existing therapy, with

imatinib therapy selecting for resistant clones.[24] For example, in patients with advanced disease, imatinib-resistant clones have been detected prior to the initiation of therapy.[75] Although mutations are rarely detected in patients with chronic-phase CML, it is notable that the rate of disease progression peaks in the first two years during imatinib therapy and then trends downward.[24] If mutations precede imatinib therapy, the rate of emergence of resistance to the drug would depend on the size of the mutant clone at the start of therapy and its doubling time. As most mutated and unmutated Bcr-Abl clones have similar doubling times [76], it would be predicted that patients harboring a mutant clone that is below the level of detection by PCR should be at highest risk of relapse over the first years of therapy. This prediction is consistent with the clinical data.[24]

1.7 The 2nd Generation of BCR-ABL Inhibitors

Shortly after the identification of imatinib-resistant mutations, several new inhibitors have joined imatinib in the clinic. These inhibitors represent two different structural classes. The first class is exemplified by dasatinib (Sprycel, BMS-354825), a broad-spectrum kinase inhibitor that was developed as an inhibitor of Src family kinases.[77] One of the tyrosine kinase families that dasatinib inhibits are the Abl kinases, including c-Abl and Bcr-Abl.[77] It is >100-fold more potent than imatinib against the Abl kinases and retains this low nanomolar inhibitory activity against the common imatinib-resistant mutations, with the exception of the T315I mutation.[78] Crystallography and nuclear magnetic resonance have each shown striking differences between dasatinib and imatinib.[72,79] These studies show that dasatinib binds at the active site of the Abl kinase domain in a vastly different manner to imatinib. Specifically, it binds the active

conformation of Abl exclusively as opposed to the inactive conformation to which imatinib binds (Figure 1.3).[72,79] Therefore, dasatinib would not be susceptible to resistance caused by mutations that favor the active conformation of the Abl kinase domain. In addition, there is no distortion of the P-loop upon binding, as occurs with imatinib.[72] Lastly, due to its smaller size, there are fewer direct contacts points, perhaps affording less opportunity for resistant mutations. However, one direct contact point is T315, which explains the resistance of T315I to dasatinib.

Another kinase inhibitor, nilotinib (AMN107), was developed by modifying the chemical structure of imatinib to optimize potency against the Abl kinases. In preclinical testing, nilotinib showed 10 to 30-fold increased potency over imatinib against the major resistant mutants, except T315I.[80] Nilotinib retains the kinase specificity profile of imatinib, with inhibition confined to Abl kinases, Arg, Kit, and PDGFR kinases. Nilotinib, in contrast to dasatinib, binds the kinase domain of Abl kinases in the inactive conformation.[80]

Dasatinib and nilotinib have progressed rapidly through clinical trials and dasatinib has been FDA approved for imatinib-resistant patients with CML. In a Phase I clinical trial of dasatinib in patients with CML with imatinib-resistance or intolerance, complete hematologic responses were achieved in 37 of 40 patients with chronic-phase CML.[81] Although early, the responses are durable in 95% of such patients with a median follow-up of 12 months.[81] Similar results have been observed with nilotinib.[82] Response rates of patients with advanced-phase disease are also quite high, but similar to the experience with imatinib, relapses are commonplace in this population. Furthermore, dasatinib and nilotinib both seem well tolerated, although each have unique side effects. For example,

pleural effusions occur in some patients treated with dasatinib, and elevated liver enzymes and prolongation of QTc intervals occur in some patients treated with nilotinib.[82-85]

In the clinical trials of dasatinib and nilotinib, one of the most common mechanisms of resistance has been the emergence of clones with the T3151 mutation in Bcr-Abl.[83-86] Therefore, T315I remains a recurring problem for all the current Abl kinase inhibitors. Despite this challenge, preclinical T315I inhibitors have been reported, and at least one has been administered to patients.[87-89] The aurora kinase inhibitor MK-0457 (VX-680) was observed to have affinity for the T315I mutant.[90] As MK-0457 was already in clinical trials for patients with solid tumors, its testing in patients with CML was expedited. Although responses have been observed in patients with the T315I mutation.[87] it is not clear whether these represent a specific effect or are due to anti-proliferative effects from aurora kinase inhibition. Other pre-clinical compounds have also been reported to have specific activity against T315I and might soon be tested clinically.[88,89] One could imagine a scenario where combinations of inhibitors could be used to circumvent resistance entirely, although persistence of hematopoietic stem cells expressing Bcr-Abl is still probable with this approach.

1.8 Application of the Imatinib Paradigm to Other Malignancies

A key lesson learned from imatinib is that the discovery of the primary genetic abnormality in a malignancy coupled with the development of an agent that targets that abnormality can lead to therapeutic success. With that in mind, a major goal for cancer research should be to reclassify all forms of cancer according to their genotypes, rather than being classified solely on histology. Genotypic classification of malignancy is further supported by the successful use of imatinib in diseases such as gastrointestinal stromal

tumors (GISTs) and hypereosinophilic syndrome (HES). Both of these have an imatinib-susceptible kinase at their core.[91,92] As more cancers are genetically classified, those cancers will become amenable to hypothesis-driven testing of targeted therapeutic approaches.

The introduction of imatinib has generated a great deal of excitement about kinase-targeted therapy for the treatment for human cancer. Several prominent examples of oncogenic kinases associated with major malignancies are known and researchers and clinicians are moving quickly to target these abnormalities. For example, the use of Egfr inhibitors to treat patients with non-small-cell lung cancer has shown remarkable response when correlated to *EGFR* mutation status.[93,94] In AML, fms-related tyrosine kinase 3 (*Flt3*) mutations are present in up to one-third of patients, but additional mutations are required to cause the disease.[95] Clinical data with Flt3 inhibitors has shown that some patients respond, but it is not likely that these inhibitors can be used as monotherapy because of the genetic complexity of AML.[96-98] Ongoing clinical trials are examining Flt3 inhibitors as a component of combination therapy. By contrast, myeloproliferative disorders such as polycythemia vera have become prime candidates for kinase inhibitor monotherapy after it was observed that a high frequency of individuals with this disease have a V617F activating mutation in the Jak2 kinase.[99-101] It is hoped that treating these myeloproliferative disorders at early stages, akin to the chronic-phase of CML, will have similar results to those obtained with imatinib.

1.9 Conclusion: An Outlook for the 21st Century

It is worth remembering the impact four decades of discovery have had on patients with CML by recounting a patient's story. One patient seen at OHSU is a 55 year-old

gentleman, husband and father, who was diagnosed in 1997 with chronic-phase CML. He was subsequently treated with hydroxyurea and interferon-α and underwent an autologous stem cell transplant in 1998. Over the following year, his cytogenetics displayed an increasing percentage of Ph chromosome–positive metaphases, despite treatment with interferon-α. In late 2000, 20 of 20 metaphases were Ph chromosome positive, and he had an additional copy of the Ph chromosome along with trisomy 8. Based on these findings he was enrolled in a clinical trial of imatinib and rapidly achieved a complete cytogenetic response. After two years of therapy, he lacked detectable *BCR-ABL* transcripts by quantitative RT-PCR. In 2003, he had cytogenetic relapse and subsequent detection of the H396R mutant of Bcr-Abl. This development made him a prime candidate for a 2nd generation inhibitor, as both nilotinib and dasatinib are predicted to have high efficacy in patients with this mutation. Over the last 3 years, he has been treated with dasatinib and has maintained a major cytogenetic response. In February of 2007, this gentleman reached a full decade of living with CML, during which he has been able to continue working and maintain his normal lifestyle when previously, survival of patients with CML beyond five years was uncommon. Ultimately, our greatest goal is to continue this kind of tangible improvement in cancer therapies. We now look forward to further discoveries during the 21st century that will continue the tradition of translation of basic research findings such as the discovery of the Ph chromosome into clinically meaningful therapies.

CHAPTER 2

MUTATIONS OF THE BCR-ABL KINASE DOMAIN OCCUR IN A MINORITY OF PATIENTS WITH STABLE COMPLETE CYTOGENETIC RESPONSE TO IMATINIB

Mutations of the BCR-ABL kinase domain occur in a minority of patients with stable complete cytogenetic response to imatinib

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2.1 Abstract

Residual leukemia is demonstrable by RT-PCR in most patients with chronic myeloid leukemia (CML) who obtain a complete cytogenetic response (CCR) to imatinib. In patients who relapse during imatinib therapy a high rate of mutations in the kinase domain of *BCR-ABL* have been identified, but the mechanisms underlying disease persistence in patients with a CCR are poorly characterized. To test whether kinase domain mutations are a common mechanism of disease persistence we studied patients in stable CCR. Mutations were demonstrated in 8 of 42 (19%) patients with successful amplification and sequencing of *BCR-ABL*. Mutation types were those commonly associated with acquired drug resistance. Four patients with mutations had a concomitant rise of *BCR-ABL* transcript levels, two of whom subsequently relapsed; the remaining four did not have an increase in transcript levels and follow-up samples, when amplifiable, were wild type. *BCR-ABL* kinase domain mutations in patients with a stable CCR are infrequent, and their detection does not consistently predict relapse. Alternative mechanisms must be responsible for disease persistence in the majority of patients.

2.2 Introduction

More than 80% of newly diagnosed patients with chronic-phase chronic myeloid leukemia (CML) achieve a complete cytogenetic response (CCR).[24] Although these responses are generally durable, residual disease usually remains detectable by reverse transcriptase polymerase chain reaction (RT-PCR) for *BCR-ABL*. Furthermore, recurrence almost invariably occurs after discontinuation of therapy, indicating that the residual *BCR-ABL*-positive cells have full leukemogenic potential.[29,30,33] A recent study found *BCR-ABL* kinase domain mutations in CD34+ cells of 5/13 (38%) patients with a CCR [102], suggesting that disease persistence may be caused by similar mechanisms as acquired imatinib resistance.[66,70] However, the relapse rate in this small cohort of patients was high and the majority of patients had residual leukemia cells detected by FISH, suggesting a potential bias toward patients with a high-risk of relapse. We therefore analyzed the incidence of kinase domain mutations in a more representative group of patients with a stable CCR.

2. 3 Materials & Methods

2.3.1 Patient Sample Collection and Processing. This study was approved by the Institutional Review Board of Oregon Health & Science University. Informed consent was obtained from all patients. Bone marrow samples were collected on patients with a stable CCR, defined as having a CCR on consecutive tests with at least 20 analyzable metaphases, at least three months apart. Prospectively collected fresh bone marrow samples were processed by isolating mononuclear cells (MNC) by density gradient centrifugation and selecting CD34+ cells using immunomagnetic columns (Miltenyi, Auburn, CA). For samples stored at -80°C verified to meet our definition of stable CCR,

MNC were analyzed directly. RNA was extracted using the RNAqueous kit (Ambion, Austin, TX) and reversely transcribed into cDNA using random hexamer primers and Superscript™ reverse transcriptase (Invitrogen, Carlsbad, CA), followed by quality assessment by one-step RT-PCR for *ABL* as described.[103]

2.3.2 Mutation Screening. Mutation screening was done in duplicate, using the following parallel procedures for each sample: direct sequencing of *BCR-ABL* PCR products as described [104] and denaturing high-performance liquid chromatography (D-HPLC) as described.[105] Both approaches begin with a PCR reaction spanning both the breakpoint and kinase domain of *BCR-ABL*. The nested step in the direct sequencing procedure also spans these regions to maximize specificity, whereas the D-HPLC procedure has the modification that four overlapping amplicons covering only the kinase domain of ABL are used (codons 221-292, 277-342, 325-387, 372-434, primers available on request). Sensitivity of D-HPLC has been shown to be two-fold greater than conventional sequencing, detecting mutant transcripts that represent at least 15% of the PCR product.[105] For increased sensitivity, subcloning of PCR products was performed using the TOPO-TA cloning kit (Invitrogen) in samples amplified with the direct sequencing procedure. Sequencing was performed for 10-20 individual clones, with a positive mutation scored when at least two clones were positive for the same mutation. This increased the sensitivity for mutants comprising only 10-20% of the total. Quantitative RT-PCR (qPCR) for *BCR-ABL* was performed as described, and results were expressed as the percent ratio *BCR-ABL/Glucose-6-Phosphate Dehydrogenase*.[106]

2.4 Results & Discussion

2.4.1 Mutation Detection Sensitivity.

A recent report found a high rate of kinase domain mutations in CD34+ cells isolated from patients with a CCR.[102] In addition, higher levels of *BCR-ABL* mRNA have been demonstrated in CD34+ cells compared to MNCs in patients with CCR.[107] These findings suggest that the likelihood of detecting a kinase domain mutation may be higher in CD34+ cells. To determine the optimal cell compartment for screening, we compared *BCR-ABL* amplification and mutation detection in CD34+, CD34-negative and MNC samples. *BCR-ABL* was amplifiable in 42 of 66 samples (64%). Unselected MNC were analyzed in the initial 18 of these 42 patients, while CD34+ cells were selected in the following 24 . In samples subjected to CD34+ selection, *BCR-ABL* could be amplified from both CD34+ and CD34-negative cells in 9 patients, in 12 patients only from CD34-negative cells and in 3 patients only from CD34+ cells (Table 2.1). The higher rate of failure in CD34+ cells is likely the result of low cell numbers. Overall, the probability of detecting a mutation was not greater in CD34+ compared to CD34-negative or unselected MNC samples (1/11 vs. 8/39, $P = 0.66$, Fisher's exact test). Thus, in our hands enrichment for CD34+ cells does not increase the probability of mutation detection.

Table 2.1. Comparison of kinase domain mutation detection in CD34 separated samples versus unselected mononuclear cell samples.

Cell Compartment	Patients Tested	BCR-ABL+ (% tested)	Mutation+ (% amplified)
CD34+	31	11 (35%)	1 (9%)
CD34-	31	21 (68%)	4 (19%)
CD34+/- (combined)	31	24 (77%)	4 (17%)
MNC	35	18 (51%)	4 (22%)
Results Combined	**66**	**42 (64%)**	**8 (19%)**

At a technical level, two additional factors influence the sensitivity and specificity of mutation detection in the setting of low level residual disease. Firstly, *BCR-ABL* may or may not be amplifiable. Secondly, the sequencing quality will influence the minimal proportion of mutant allele that is detected. To address some of these issues, we analyzed all specimens by two independent methods. The first approach consisted of direct sequencing of PCR products using two sets of nested primers spanning the *BCR-ABL* breakpoint for amplification and primers spanning the *ABL* kinase domain for sequencing. The second approach used a primer set spanning the *BCR-ABL* breakpoint for the first round of PCR and four independent primer sets spanning overlapping portions of the *ABL* kinase domain for the second round, the product of which is subjected to D-HPLC. In 24/66 samples (36%) *BCR-ABL* was undetectable with either method. Twenty-three samples (35%) were informative only by D-HPLC, 12 (18%) with both methods and 7 (11%) only by direct sequencing (Figure 2.1). In the latter, failure was mostly due to incomplete D-HPLC results (n = 5), when the amplifications failed for 1, 2 or 3 amplicons. Only two samples amplifiable in the direct sequencing procedure failed D-HPLC outright (Table 2.2). The lower rate of informative results for the direct sequencing procedure is explicable by the use of two breakpoint-spanning primer pairs rather than a second primer pair spanning only the *ABL* kinase domain. While this approach is likely to increase specificity by avoiding second-step amplification of *ABL* from carry-over cDNA it reduces amplification efficacy due to the longer amplicons. The failure of D-HPLC in samples that were informative by direct sequencing is not readily explicable. Attempts are underway in our laboratory to further optimize conditions for a situation with generally low levels of *BCR-ABL*, such as patients in CCR. In any case, from the standpoint of

informativity, D-HPLC was superior to direct sequencing. It is important to note that in our series of patients with low levels of *BCR-ABL* this superiority is the result of a higher rate of successful amplification compared to direct sequencing. When both methods were informative there was concordance of kinase domain sequencing results, though results in CD34+ and negative cell compartments were occasionally different in this subgroup (Table 2.3). Given that D-HPLC has been shown to detect mutant amplicons as low as 15% of total *BCR-ABL* compared to 30% for direct sequencing [105], it is possible that our failure to detect a difference between the two methods is related to the low incidence of mutations. To determine whether the sensitivity of detecting mutations could be improved by subcloning, we sequenced 10-20 individual PCR clones from the samples that were informative by direct sequencing (Table 2.4). However, only one additional mutation (C305S) was detected, suggesting that subcloning is not generally warranted.

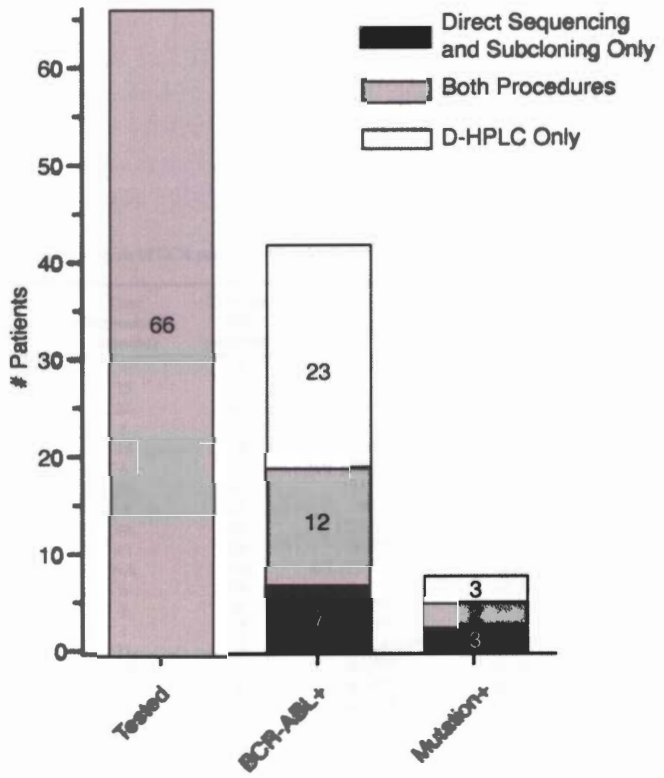

Figure 2.1. Amplification and mutation detection in patient samples by direct sequencing and D-HPLC procedures. Successful amplification in patients is compared for the direct sequencing procedure versus D-HPLC. Of 66 patients, 24 failed to amplify with either method. Of the 42 patients with detectable *BCR-ABL*, D-HPLC was informative in 35, direct sequencing in 19 and both procedures in 12. Of the eight patients with a mutation, D-HPLC detected the mutation in three, direct sequencing in three (including one mutation detected only by subcloning) and both procedures in two.

Table 2.2. Demographics and kinase domain mutation analysis of CCR patients with amplifiable BCR-ABL.

Patient No.	Age at Study (years)	Sex	Disease Stage at Diagnosis	MNC FISH	Time Diagnosis-IM (months)	Time on IM (months)	D-HPLC Mutation Result	Direct Sequencing Result	Subcloning Result	Study – Last Follow-up (months)	Current Cytogenetic Status	Current FISH Status
1	85	M	AP	0%	96	48	No Amp	WT^{MNC}	WT (13/16)^{MNC}	12	4% Ph+	0%
2	63	F	CP	6%	35	49	No Amp	WT^{MNC}	WT (18/19)^{MNC}	12	CCR	1%
3	84	F	CP	0.5%	26	40	WT^{MNC}	WT^{MNC}	WT (15/15)^{MNC}	11	5% Ph+	0.5%
4	80	M	AP	0%	5	31	WT (325-387)^{MNC}	$T315I^{MNC}$	T315I (15/15)^{MNC}	12	CCR	0%
5	64	M	CP	0%	15	28	WT	No Amp	NA	28	CCR	0%
6	51	M	CP	0%	6	39	WT (277-434)^{MNC}	$Y253H^{MNC}$	Y253H (16/16)^{MNC}	22	CCR	0%
7	39	M	CP	0%	96	43	$T315I^{MNC}$	No Amp	NA	29	CCR	0%
8	59	M	CP	0.5%	4	27	WT	No Amp	NA	14	CCR	0.5%
9	69	F	AP	0.5%	46	32	$F359V^{MNC}$	No Amp	NA	5	100% Ph+	14.5%
10	69	M	AP	0%	41	29	WT^{MNC}	No Amp	NA	NA	NA	NA
11	NA	NA	NA	NA	NA	NA	WT (277-434)^{MNC}	WT^{MNC}	WT (16/19)^{MNC}	36	36% Ph+	NA
12	58	F	CP	NA	6	19	WT^{MNC}	No Amp	NA	27	CCR	NA
13	46	M	CP	NA	2	8	WT^{MNC}	No Amp	NA	18	CCR	NA
14	57	F	CP	NA	1	12	WT^{MNC}	WT^{MNC}	WT (13/15)^{MNC}	18	CCR	NA
15	59	M	CP	NA	31	13	WT (325-434)^{MNC}	WT^{MNC}	WT (10/11)^{MNC}	21	CCR	NA
16	52	M	CP	NA	15	11	WT^{MNC}	No Amp	NA	NA	NA	NA
17	36	F	CP	NA	0	8	WT^{MNC}	No Amp	NA	18	CCR	NA
18	78	M	CP	NA	45	23	WT^{MNC}	No Amp	NA	30	CCR	NA
19	54	M	CP	0%	0	17	WT^{34-}	WT^{34-}	WT (15/16)$^{34-}$	21	CCR	0%
20	27	F	CP	0.5%	0	28	$G250E^{34-}$	$G250E^{34-}$	G250E (17/17)$^{34-}$	20	CCR	0%
21	68	M	AP	1.5%	7	46	WT (277-434)$^{34-/+}$	WT^{34+}	WT (17/17)$^{34+}$	12	10% Ph+	1.5%
22	49	M	CP	0%	2	18	$WT^{34-/-}$	WT^{34+}	WT (13/15)$^{34+}$	13	CCR	0.5%
23	56	M	CP	0%	19	34	WT^{34-}	WT^{34-}	WT (20/20)$^{34-}$	12	CCR	0%
24	50	M	CP	1%	3	28	$WT^{34-/-}$	$WT^{34-/-}$	WT (17/18)$^{34+}$	12	CCR	0%
25	72	F	CP	0%	19	34	WT^{34+}	WT^{34-}	WT (13/13)$^{34-}$	9	CCR	0%
26	44	M	CP	0.5%	5	41	WT^{34-}	No Amp	NA	20	CCR	0%
27	43	F	CP	0%	0	12	WT^{34-}	No Amp	NA	12	CCR	0%
28	54	M	CP	0%	3	38	WT^{34-}	No Amp	NA	17	CCR	0%
29	44	M	CP	0%	2	53	WT^{34-}	No Amp	NA	NA	NA	NA
30	68	M	CP	0%	13	38	WT^{34-}	No Amp	NA	18	CCR	0%
31	45	M	AP	0%	112	44	WT^{34-}	No Amp	NA	NA	NA	NA
32	43	M	CP	0%	0	21	WT^{34+}	No Amp	NA	NA	NA	NA
33	64	F	CP	0%	144	41	WT^{34-}	No Amp	NA	19	CCR	0%
34	52	F	CP	0%	3	18	$WT^{34-/-}$	WT^{34-}	C305S (3/20)$^{34-}$	17	CCR	0%
35	43	M	CP	0%	1	43	$Y253F^{34-}M244V^{34-/+}$	$M244V^{34-}$	M244V (20/20)$^{34-}$	14	CCR	0%
36	24	F	CP	0%	43	63	$G321E^{34+}E355G^{34-}$	WT^{34+}	WT (16/16)$^{34+}$	15	CCR	0.5%
37	24	M	CP	1%	1	36	$WT^{34-/}$	WT^{34+}	WT (15/15)$^{34+}$	NA	NA	NA
38	55	M	CP	0%	10	53	WT^{34+}	No Amp	NA	19	CCR	0%
39	70	M	CP	0%	29	31	$WT^{34-/}$	No Amp	NA	12	CCR	0%
40	41	F	CP	0%	25	31	$WT^{34-/-}$	No Amp	NA	11	CCR	0%
41	56	M	CP	0%	19	50	WT^{34-}	No Amp	NA	NA	NA	NA
42	51	F	CP	0%	49	65	WT^{34-}	No Amp	NA	NA	NA	NA

Table 2.2 legend: Mononuclear cell samples were tested from patients 1-18; CD34-postive and CD-34 negative cells were selected in patients 19-42. MNC: Result obtained from mononuclear cell sample. 34+: Mutation result obtained in CD34-positive cells. 34-: Mutation result obtained in CD34-negative cells. Codons successfully analyzed in the D-HPLC procedure are included in parentheses when amplification failed for part of the 4 amplicons covering the kinase domain. For subcloning results the number of individual clones with the indicated result are shown in parentheses; mutations were dismissed if not present in more than one subclone. No Amp: No PCR amplification. NA: No sample available.

Table 2.3. Comparison of mutation analysis in samples with informative results in both procedures.

Patient No.	CD34+ D-HPLC result	CD34+ Direct Sequencing Result	CD34-/MNC D-HPLC Result	CD34-/MNC Direct Sequencing Result
3	NA	NA	WT	WT
14	NA	NA	WT	WT
19	ABL-	ABL-	WT	WT
20	ABL-	ABL-	G250E	G250E
22	WT	No Amp	WT	WT
23	ABL-	ABL-	WT	WT
24	WT	WT	WT	WT
25	ABL-	ABL-	WT	WT
34	WT	No Amp	WT	WT (C305S)
35	Y253F/M244V	No Amp	M244V	M244V
36	WT	WT	G321E/E355G	No Amp
37	WT	WT	WT	No Amp

Results denoted as ABL- were not amplifiable with *c-ABL* one-step PCR and thus discounted as negative due to low cell numbers. The single additional mutation found through subcloning is indicated in parentheses. NA: No sample available. No Amp: No BCR-ABL PCR amplification.

Table 2.4. Detection of mutations in CCR patients by direct sequencing, subcloning, or D-HPLC.

Procedure	Patients Tested	BCR-ABL+ (% tested)	Mutation+ (% amplified)
Nested RT-PCR & Direct Sequencing	66	19 (29%)	4 (21%)
Nested RT-PCR & Subcloning	19*	19*	5 (26%)**
Nested RT-PCR & D-HPLC	66	35 (53%)	5 (14%)**

*Samples subcloned were those that were amplifiable in the direct sequencing procedure.
**P = 0.29, Fisher's exact test.

2.4.2 Frequency of kinase domain mutations.

Sixty-six patients with stable CCR were enrolled and *BCR-ABL* amplification was successful in 42 (63%). In the remaining 24 patients *BCR-ABL* was undetectable, while *ABL* was detected by one-step PCR, consistent with good quality cDNA. The *BCR-ABL*-positive patients had a median age of 54 (range, 24-85) years, median disease duration of 46 months (range, 8-185) and median time on imatinib of 32 months (range, 8-65) (Table 2.2). At the time of starting imatinib, 36 patients had been in chronic- and 6 in accelerated-phase. At the time of mutation screening, only three patients were within 12 months of diagnosis. Thus, our cohort was comprised almost entirely of patients with long-standing disease. Altogether, we detected nine different point mutations in the *BCR-ABL* kinase domain of 8 of 42 patients (19%) (Table 2.5). Seven patients were found to harbor mutations associated with clinical resistance to imatinib, including G250E, T315I, Y253H, Y253F, M244V, F359V and E355G.[68,70,71,74] G321E was identified in one patient. This mutation was also found in a CCR patient studied by Chu et al. and was shown to confer intermediate imatinib-resistance.[102] One patient harbored a C305S mutation, which has not previously been described in patients or by *in vitro* mutagenesis screens.[108] In contrast to

all other mutations, C305S was detected only after subcloning and failed to confer IL-3 independence when transfected into Ba/F3 cells (data not shown), suggesting that it may be kinase inactive and thus irrelevant. In four patients with mutations, additional aliquots of the same bone marrow sample were available for analysis. In three patients the mutation was confirmed and in one *BCR-ABL* amplification failed. In total, mutations were present in 8 of 66 (12%) patients with stable CCR and in 8 of 42 (19%) of those patients with stable CCR who had amplifiable *BCR-ABL*.

Table 2.5. Results of kinase domain mutation analysis in CCR patients.

Patient No.	Age at Study (years)	Time on IM (months)	D-HPLC Mutation Result	Direct Sequencing Result	Subcloning Result	Time to Last Follow-up (months)	Current Cytogenetic Status	Follow-up Sample Mutation Status
4	80	31	WT (325-387)^{MNC}	T315I^{MNC}	T315I (15/15)^{MNC}	12	CCR	WT
6	51	39	WT (277-434)^{MNC}	Y253H^{MNC}	Y253H (16/16)^{MNC}	22	CCR	WT
7	39	43	T315I^{MNC}	No Amp	NA	29	CCR	No Amp
9	69	32	F359V^{MNC}	No Amp	NA	5	100% Ph+	NA
20	27	28	G250E^{34-}	G250E^{34-}	G250E(17/17)$^{34-}$	20	CCR	G250E
34	52	18	WT$^{34-/+}$	WT^{34-}	C305S (3/20)$^{34-}$	17	CCR	NA
35	43	43	Y253F^{34+} M244V$^{34-/+}$	M244V^{34-}	M244V (20/20)$^{34-}$	14	CCR	M244V
36	24	63	G321E^{34-} E355G^{34-}	WT^{34+}	WT (16/16)$^{34+}$	15	CCR	WT

MNC: Result obtained from mononuclear cell sample. 34+: Mutation result obtained in CD34-positive cells. 34-: Mutation result obtained in CD34-negative cells. Codons successfully analyzed in the D-HPLC procedure are included in parentheses when amplification failed for part of the 4 amplicons covering the kinase domain. For subcloning results the number of individual clones with the indicated result are shown in parentheses; mutations were dismissed if not present in more than one subclone. No Amp: No PCR amplification. NA: No sample available.

2.4.3 Follow-up of patients with mutations.

Four of eight patients with a mutation showed a concomitant rise of *BCR-ABL* transcript levels of more than two-fold within six months after sequencing (Figure 1.2A, #9, 20, 35 and 36). This cutoff has been proposed by Branford and colleagues as predictive for detection of kinase domain mutations.[68] Of these four, two (#9 and 36) had subsequent cytogenetic relapse and one (#9) died from disease progression. The remaining two patients with a rise in *BCR-ABL* transcript levels maintained a CCR, one (#20, G250E) with a dose increase of imatinib from 600 to 800 mg daily. In the other four patients (of the eight with mutations), stable levels of *BCR-ABL* transcript were observed, and follow-up specimens, when amplifiable, were consistently wild type (Figure 1.2B).

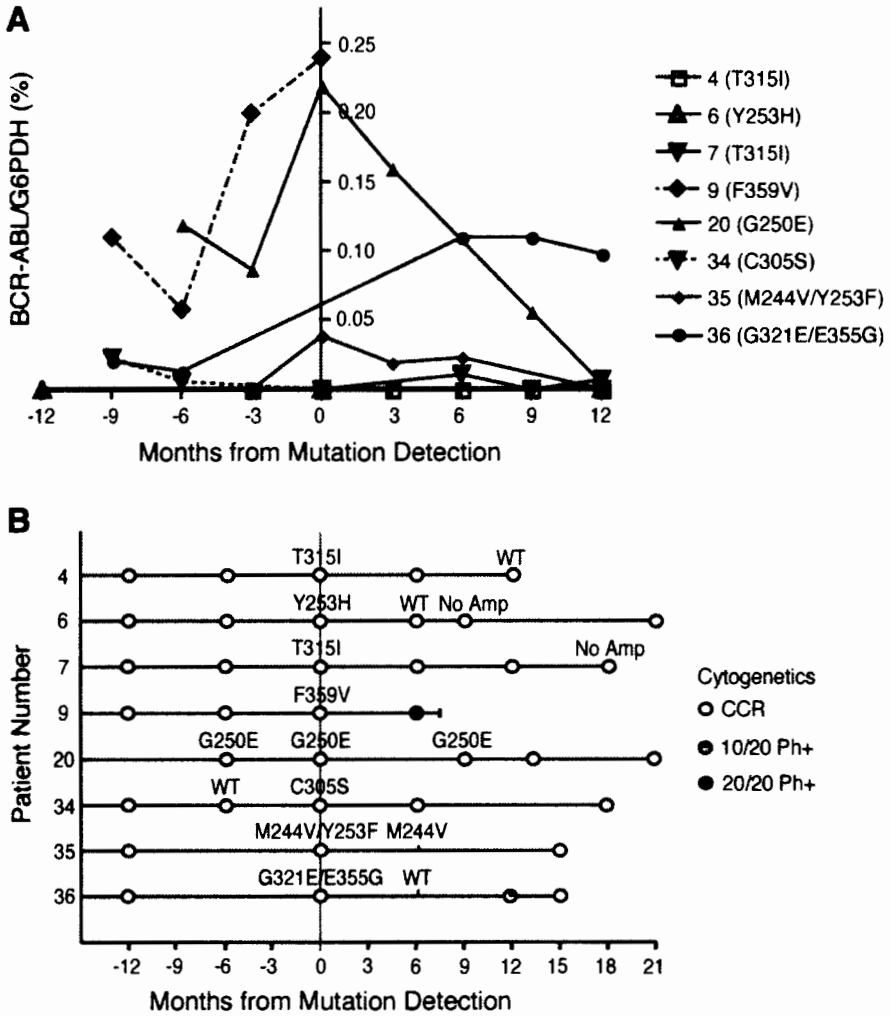

Figure 2.2. Follow-up data for patients with a kinase domain mutation. **(A)** Available quantitative RT-PCR data for *BCR-ABL* in patients with a kinase domain mutation was graphed for the period of 12 months before and after the original detection of a mutant. **(B)** Clinical timelines corresponding to the quantitative RT-PCR data are shown to indicate the cytogenetic and mutation analysis during the study period.

2.4.4 Follow-up of patients without detectable mutations at initial study.

Our data suggested that some kinase domain mutations may be detected only transiently. We reasoned that patients with stable CCR may have occasional peaks in *BCR-ABL* mRNA due to transient amplification of kinase domain mutant clones. We

therefore reviewed the qPCR data for the 58 patients without a detectable mutation at initial study in the 12 months before and after the analysis (all patients without mutation detection, including those that failed to amplify for *BCR-ABL* in the initial analysis, but excluding the previously discussed patients with mutations). Patients found in the original sample to have undetectable *BCR-ABL* were included in this analysis because patients with undetectable *BCR-ABL* often fluctuate between detectable and undetectable levels over time.[109] Of 39 patients with available data during this period (median number of qPCR tests 5, range 1-9) 32 had stable transcript levels, 2 patients had a steady decrease of transcripts from start of treatment, and 5 patients displayed a rise in *BCR-ABL* levels (median 9.7-fold, range 3.2-25) compared to the previous result (Figure 1.3A). Sequencing showed wild type *BCR-ABL* in all five patients at the time of the increase (Figure 1.3B). Chart review revealed inconsistent compliance with imatinib dosing in three of five patients (#2, 21 and 25). For example, patient #2 had ceased medication for two months prior to the rise in transcript to a peak of 2.2%, after which dosing was followed consistently and the qPCR level decreased to 0.01%. No reason for the peak could be established in the remaining two cases. Thus, noncompliance is also an important factor that can lead to a rise in *BCR-ABL* transcript levels and should be part of the diagnostic considerations.

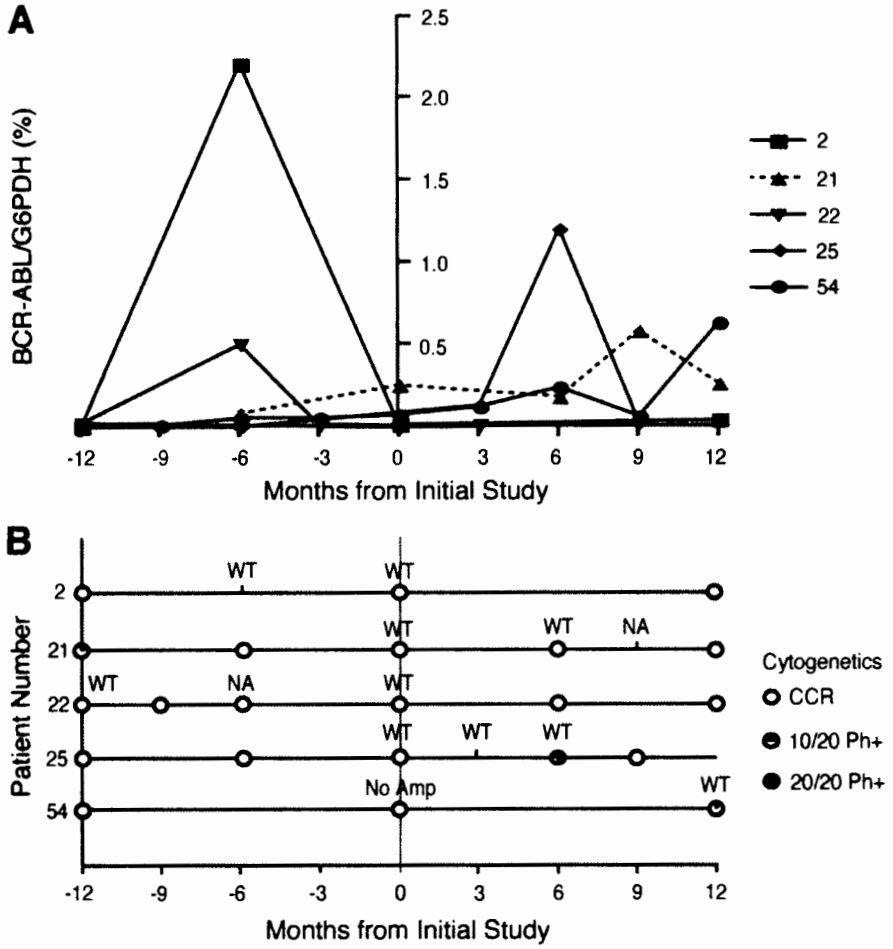

Figure 2.3. Follow-up analysis for patients without a kinase domain mutation at initial study. **(A)** Available quantitative RT-PCR data for the five patients with an increase in transcript levels during a period of 12 months before and after initial study, when negative for *BCR-ABL* or no mutations were detected. **(B)** Clinical timelines matched to the quantitative RT-PCR data are displayed to show the results of kinase domain mutation analysis corresponding to the time points with increased *BCR-ABL* transcript.

2.5 Conclusions

Our data suggest that the mere detection of a kinase domain mutation in a CCR patient, without a concomitant rise in *BCR-ABL* transcripts, does not predict for relapse, and that in the majority of patients with a stable CCR molecular persistence is not mediated by kinase domain mutations. It is unclear, why in some patients kinase domain

mutant clones fail to cause relapse. One possibility is that these transient clones lack self-renewal capacity and are thus unable to maintain long-term hematopoiesis. In regards to the overall prevalence of kinase domain mutations in CCR patients, our results appear to differ from the report of Chu and colleagues, who found kinase domain mutations in 38%, two-fold higher than the rate in our study.[102] Although this difference is not statistically significant (5/13 vs. 8/42, P = 0.26, Fisher's exact test), this may be due to the relatively small cohort sizes. Chu et al. analyzed CD34+ in all but one patient and multiple clones were routinely sequenced. However, in our study, mutation detection was not more frequent in CD34+ cells and sensitivity was not significantly improved by subcloning, excluding technical differences as the reason for the discrepancy. More likely, the cohort studied by Chu et al. was at higher risk for relapse and kinase domain mutations compared to our patients. This is supported by the fact that the median time on imatinib in our cohort was 32 months compared to six months in the study by Chu et al., suggesting that our cohort was enriched for patients with a stable response to imatinib. In the IRIS trial a reduction of relapse risk has been demonstrated with CCR duration.[110] Consistent with this, only 5/49 (10%) patients with available cytogenetic follow-up have lost a CCR at a median of 15 months (range, 5-36). Thus, in the majority of CCR patients, mechanisms other than kinase domain mutations must account for disease persistence. These potential mechanisms of disease persistence include insufficient kinase inhibition in leukemic stem cells due to drug efflux or high levels of Bcr-Abl expression, or Bcr-Abl kinase independent mechanisms such as stem cell quiescence.[111] Elucidating these mechanisms will be crucial for developing strategies that aim at eradication of residual leukemia.

CHAPTER 3

BCR-ABL SH3-SH2 MUTATIONS IN CHRONIC MYELOID LEUKEMIA PATIENTS DURING ABL KINASE INHIBITOR THERAPY.

BCR-ABL SH3-SH2 mutations in chronic myeloid leukemia patients during ABL kinase inhibitor therapy.

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D.W.S. performed the research, analyzed the data, wrote the paper and helped design the research; O.H., S.W., T.B. and L.T. participated in performing the research; R.D.P. managed the database and contributed samples; G.S.F. helped supervise the project, B.J.D. managed the study patients, and helped in the research design and supervising the project: M.W.D. designed the research, supervised the project, managed the study patients and helped write the paper.

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3.1 Abstract

Point mutations in the kinase domain of *BCR-ABL* are the most common mechanism of drug resistance in CML patients treated with kinase inhibitors, including imatinib, dasatinib and nilotinib. It has also been shown through in vitro mutagenesis screening that mutations outside the kinase domain in the neighboring linker, SH2, SH3 and Cap domains can confer imatinib resistance. These domains in c-Abl have an autoinhibitory function on the kinase domain and mutations in this region can result in an activated enzyme. Despite these observations, mutations in these domains have not yet been reported in CML patients being treated with Abl kinase inhibitors. We characterize for the first time mutations in the regulatory domains of Bcr-Abl during imatinib treatment. Mutations were detected in 7 of 98 (7%) patients at varying levels of response, while kinase domain mutations were detected in 29 (30%). Despite detection of mutations outside the kinase domain in a significant portion of patients, only one proved to be substantially imatinib-resistant in Ba/F3 cells. The mechanism of resistance of this mutation, T212R, may be linked to increased kinase activity. Altogether, we find imatinib-resistant mutations in the SH3-SH2 domains to be a rare, but valid mechanism of resistance in patients treated with imatinib.

3.2 Introduction

Chronic myeloid leukemia (CML) is caused by the Bcr-Abl fusion protein expressed from a reciprocal translocation between chromosomes 9 and 22. Bcr-Abl has constitutive tyrosine kinase activity that produces the myeloproliferative phenotype of chronic-phase CML. The treatment of CML patients with imatinib, a tyrosine kinase inhibitor with activity against Bcr-Abl, leads to disease control in almost all patients in chronic-phase.[24] However, the disease persists at low levels detectable only by RT-PCR for *BCR-ABL* and imatinib cessation invariably leads to relapse.[29,30] In the setting of CML progression beyond the chronic-phase, imatinib responses are typically transient and development of imatinib resistance is common.[36] Point mutations in the kinase domain of Bcr-Abl are the most common mechanism of drug resistance in CML patients treated with imatinib.[112] Although common, kinase domain mutations do not explain relapse or nonresponse in all patients treated with imatinib.

Other domains in Bcr-Abl may also be important for causing resistance. It has been shown through in vitro mutagenesis screening that mutations outside the kinase domain in the neighboring linker, SH2, SH3 and Cap domains can confer imatinib resistance.[108] These domains in c-Abl have an autoinhibitory function on the kinase domain, as mutations in these domains were shown to activate the otherwise autoinhibited c-Abl.[52,113] This was further elucidated by crystal structures of the Cap-SH3-SH2-kinase domain fragment of c-Abl.[53] The results from the in vitro mutagenesis screening and the crystal structures implied that residues that are critical for c-Abl autoinhibition are capable of rendering Bcr-Abl resistant to imatinib in vitro, and mechanisms that govern Bcr-Abl regulation might be identical to those observed in c-Abl.[114] Despite this, mutations outside

the kinase domain have not yet been reported in CML patients being treated with imatinib. The focus of this study was to investigate the possibility of mutations in the regulatory domains of Bcr-Abl in CML patients. Several mutations were found in these domains, but only one was found to be substantially imatinib resistant. This finding illustrates that mutations do occur outside the kinase domain in Bcr-Abl in patients with CML treated with imatinib. When such a mutation is detected serially over time in a patient, it may represent a mechanism of resistance and contribute to relapse.

3.3 Patients, Materials and Methods

3.3.1 Patient Samples. This study was approved by the Institutional Review Board of Oregon Health & Science University. Peripheral blood or bone marrow aspirate samples were obtained with informed consent from all patients. Mononuclear cells (MNCs) were isolated by density gradient centrifugation. Aliquots of cells were used for RNA extraction (RLT reagent, Qiagen, Valencia, CA).

3.3.2 Mutation Analysis. RNA was isolated from all samples using the RNeasy kit (Qiagen). Random hexamer primed cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). Mutation screening was done by direct sequencing of *BCR-ABL* products generated by nested PCR using 2 sets of primers on *BCR* and *ABL* as described.[104] All PCR reactions were set-up in DNA-free enclosures.

3.3.3 Generation of mutant alleles. Mutagenesis of the native BCR-ABL cDNA in the pSRα mammalian expression vector was performed using the Quik Change mutagenesis kit (Stratagene, La Jolla, CA). Correct mutagenesis was confirmed by sequence analysis covering the entire *BCR-ABL* sequence for each mutant.

3.3.4 Ba/F3 cell retroviral transduction. Infectious virus particles packaging WT and mutant $p210^{BCR-ABL}$ were produced by transient transfection of HEK293 T17 cells and supernatant collection after 48 hours incubation at 37°C. Parental Ba/F3 cells were then infected with retroviral supernatants. Isolation of Bcr-Abl expressing cells was performed by 2 weeks of neomycin selection followed by the additional withdrawal of IL-3.

3.3.5 Cell proliferation assays. Exponentially growing Ba/F3 cells expressing WT or mutant Bcr-Abl were plated (in quadruplicate) at 4×10^3 per well in 96-well plates and exposed to increasing doses of imatinib, nilotinib, or dasatinib. After 72 hours, the methanethiosulfonate (MTS)-based viability assay was performed and analyzed as described.[115] The average of 4-10 experiments was reported from the mean inhibition of growth at each dose.

3.3.6 Phosphotyrosine immunoblot analysis. Ba/F3 cells expressing WT or mutant Bcr-Abl were plated at 4×10^6 per well in 6-well plates and exposed to concentrations of imatinib, nilotinib or dasatinib that matched the cell proliferation experiments. Cells were incubated for 3 hours at 37°C, pelleted and lysed directly in SDS-PAGE loading buffer for 5 minutes at 95°C. Bcr-Abl phosphorylation and expression were detected by immunoblot with mouse monoclonal phosphotyrosine antibody 4G10 (Upstate Biotechnology, Waltham, MA) and rabbit Abl antibody K12 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

3.3.7 Kinase Assays. Transfection of HEK293 cells, immunoprecipitation of Abl protein and Abl in vitro kinase assay were carried out as described previously.[52,116] The relative concentration of immunoprecipitated Abl protein was determined by immunoblotting (anti-Abl Ab-3, Oncogene Science, Cambridge, MA) and subsequent quantification using

the Li-cor Odyssey system and normalized for c-Abl wild-type. Full-length mutant and native Bcr-Abl proteins were expressed in SF9 cells by baculoviral transduction as described.[76] A 6x-Histidine tag at the N-terminus allowed for purification with Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) as described.[76] Protein was eluted with 250mM imidazole, dialyzed overnight and stored at –80°C. Kinase assays were performed in triplicate using graded concentrations of N-terminal biotin-linked peptide substrate (biotin-EAIYAAPFAKKK-amide), approximately 10nM enzyme and 50μM ATP/[γ-^{32}P]ATP. Reactions were terminated after 10 minutes by addition of guanidine hydrochloride and transferred to a streptavidin-coated membrane (SAM^2 Biotin capture membrane, Promega, Madison, WI). Membranes were washed according to manufacture's instructions and phosphate incorporation measured by scintillation counting.

3.3.8 Quantitative RT-PCR (qRT-PCR). qRT-PCR was performed on peripheral blood or bone marrow aspirate specimens as described.[106] Briefly, *BCR-ABL* and *G6PDH* transcripts were quantified using real-time quantitative PCR and fluorescent resonance energy transfer hybridization probes in a LightCycler instrument (Roche Applied Science, Indianapolis, IN). The relative *BCR-ABL* and *G6PDH* transcript levels were quantified and reported as the ratio of *BCR-ABL* to *G6PDH* in percent.

3.4 Results

3.4.1 Prevalence of BCR-ABL SH3-SH2 domain mutations.

Mutation screening in CML patients treated with imatinib yielded 9 novel mutations in the regulatory domains, confirmed by bidirectional sequencing. These were dispersed in the Cap domain (R47C), SH3 (K84N), SH3-SH2 connector (E123Q), SH2

(G144E, S154N, A196V, T212R) and SH2-kinase linker (N231D, N231I) domains (Table 3.1). These occurred in 7 of the 98 patients tested (7%), with two having a second SH3-SH2 domain mutation. Patient #2 had two mutations, T212R and S154N. Patient #4 with CML blast crisis had mutations in both the SH3-SH2 domains (R47C, K84N) and the kinase domain (Q346R, H396P). Whereas SH3-SH2 domain mutations occurred in 7%, kinase domain mutations occurred in 29 of the 98 patients tested (30%).

Table 3.1. Characteristics of patients with mutation detection in the regulatory domains of Bcr-Abl.

Patient No.	Age/ Sex	Disease Duration (months)	Time on IM (months)	Disease Phase	Kinase Domain Sequencing	SH3-SH2 Domain Sequencing	Cytogenetics/ FISH/qRT-PCR
1	52/F	21	18	CP	WT	N231D	0%/0%/0
2	84/F	66	40	CP	WT	T212R, S154N	0%/0.5%/NA
3	38/F	82	42	CP	WT	A196V	0%/0%/0.057
4	72/F	87	67	BC	H396P, Q346R	K84N, R47C	10%/12.5%/NA
5	54/F	47	27	AP	E292V	N231I	0%/7.5%/0.053
6	54/M	45	26	CP	WT	G144E	0%/0.3%/0.019
7	37/F	26	22	CP	WT	E123Q	5%/2.5%/0.18

Patients of each disease stage and with differing degrees of imatinib response were tested. Imatinib response was classified on the basis of achievement of a complete cytogenetic response (CCR) at any point during imatinib treatment. CCR patients were those with absence of the Philadelphia chromosome upon bone marrow cytogenetics of the index sample, relapsed patients were defined by the loss of a previous CCR and nonresponders were those not reaching a CCR. SH3-SH2 domain mutations were found in 5 of 38 (13%) with a CCR, 2 of 22 (9%) relapsed patients and 0 of 38 nonresponders (Table 3.2). By comparison, kinase domain mutations were detected in 9 of 38 (24%) with a CCR, 7 of 22 (32%) relapsed patients and 13 of 38 (34%) nonresponders.

Table 3.2. Summary of mutation analysis in CML patients according to imatinib response.

Imatinib response (at Study)	Total Number of Patients	Number with kinase domain mutation	Number with Regulatory Domain mutation
Nonresponder	38	13 (34%)	0 (0%)
CCR	38	9 (24%)	5 (13%)
Relapse	22	7 (32%)	2 (9%)
Total	**98**	**29 (30%)**	**7 (7%)**

3.4.2 Structural modeling of mutations in Abl regulatory domains.

The locations of the mutations detected in the Cap, SH2, SH3 and linker domains are shown in the crystal structure of autoinhibited c-Abl (Figure 3.1A).[53] The location of some mutations may imply that they activate the Abl kinase by destabilizing intramolecular interactions. For example, A196V occurs at the interface between the SH2 domain and the N-terminal Cap that latches the SH3-SH2 domain clamp in the

autoinhibited conformation. Another example is residue 231, at which N231D and N231I may destabilize the packing of SH3, the kinase domain linker and the kinase domain in the autoinhibited conformation. On the other hand, T212R is a mutation in the SH2 domain that does not have obvious consequences in the autoinhibited conformation, as this residue is solvent exposed in this structure. To explain the effect of T212R, we modeled this mutation in disinhibited conformation of c-Abl, in which the SH2 domain changes its position substantially in reference to the kinase domain.[117] In this conformation, T212R occurs near the new interface formed between SH2 and N-lobe of the kinase domain (Figure 3.1B). The threonine side chain at this position does not interact with the kinase domain, whereas arginine appears to be able to form a new electrostatic interaction with E275 (Figure 3.1C). Thus, we hypothesized that the unique propensity for imatinib resistance and Abl kinase activation may stem from stabilization of the disinhibited conformation.

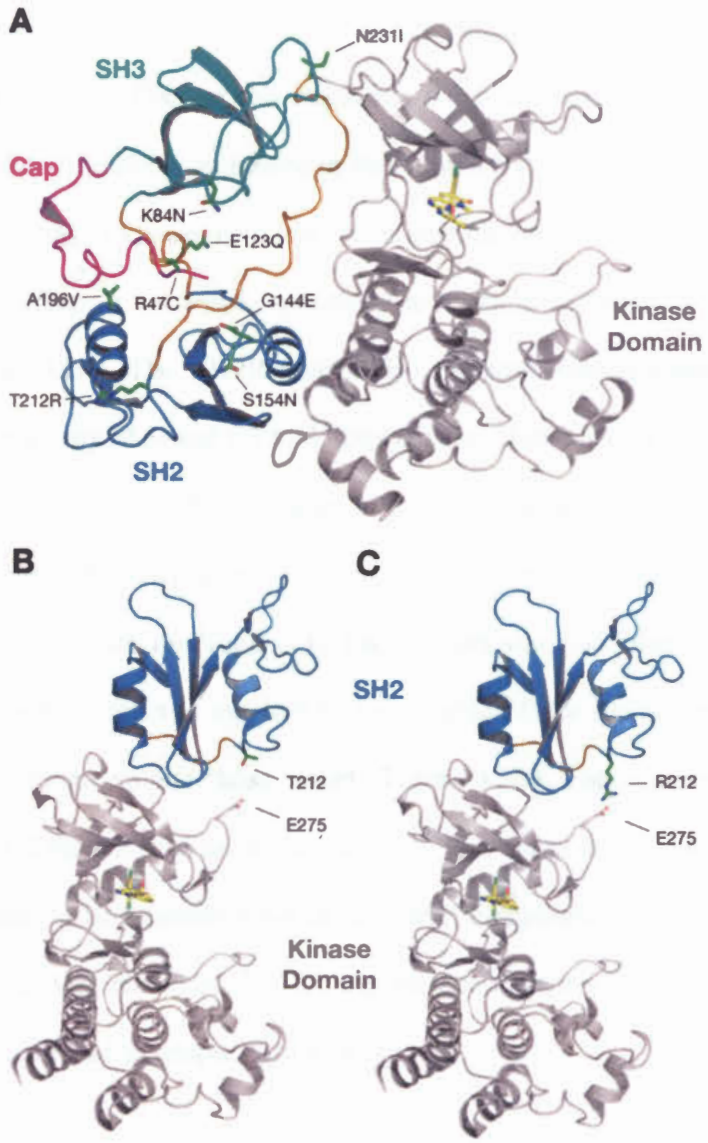

Figure 3.1. Locations of patient regulatory domain mutations in the autoinhibited and disinhibited conformations of Abl. (A) The crystal structure of autoinhibited Abl in complex with the kinase inhibitor PD166326 (PDB entry 2FO0)[117] displayed with the locations of the mutated residue side chains shown in green stick format. The mutations are dispersed across the SH2 (sky blue), SH3 (cyan) and linker domains (orange). (B) The threonine of residue 212 (green) is shown near the interface between SH2 and the kinase domain's N-terminal lobe in the disinhibited conformation of Abl (PDB entry 1OPL, molecule B).[117] (C) In the T212R mutant, arginine is in position to hydrogen bond with the kinase domain at E275 (gray). The kinase domain is shown in gray with bound inhibitor in yellow. Stick format atoms are color-coded for nitrogen (dark blue), oxygen (red) and sulfur (orange). The figure was created using PyMol.[73]

3.3.3 Kinase inhibitor sensitivity of SH3-SH2 domain mutations.

To measure the effect of the mutations we discovered in patients, each was introduced into Bcr-Abl, stably expressed in Ba/F3 cells and tested for cell proliferation in graded concentrations of kinase inhibitors. With imatinib only the T212R mutant of Bcr-Abl had a large effect in decreasing sensitivity to imatinib compared to native Bcr-Abl (2.4-fold) (Figure 3.2A). The T212R mutant also conferred similar changes in nilotinib and dasatinib sensitivity (2.5- and 1.5 fold, respectively) (Table 3.3). The E123Q mutation also caused a statistically significant change in imatinib sensitivity, but was only 1.3-fold resistant to imatinib. The other patient mutations did not significantly change sensitivity to imatinib, nilotinib or dasatinib (Table 3.3). Each mutant was also tested for sensitivity of Bcr-Abl autophosphorylation to imatinib by immunoblot. Once again, the T212R mutant was uniquely resistant among those tested (Figure 3.2B). For comparison, the most resistant SH3-SH2 mutant from an in vitro screen[108] was tested in our assays. This T224A mutant was found to be imatinib resistant to a level comparable to E123Q (Table 3.3). Thus, among SH3-SH2 mutations, T212R was the one mutant with enough resistance to suspect that it might lead to relapse during therapy.

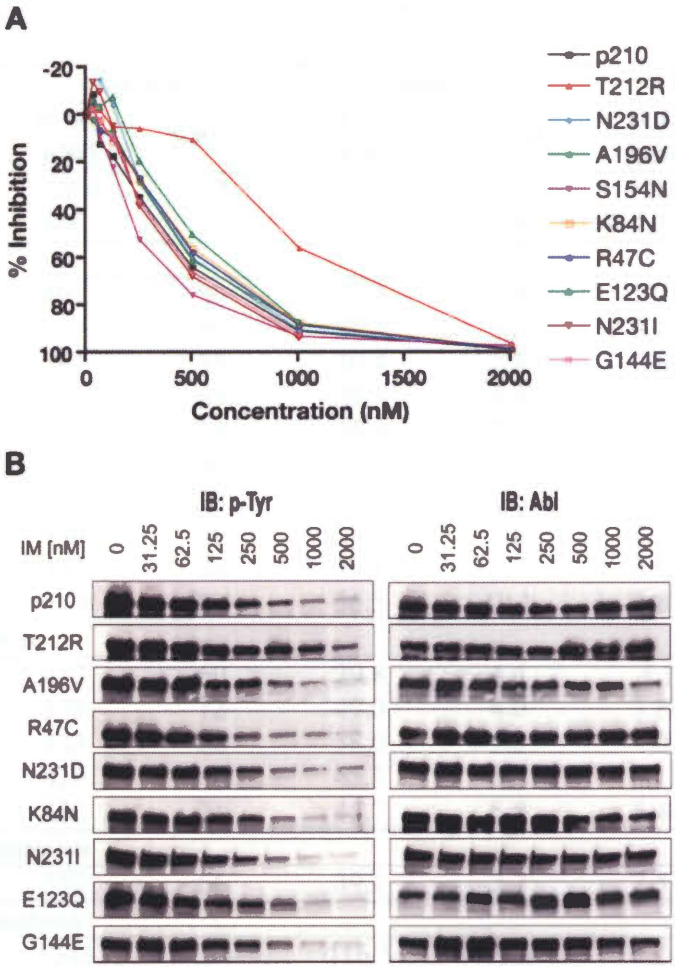

Figure 3.2. Imatinib sensitivity of regulatory domain mutations in Bcr-Abl. (A) SH3-SH2 mutations were introduced into Bcr-Abl and stably expressed in Ba/F3 cells. These cell lines were exposed to graded concentrations of imatinib and cell proliferation measured at 72 hours by MTS assay. Wild type Bcr-Abl (p210) is shown in comparison to the patient mutations. T212R was the only mutant with substantial resistance to imatinib in cell proliferation. Graphs represent the average of 4-10 experiments. (B) Cell lines were exposed to graded concentrations of imatinib matching the cell proliferation experiments for four hours and lysed directly into SDS-page loading buffer. Immunoblots were performed for Bcr-Abl autophosphorylation and expression. T212R was the only mutant to display detectable resistance of Bcr-Abl autophosphorylation to imatinib.

Table 3.3. Summary of Ba/F3 cell proliferation experiments.

	Imatinib		Nilotinib		Dasatinib	
	IC50 (nM)	S.E.M.	IC50 (nM)	S.E.M.	IC50 (nM)	S.E.M.
Ba/F3	>2000	NA	>2000	NA	>100	NA
p210	406	51	34.3	2.8	2.53	0.49
Patient mutants						
N231D	401	50	38.2	3.8	3.35	0.78
T212R	957	96	84.4	5.6	3.75	0.36
A196V	381	55	30.4	2.8	2.62	0.52
S154N	256	69	28.3	6.9	2.61	0.64
K84N	396	34	35.8	3.2	3.15	0.74
R47C	392	46	33.2	3.1	2.71	0.56
E123Q	510	33	35.7	3.6	1.98	0.18
N231I	340	70	38.2	5.8	2.24	0.33
G144E	373	44	39.1	3.7	2.89	0.39
Q346R	1078	261	56.5	10.1	3.81	0.92
Activating mutants						
p190	412	60	29.8	4.4	4.1	1.65
Abl PP	378	72	49.0	4.0	3.86	1.05
K51A	433	68	38.0	5.5	4.00	1.21
W99A	423	137	45.3	137.1	4.18	0.97
Y139D	430	71	45.7	5.8	5.48	2.20
In vitro screen mutants						
T224A	559	66	39.1	2.7	2.57	0.30
T212R double mutants						
E275K	1343	134	116.5	16.4	3.19	0.36
T212R/E275K	~2000	NA	176.3	10.4	4.67	1.39
E275V	583	82	24.1	0.9	1.33	0.09
T212R/E275V	1261	55	53.9	5.2	17.12	5.20
T212R/E275A	951	128	57.3	6.7	1.82	0.15
T212R/S154N	403	73	36.6	1.8	1.79	0.19

3.4.4 Mutations in SH3-SH2 activate the kinase activity of c-Abl.

Due to the locations of the mutations discovered in the autoregulatory domains of the Abl kinase, we hypothesized that their mechanism of resistance may be rooted in deregulating kinase activity. To test the effects of the mutations on Abl kinase function, we introduced them into c-Abl and overexpressed the mutant proteins in HEK293 cells. SH3-SH2 domain mutations were tested alone and in combination with the P223E/P230E

double mutations that constitutively activate c-Abl (Abl PP).[113] Immune complex in vitro kinase assays showed that the T212R mutation conferred the most activation of c-Abl, to a level comparable to the Abl PP double mutant (Figure 3.3). The R47C and A196V mutants also activated the kinase activity of c-Abl, but to a lesser extent. All three mutations tested displayed an additive increase in kinase activity when combined with the PP mutations. In summary, several mutations in the SH3-SH2 domains, T212R in particular, activate the kinase activity of the native Abl kinase.

Because T212R displayed the largest increase in both imatinib resistance and c-Abl kinase activity, we sought to investigate the consequence of this mutation in full-length Bcr-Abl. T212R and native Bcr-Abl were purified from insect cells. These were dephosphorylated and used in kinase assays with varying concentrations of optimal substrate peptide. Preliminary experiments showed a small increase in the kinase activation of the T212R mutant compared to native Bcr-Abl (Figure 3.3B). Thus far the difference has been consistent in two experiments, but the change is not statistically significant and is currently being repeated. The protein levels were measured by Bradford assay and shown to be equivalent by immunoblot (Figure 3.3C). Thus, preliminary data suggests that the T212R mutant also increases the kinase activity of full-length Bcr-Abl.

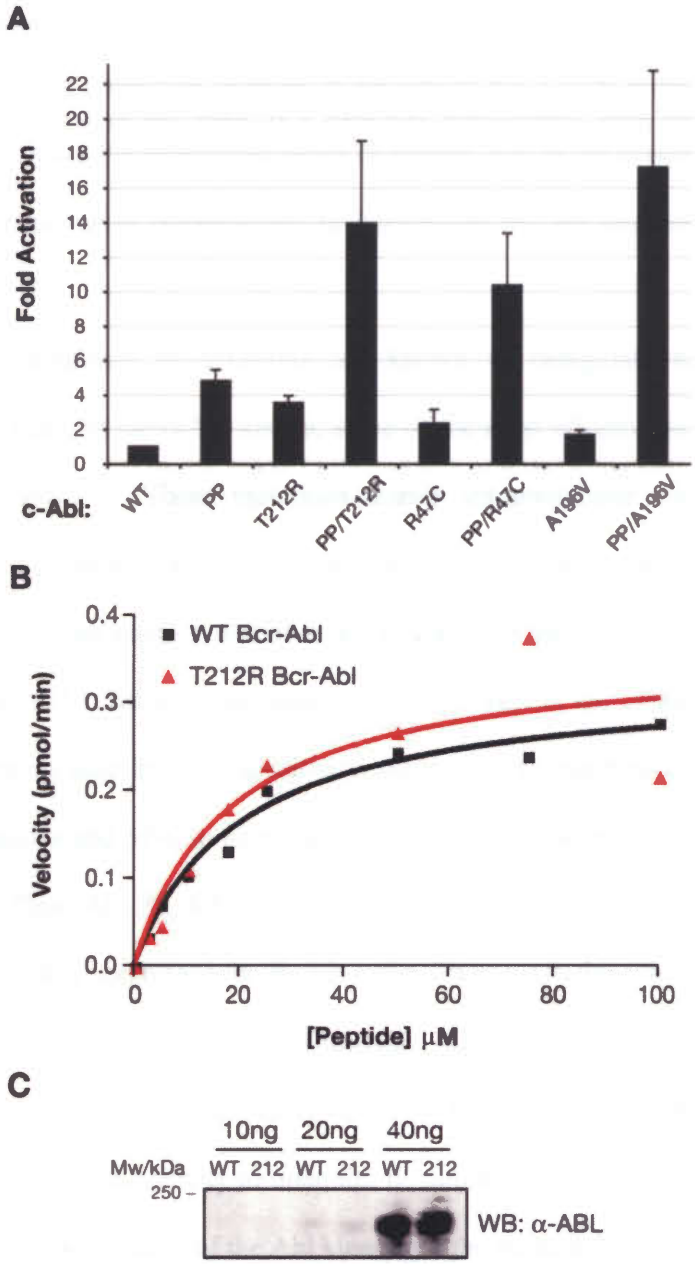

Figure 3.3. Kinase assay of SH3-SH2 mutants to test for propensity for kinase activation. (A) Immunoprecipitated c-Abl proteins expressing patient SH3-SH2 domain mutations alone or together with the Abl-activating PP (P223E/P230E) mutations were assayed for activity by in vitro kinase assays with an optimal substrate peptide. The graphs show catalytic activity relative to c-Abl for two experiments done in duplicate (mean with S.D.). T212R was the most activating mutation. A196V and R47C also caused an increase in kinase activity compared to wild type c-Abl. All three patient mutations tested conferred an additive increase in kinase activity when combined with Abl PP. Data from Oliver Hantschel. (B) T212R and native full length Bcr-Abl were expressed and purified from

baculovirus infected SF9 cells. Kinase assays were performed in triplicate and the enzyme velocity plotted as a function of peptide concentration. Preliminary data from two experiments shows a consistent increase in activity of the T212R mutant, but this is not statistically significant at this time. (C) Anti-Abl immunoblot with purified native and T212R Bcr-Abl shows the normalized levels of protein used in the kinase assay.

3.3.5 Mutations known to constitutively activate c-Abl are not Imatinib resistant in the context of Bcr-Abl.

Several mutations in SH3-SH2 are known to deregulate the autoinhibitory mechanisms in place in the c-Abl kinase, some of the most effective are Abl PP, K51A, W99A and Y139D.[52,113] These mutations disrupt intramolecular contacts within the autoinhibited conformation of c-Abl.[53] In the context of Bcr-Abl, the p190 fusion protein that is associated with acute lymphoblastic leukemia is known to have higher kinase activity than the p210 fusion. After discovering mutations in the domains in Bcr-Abl in patients and testing their imatinib sensitivity, we wished to compare these to the known activating mutations and p190 to gain insight into the relationship between kinase activity and resistance. Thus, Abl PP, K51A, W99A and Y139D were introduced into full length *BCR-ABL* and stably expressed in Ba/F3 cells. These cell lines were tested for cell proliferation in graded concentrations of imatinib. All of these mutants displayed nearly identical imatinib sensitivity as the native Bcr-Abl (Table 3.3). The p190 Bcr-Abl protein also produced a similar sensitivity to imatinib. In summary, neither mutations that disrupt the autoinhibited conformation of the Abl kinase nor the more active p190 Bcr-Abl change imatinib sensitivity.

3.3.6 *Mutation analysis of residue 275 in the T212R mutant Bcr-Abl.*

To test the hypothesized interaction of T212R with E275 a series of double mutants were made in attempt to cancel the observed effect of T212R on imatinib

sensitivity. We reasoned that E275K would prevent the formation of the electrostatic interaction and create a repulsive interaction between the two positively charged side chains. Moreover, E275V and E275A were predicted to also eliminate the electrostatic interaction. Thus, T212R/E275K, T212R/E275V and T212R/E275A Bcr-Abl double mutants were stably expressed in Ba/F3 cells and tested for imatinib sensitivity in cell proliferation assays. Cells expressing the E275K mutant were 3.3-fold more imatinib resistant than cells expressing native Bcr-Abl (Figure 3.4). In the T212R/E275K double mutant there was an additive effect of increased resistance to imatinib (4.9-fold), in contrast to the hypothesized effect. In addition, the E275V mutant was also imatinib resistant (1.4-fold) and the T212R/E275V mutant had additive resistance of each single mutant (3.1-fold). The T212R/E275A mutant had the same imatinib sensitivity as T212R alone, indicating that E275A does not have an effect on resistance. On the other hand, the other mutation found in patient #2, S154N reverted the sensitivity of T212R back to that of wild type Bcr-Abl. In summary, the compensatory mutations made at E275 indicate that T212R does not interact with this residue.

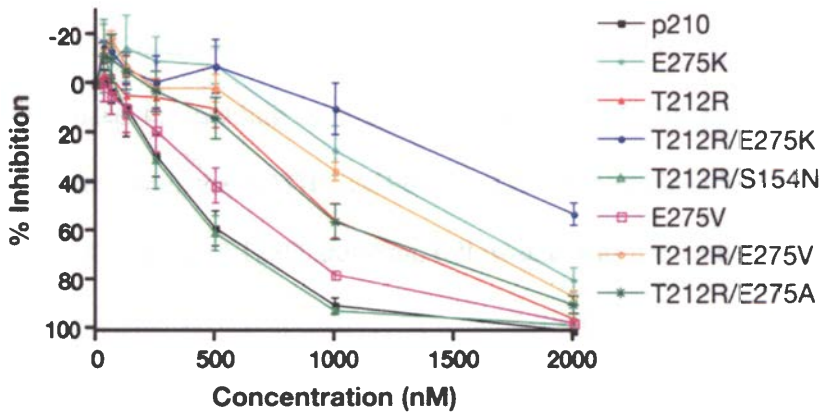

Figure 3.4. Compensatory mutation analysis in the T212R mutant. The E275K, E275V, E275A and S154N were tested in native and T212R Bcr-Abl by Ba/F3 cell proliferation assays, as before. E275K and E275V displayed substantial imatinib resistance alone and this effect was additive to that of T212R in the double mutants. T212R/E275A had the same sensitivity to that of the T212R single mutant. In the T212R/S154N double mutant, the sensitivity of imatinib was reverted back to that of wild type Bcr-Abl.

3.3.7 Follow-up analysis of CML patients with an SH3-SH2 domain mutation.

Patients in whom an SH3-SH2 domain mutation was discovered were observed for their clinical response to imatinib before and after the detection of the mutation. A graph of the qRT-PCR values of *BCR-ABL* is shown in Figure 3.5A. Consistent with the cell line results, patient #2, harboring the T212R mutation, had a large increase in *BCR-ABL* transcript level beginning at 29 months. Notably, patient #4 with blast crisis was at a very high level of *BCR-ABL* at the time when four mutations were detected, but was subsequently switched to dasatinib, resulting in a reduction in transcript to an undetectable level. Among the remaining patients with detection of a mutation in SH3-SH2, only patient #3 with the A196V mutant has had an increase of *BCR-ABL* transcript over baseline levels. The cytogenetics and mutation analysis before and after mutation analysis is shown in Figure 3.5B. Patient #2 had detection of T212R at two follow-up time points,

whereas the other mutations have not been found on follow-up samples, when available. Accordingly, patient #2 had cytogenetic relapse, first at 12 months and worsening at 30 months after T212R detection. Patient #4 with the R47C, K84N, Q346R and H396P mutations, responded to dasatinib with an undetectable *BCR-ABL* transcript level at 1 month. The patients with the N231D, A196V, or G144E mutations have maintained a CCR. For patient #7 with the E123Q mutation, follow-data was not available.

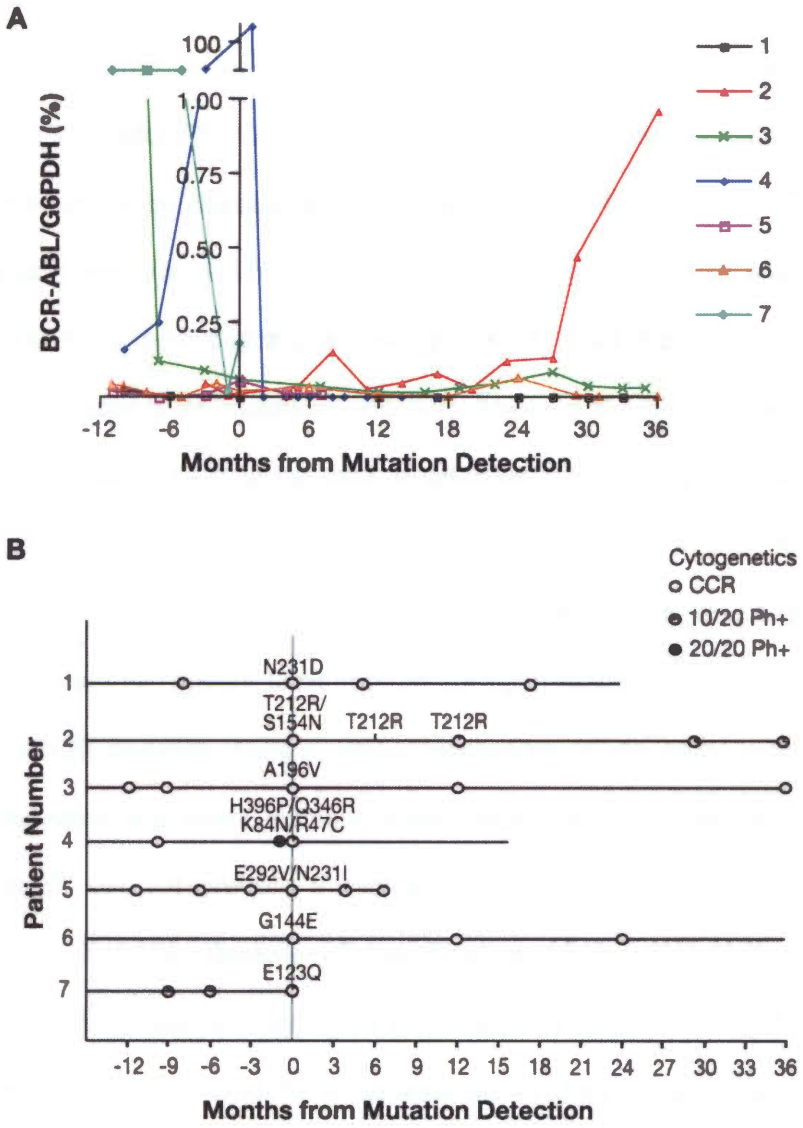

Figure 3.5. Follow-up data for patients with detection of a regulatory domain mutation. (A) Available qRT-PCR data for *BCR-ABL* in patients with a SH3-SH2 domain mutation was graphed for the period of 12 months before to 36 months after the index sample with mutation detection. (B) Clinical timelines corresponding to the qRT-PCR data are shown to indicate the cytogenetic and mutation analysis during the study period. All patients were taking imatinib (IM) during the period shown, unless switched to dasatinib (das) when noted.

3. 4 Discussion

We have described the novel occurrence of mutations in the Cap, SH3, SH2 and SH2-kinase linker domains of Bcr-Abl in CML patients while on imatinib therapy. Overall, 7 of 98 patients (7%) in our cohort had mutations in the regulatory domains of Bcr-Abl. However, when characterized for imatinib sensitivity, only the T212R mutant was substantially resistant. This result from Ba/F3 cells correlated well with the clinical follow-up data from these patients, as only patient #2 with the T212R mutation has subsequently relapsed after mutation detection. Most of the patients with SH3-SH2 domain mutations had a CCR at the time of detection. This highlights a trend found in previous work of spurious mutations to be common in patients with a CCR.[102,118] Thus, detection of a previously uncharacterized *BCR-ABL* mutation in a patient without other signs of relapse must be regarded to be of uncertain significance.

The T212R mutation poses a unique mechanism of resistance with its location distant to the active site where imatinib binds. Surprisingly, even activating mutations such as Abl PP did not produce a comparable effect on imatinib resistance when introduced into Bcr-Abl. Because of the function of the SH3-SH2 apparatus in autoinhibition of Abl kinase activity, it was postulated that the mechanism of resistance of the discovered mutations would be a consequence of their effect on kinase activity. Consistent with this notion, the T212R mutation that was the most resistant also activated c-Abl kinase activity most strongly. Our hypothesis from structure modeling to explain the difference between T212R and the other patient mutations was that T212R would stabilize the disinhibited conformation, while the others might destabilize the autoinhibited conformation. Although this model might explain the unique properties of T212R,

attempts to test the model through reversion mutations at E275 were unsuccessful. Considering the relatively low resolution of the disinhibited structural model, it is possible that T212R interacts with other residues in the kinase domain, such as E276. However, at this time we cannot rule out other mechanisms of T212R's effect.

The most important clinical implication of this work is that mutations do occur in the regulatory domains of Bcr-Abl and can confer imatinib resistance. However, the results from studying our cohort suggest the frequency of this event to be $\leq$1% of patients with CML. Thus, sequencing of the full BCR-ABL PCR product in a patient without a kinase domain mutation and unexplained imatinib resistance may be warranted. Based on the intermediate level of resistance for the T212R mutant, regulatory domain mutations that are imatinib resistant should respond fully to dasatinib or nilotinib. Incorporation of SH3-SH2 sequencing into the protocols of larger studies will be necessary to validate the frequency of resistant mutations found in our work.

CHAPTER 4

CHARACTERIZATION OF BCR-ABL DELETION MUTANTS FROM PATIENTS WITH CHRONIC MYELOID LEUKEMIA

Characterization of BCR-ABL deletion mutants from patients with chronic myeloid leukemia.

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Word counts: Abstract: 151
Text: 4,548

4.1 Abstract

The Bcr-Abl oncogenic tyrosine kinase causes chronic myeloid leukemia and is the target for imatinib therapy. During imatinib treatment, cells with Bcr-Abl kinase domain mutations are selected in some patients that render decreased drug sensitivity. In addition, some patients express deletion mutants of Bcr-Abl, apparently due to missplicing. Most commonly these deletion mutants lack a significant portion of the kinase domain that includes the P-loop. We describe a screen for such mutations in patients with CML and demonstrate that they are not oncogenic and are catalytically inactive. We hypothesized that coexpressed Bcr-Abl deletion mutants would have a dominant-negative effect on the native form. Upon coexpression of native and deletion mutant Bcr-Abl in Ba/F3 cells, growth factor independence is attained and signaling is activated normally. Despite this, these cells have increased imatinib sensitivity compared to cells expressing only native Bcr-Abl. We believe these misspliced Bcr-Abl proteins hetero-dimerize and -tetramerize with the native form to increase imatinib sensitivity.

4.2 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease caused by Bcr-Abl, a chimeric tyrosine kinase that is the result of the 9;22 reciprocal translocation, cytogenetically evident as the Philadelphia chromosome (Ph).[2] CML cells exhibit increased proliferation and reduced apoptosis, a phenotype thought to reflect the cellular consequences of Bcr-Abl-induced activation of numerous signaling pathways.[50] The Bcr-Abl dependence of CML cells was the rationale for the clinical development of imatinib (also known as Gleevec, STI571), an Abl kinase inhibitor that has become standard therapy for CML in all phases. Patients with chronic phase CML respond very well to imatinib, with a cumulative complete cytogenetic response rate of 87% and predicted event-free survival of 83% at 5 years.[24] However, some patients have emergence of resistant clones with point mutations in the Bcr-Abl kinase domain that decrease imatinib sensitivity. This is relatively rare in chronic phase, but is a common occurrence in patients with accelerated phase or blast crisis.[66] As a result, strategies to combat drug resistance have focused on the development of more potent inhibitors with activity against kinase domain mutant Bcr-Abl.[78,80]

The L248V mutation in the ATP-binding loop (P-loop) has been shown to confer a high level of imatinib resistance.[119] It has recently been reported that L248V introduces a secondary 5' donor splice site into ABL exon 4.[120] Alternative splicing at this site results in the co-expression of a mutant with deletion of residues 248-274 (Δ248-274), which lacks part of the kinase domain's N-terminal lobe. The lack of critical structures including the P-loop leads to the prediction that Δ248-274 is kinase inactive. Missplicing of BCR-ABL mRNA has also been observed in a significant portion of patients without the L248V

mutation, indicating that co-expression of deletion mutants and native Bcr-Abl may be common.[121,122]

Bcr-Abl forms a dimer and/or tetramer through the coiled-coil domain in the N-terminus of Bcr.[54] This is thought to promote Bcr-Abl activation and oncogenesis by facilitating transphosphorylation.[55] Although the effects of co-expressing kinase inactive (deletion mutant) and native Bcr-Abl on kinase activity and imatinib sensitivity are unknown, it is conceivable that the deletion mutants have a dominant-negative effect on the native Bcr-Abl by formation of heterodimers with decreased transphosphorylation. Here, we report the frequency of deletion mutants in patients on imatinib therapy, confirm that they are inactive and characterize their properties when coexpressed with native Bcr-Abl.

4.3 Patients, Materials and Methods

4.3.1 Patient Samples. This study was approved by the Institutional Review Board of Oregon Health and Science University and informed consent was obtained from all patients. Mononuclear cells (MNCs) were isolated from bone marrow aspirates or peripheral blood samples by density gradient centrifugation. Aliquots of cells were used for RNA extraction (RLT reagent, Qiagen, Valencia, CA).

4.3.2 Mutation Analysis. RNA was isolated with the RNeasy kit (Qiagen). Superscript reverse transcriptase was used to synthesize cDNA with random hexamer primers (Invitrogen, Carlsbad, CA). Mutation screening was performed by automated sequencing of *BCR-ABL* products generated by nested PCR using 2 sets of primers on *BCR* and *ABL* as described.[104] In three patients PCR products were subcloned using the TOPO-TA

cloning kit (Invitrogen) and a minimum of 16 individual colonies were sequenced. Mutations occurring in only one subclone were discarded.

4.3.3 Generation of mutant alleles. To generate expression constructs the AatII/KpnI *ABL* kinase domain fragments were cut from pCR4-TOPO containing deletion mutations and pasted into full-length *BCR-ABL* in pGEM5. The *BCR-ABL* variants were then cloned into pENTR-1A, an entry vector for the Gateway Cloning System (Invitrogen), followed by recombination-mediated introduction into the retroviral expression vector MSCV-IRES-GFP (MIGR1) appropriately modified using the Gateway Vector Conversion System (Invitrogen). To generate *c-ABL* expression constructs, deletion mutation were introduced by site-directed mutagenesis using the Quik-Change site directed mutagenesis kit (Stratagene, La Jolla, CA) and pSGT-c-ABL 1b as template.[113] All deletions were confirmed by sequencing.

4.3.4 Cell lines. Ba/F3 cells were maintained in RPMI 1640 supplemented with 10% FBS, 200mM L-glutamine, penicillin (200U/ml), streptomycin (200mg/ml) and WEHI-conditioned media as a source of interleukin 3 (IL-3). TonB210 cells were kindly provided by George Daley (Children's Hospital, Boston, MA) and maintained in the same media with 10% tetracycline-free FBS (Invitrogen). For the TonB210 cell experiments, Bcr-Abl was induced by addition of 1mg/ml doxycycline (Dox) to the culture. The Ba/F3 cells expressing native Bcr-Abl that were used for co-expression experiments have been described previously.[123] Wild type and mutant BCR-ABL-MIGR1 constructs were used for production of retrovirus by transient transfection of 293 T17 cells (ATCC, Manassas, VA). Supernatant was collected after 48 hours and used to infect parental Ba/F3 or

TonB210 cells. Isolation of Bcr-Abl expressing cells was performed by cell sorting for GFP expression using a FACS Aria (BD Biosciences, San Diego, CA).

4.3.5 Immunoblotting. Cells expressing wild type or mutant Bcr-Abl were pelleted and lysed directly in SDS-PAGE loading buffer for 5 minutes at 95°C. Bcr-Abl and c-Abl autophosphorylation were detected by immunoblot with mouse monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Waltham, MA). After 10 min stripping of blots with 7M Guanidine-HCl, Bcr-Abl expression was detected with rabbit anti-Abl antibody K12 (Santa Cruz Biotechnology, Santa Cruz, CA). Loading controls were performed by immunoblot with anti-actin (Ab-1) mouse monoclonal (JLA20) antibody (Calbiochem, San Diego, CA).

4.3.6 Kinase assays. Transfection of HEK293 cells, immunoprecipitation of Abl protein and Abl in vitro kinase assay was carried out as described previously.[52,116] The relative concentration of immunoprecipitated Abl protein was determined by immunoblotting (anti-Abl Ab-3, Oncogene Science, Cambridge, MA) and subsequent quantification using the Li-cor Odyssey system and normalized for c-Abl wild type.

4.3.7 Cell proliferation assays. Cell line dependence on IL-3 and sensitivity to imatinib were tested independently in MTS-based viability assays (CellTiter 96 Aqueous One Solution Reagent; Promega, Madison, WI). Exponentially growing Ba/F3 cells expressing wild type or mutant Bcr-Abl were plated in quadruplicate at 4 x 10^3 per well in 96-well plates in the presence of either decreasing IL-3 concentrations or increasing doses of imatinib. After 72 hours the absorbance at 490nm was read on a Benchmark Plus Microplate Spectrophotometer (Biorad, Hercules, CA). The mean of two independent cell

lines is reported for each condition as the percentage absorbance compared to the control concentration (1ng/ml for IL-3, no imatinib).

4.3.8 Quantitative RT-PCR (qRT-PCR). qRT-PCR was performed on peripheral blood or bone marrow aspirate specimens as described.[106] In brief, *BCR-ABL* and *G6PDH* transcripts were quantified with real-time quantitative PCR and fluorescent resonance energy transfer hybridization probes in a LightCycler instrument (Roche Applied Sceince, Indianapolis, IN). The results are reported as the ratio of *BCR-ABL* to *G6PDH* in percent.

4.4 Results

4.4.1 Characterization of abnormal BCR-ABL transcripts from CML patients.

In sequencing of *BCR-ABL* for kinase domain mutations, we have occasionally observed smaller bands in addition to the expected PCR product on agarose gel electrophoresis and/or overlapping sequence on high-quality kinase domain sequence traces (data not shown). This prompted us to perform a systematic analysis with a retrospective screen of randomly selected patient samples. To analyze the entire PCR product in these patients, sequencing was done using primers covering the *ABL* Cap, SH3, SH2 and kinase domains. We found 14 of 101 (14%) randomly selected patients to have a deletion mutation (Table 4.1). Five patients were found to harbor Δ184-274, corresponding to a complete loss of ABL exon 4. One of these five also had the point mutation M244V. Three patients had an alternative transcript joining *BCR* sequence to *ABL* exon 4, resulting in out of frame (OOF) *ABL* translation (Δ27-183 OOF, stop at 296) as previously described.[122] Two patients were found to have Δ248-274, previously reported to coexist with the L248V mutant.[120] In another patient, loss of *ABL* exon 7 was

detected, also resulting in a frame-shift (Δ362-444 OOF, stop at 458). The Δ342-383 mutant was detected in two patients, one of which also had the Q333R point mutation. Lastly, the Δ151-183 mutant was detected in a single patient. Thus, deletion mutants detected in patients most frequently involved splice junctions.

Table 4.1. CML patients found by direct sequencing to have a BCR-ABL deletion mutation.

Patient #	Age at study (years)/sex	Disease duration (months)	Time on IM (months)	Disease Phase	BCR-ABL sequencing	Estimated Deletion Mutant Sequence	Cytogenetics/ FISH/qRT-PCR	*Time to* last follow-up (months)	Cytogenetics/ FISH/qRT-PCR (at follow-up)
1	51/M	61	39	CP	L248V/Δ248-274	60%	cryptic/1.5%/0.11	23	cryptic/83%/73
2	72/F	142	51	CP	Δ184-274	30%	5%/9.5%/1.7	25	0%/0%/0.062
3	64/M	108	54	CP	L248V/Δ248-274	30%	5%/3.5%/0.42	0	NA
4	47/F	67	25	CP	Δ184-274	95%	65%/34.5%/2.1	53	10%/7.5%/1.8
5	66/M	32	18	AP	Δ184-274	50%	100%/70.5%/4.4	16	95%/70.5%/2.9
6	40/F	113	17	CP	Δ27-183 OOF	100%	18%/14%/0.41	39	0%/0%/0.044
7	58/M	55	29	CP	M244V/Δ184-274	60%	80%/65.5%/3.1	0	NA
8	32/F	8	5	CP	Δ184-274	100%	5%/6.5%/0.026	9	0%/4%/0.035
9	70/F	59	17	CP	Δ151-183	80%	0%/0.5%/0.047	43	0%/0%/0.016
10	67/M	84	21	AP	Δ27-183 OOF	30%	NA/NA/13	56	95%/51.5%/3.4
11	52/M	19	17	CP	Δ362-444 OOF	100%	0%/0.5%/0.033	50	0%/0%/0
12	66/F	83	28	CP	Q333R/Δ342-383	50%	15%/7.5%/0.54	30	55%/51%/1.9
13	30/M	18	16	CP	Δ342-383	50%	NA/NA/0.043	5	4%/0%/0.0075
14	53/F	92	31	AP	Δ27-183 OOF	100%	100%/79.5%/6.8	0	NA

All data refer to the time of the index sample, unless noted. OOF-Deletion results in a frame-shift. AP-Accelerated phase. NA not available.

We decided to concentrate our studies on the in-frame mutations that were most common in our patients, Δ248-274 and Δ184-274. To confirm the precise borders of these deleted sequences, we subcloned the PCR products and sequenced multiple single clones from three of the patients. For each patient, two independent samples from different time points were tested (Table 4.2). Of the three patients, two had the L248V/Δ248-274 combination and one had the Δ184-274 mutant. Subcloning of *BCR-ABL* amplicons confirmed the suspected deletion borders and revealed that both patients with the L248V and Δ248-274 mutants also expressed the Δ184-274 mutant. For patient #1, the Δ248-274 mutant was present in both samples tested, two years apart. For patient #3, the index sample was three years apart from a previous sample, which also showed the Δ248-274 mutant. Thus, both patients with the L248V mutant tested at different time points showed the continued presence of the Δ248-274 deletion.

Table 4.2. Patient characteristics and results from RT-PCR product subcloning at two independent samples over time.

Patient #	Time from index sample (months)	BCR-ABL sequencing	Subclone Frequency	Cytogenetics/ FISH/qRT-PCR
1	0	L248V	3/16	cryptic/1.5%/0.11
		Δ248-274	10/16	
		WT	3/16	
	23	L248V	6/18	cryptic/83%/73
		Δ248-274	5/18	
		Δ184-274	4/18	
		WT	2/18	
2	-12	WT	19/19	25%/7.5%/1.2
	0	Δ184-274	12/37	5%/9.5%/1.7
		WT	24/37	
3	-54	WT	19/19	NA
	-41	L248V	4/19	5%/6%/0.093
		Δ248-274	2/19	
		Δ184-274	1/19	
		WT	12/19	
	0	L248V/Δ248-274	NA	5%/3.5%/0.42

NA – not available.

4.4.2 Bcr-Abl P-loop deletion mutants are catalytically inactive.

Deletion of residues 248-274 eliminates two of the five strands of the β-sheet of the N-terminal lobe of the kinase domain and the glycine-rich loop (P-loop). Both regions provide essential residues for ATP binding and catalysis of phosphoryl transfer (Figure 4.1A).[124] In the Δ184-274 mutant, the C-terminal α-helix (αB) of the SH2 domain and the linker between the SH2 and kinase domains are missing in addition to the two beta strands of the N-lobe and the P-loop (Figure 4.1B). Given the important functional role of these motifs, we hypothesized that their deletion may result in the loss of enzymatic activity.

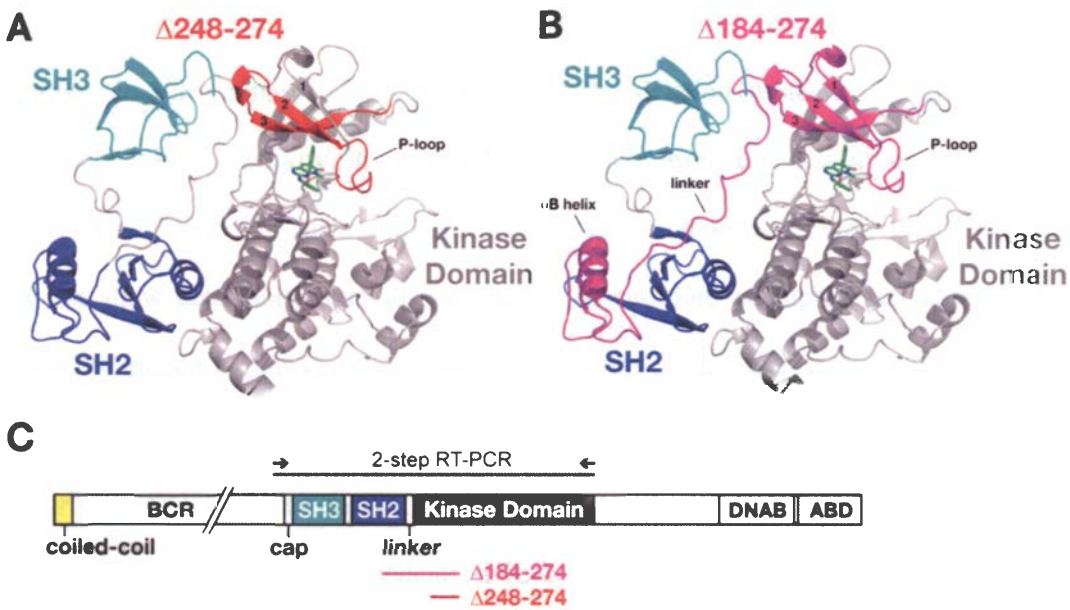

Figure 4.1. Deleted Abl structural elements in the Δ248-274 and Δ184-274 Bcr-Abl mutants. The crystal structure of autoinhibited c-Abl in complex with the kinase inhibitor PD166326 (PDB entry 1OPK) [53] is utilized to illustrate the extent of residues lost in the Bcr-Abl deletion mutants. The Abl SH3, SH2 and kinase domains are colored in cyan, blue and gray, respectively. (A) In the Δ248-274 mutant, a significant portion of the N-lobe of the kinase domain is missing (shown in red). Two of the five strands of the β-sheet are deleted (numbered 2, 3), as well as the P-loop that bridges the ATP binding site. (B) In the Δ184-274 mutant, strand 1 of the kinase domain β-sheet, the C-terminal α-helix (αB) of the SH2 domain and the linker between the SH2 and kinase domains are also missing (shown in magenta). (C) The primary structure of Bcr-Abl is shown to indicate the context of the SH2, SH3 and kinase domains within the entire fusion protein (not to scale). ABD: actin-binding domain. DNAB: DNA-binding domain. (A) and (B) were created using PyMol.[73]

To test the effects of the deletions on Abl kinase function, we introduced the deletions into c-Abl, overexpressed the mutant proteins in HEK293 cells and assayed whole cell extracts for protein tyrosine phosphorylation. Neither c-Abl$^{\Delta 184-274}$ nor c-Abl$^{\Delta 248-274}$ expressing cells showed detectable levels of cellular tyrosine phosphorylation, in contrast to a constitutively active form of c-Abl containing P223E/P230E double mutations (Abl PP) [113] (Figure 4.2A). Moreover, a slight increase in cellular tyrosine phosphorylation could be observed by overexpression of the wild type c-Abl protein, but

overexpression of the deletion mutants showed no tyrosine phosphorylation. Immune complex in vitro kinase assays showed that the deletion mutants displayed almost undetectable kinase activity, in contrast to the readily observable activity of wild type c-Abl (Figure 4.2B). Consistent with this, the immunoprecipitated deletion mutant proteins did not show detectable autophosphorylation. In addition, attempted activation of the Δ248-274 mutant by introduction of the PP mutations did not rescue any activity (Figure 4.2A-B, far right lanes). To test the mutants in the context of Bcr-Abl, the deletion mutant constructs were expressed stably in Ba/F3 cells. Unlike p210 Bcr-Abl expressing cells, deletion mutants remained dependent on IL-3 for proliferation (Figure 4.2C). Although expressed to a similar level in the Ba/F3 cells, the deletion mutants did not show detectable Bcr-Abl autophosphorylation (Figure 4.2D) and downstream target phosphorylation was absent (see Figure 4.3B). In summary, these experiments indicate that the deletion mutant proteins are catalytically inactive.

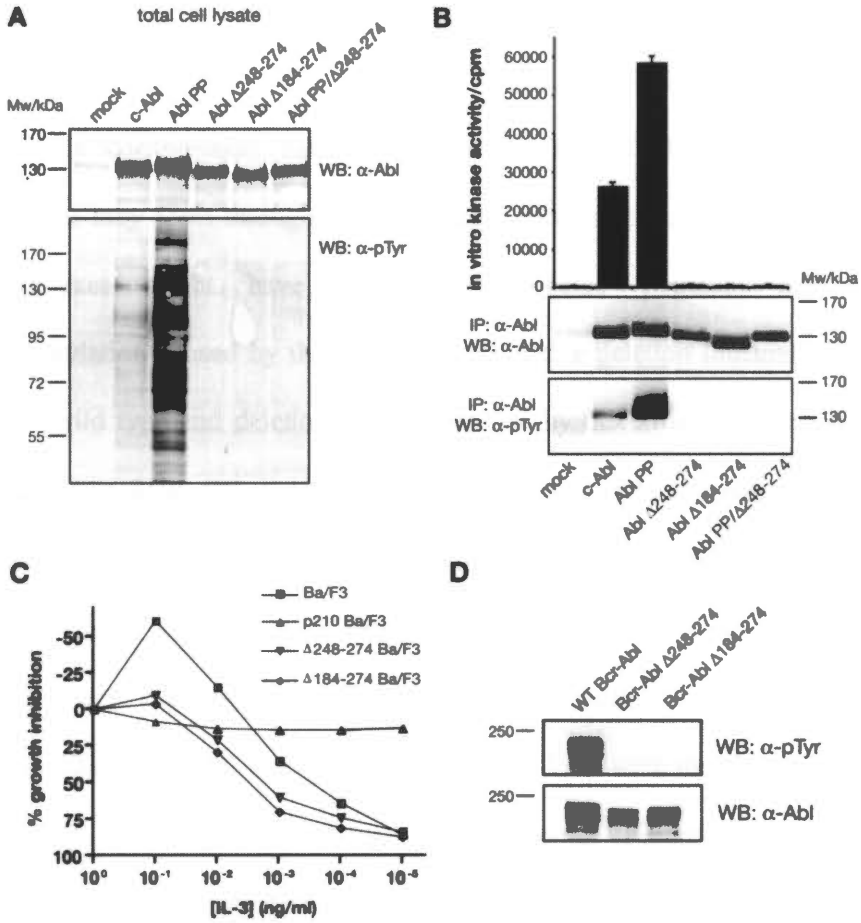

Figure 4.2. Deletion mutants are kinase dead. (A) HEK293 cells were transiently transfected with the indicated SV40-driven Abl expression constructs. Cells were lysed forty hours after transfection and total protein extracts analyzed by anti-Abl and anti-phosphotyrosine immunoblotting. (B) Abl immunoprecipitates were probed with anti-Abl and anti-phosphotyrosine antibodies (lower panels) and assayed for catalytic activity by *in vitro* kinase assays using an optimal substrate peptide. The histograph shows the in vitro kinase activity (mean with SD of two experiments done in triplicate) of the Abl constructs. Whereas native and PP c-Abl displayed kinase activity, the deletion mutants have almost undetectable activity. The PP c-Abl mutant combined with the Δ248-274 mutation was also kinase inactive. (C) Deletion mutant BCR-ABL stably infected in Ba/F3 cells were maintained in IL-3 supplemented medium. Growth factor dependence was tested by cell proliferation assay with a gradient of IL-3 in 10-fold dilution steps from the normal concentration of 1 ng/ml. Unlike p210 BCR-ABL infected cells, deletion mutants remain dependent on external growth factor like the parental Ba/F3 cells. (D) Deletion mutant Ba/F3 lines maintained in IL-3 supplemented media were lysed and tested for Bcr-Abl expression and autophosphorylation by immunoblot. Although expressed, Bcr-Abl deletion mutants do not have detectable autophosphorylation. Data in (A) and (B) from Oliver Hantschel and Ines Kaupe.

4.4.3 Coexpression of P-loop deletion mutants does not interfere with the oncogenic activity of native Bcr-Abl.

In cells coexpressing native and deletion mutant Bcr-Abl heterodimers or heterotetramers may form through the coiled-coil domain of Bcr. We hypothesized that such complexes might have decreased oncogenic potential and decreased transphosphorylation caused by the catalytically inactive deletion mutants. We therefore coexpressed wild type and deletion mutant Bcr-Abl by infecting Ba/F3-p210 cells with MIGR1 Δ248-274 *BCR-ABL* and MIGR1 Δ184-274 *BCR-ABL* retroviruses. To generate sublines with high, medium and low mutant/native Bcr-Abl ratios we sorted cells based on low (L), medium (M) and high (H) GFP expression. Cell lines coexpressing wild type and the deletion mutant constructs were still IL-3 independent at any mutant/native Bcr-Abl ratios tested. (Figure 4.3A). The deletion mutant cell lines were then tested for total Bcr-Abl expression and autophosphorylation by immunoblot (Figure 4.3B). As a positive control, p210 Bcr-Abl was expressed in Ba/F3 and Ba/F3-p210 cells to show the effect of doubling the Bcr-Abl 'dose'. In the case of Δ248-274 we observed a direct correlation between Bcr-Abl phosphorylation and the expression of deletion mutant Bcr-Abl, suggesting that the Δ248-274 mutant is efficiently transphosphorylated by native Bcr-Abl. In contrast, no such correlation was seen in the case of the Δ184-274 mutant. This may be partly due to the absence of Y226, a known major phosphorylation site, which remains intact in the Δ248-274 mutant.

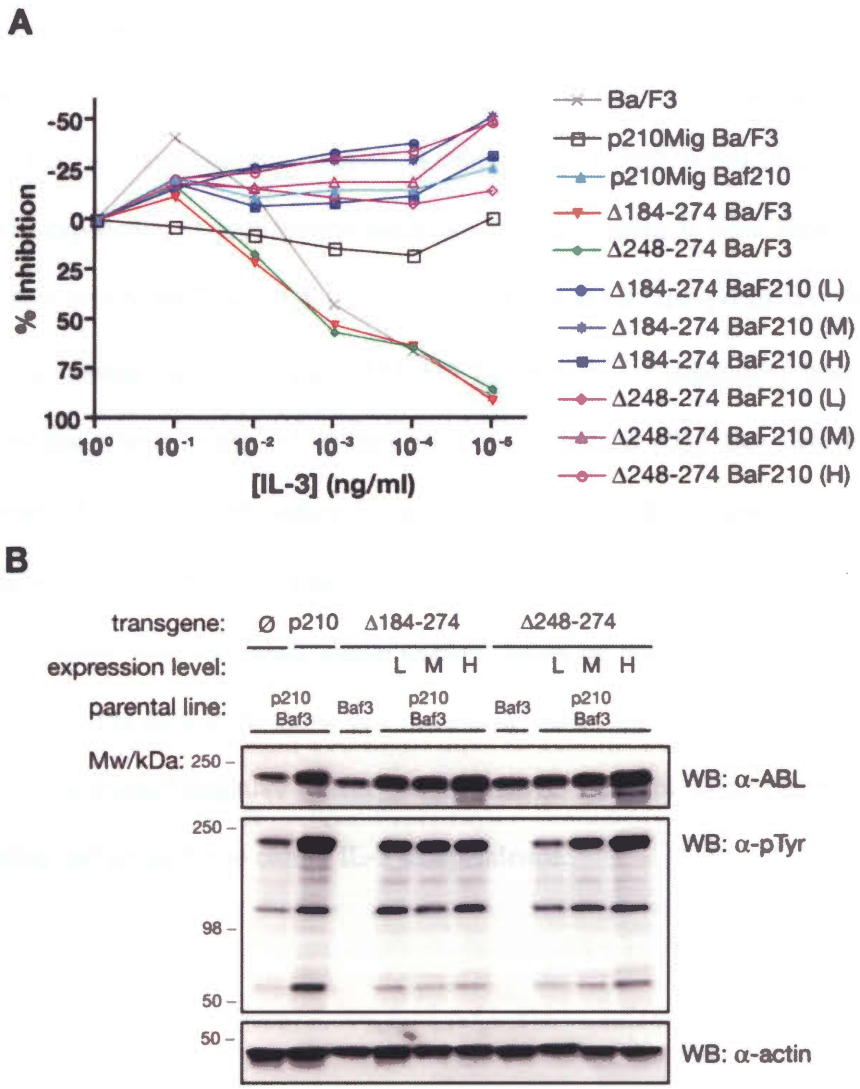

Figure 4.3. Coexpression of deletion mutants does not interfere with the oncogenic tyrosine kinase activity of wild-type Bcr-Abl. (A) Deletion mutant Bcr-Abl stably expressed in Ba/F3-p210 cells were sorted based on low (L), medium (M) and high (H) GFP expression as a surrogate for deletion mutant protein expression levels. L, M, and H deletion mutant expressing cells did not prevent the wild-type (p210) Bcr-Abl protein from producing IL-3 independence. (B) Deletion mutants in Ba/F3 or Ba/F3-p210 cells were tested for total Bcr-Abl expression and autophosphorylation by immunoblot. Native BCR-ABL was transfected into Ba/F3 and Ba/F3-p210 cells to show the effect of the p210 dose expressed. L, M and H deletion mutant expression did not reduce p210 Bcr-Abl protein autophosphorylation and transphosphorylation of the deletion mutant proteins by p210 was evident.

To confirm the effects of coexpressing the deletion mutants with native Bcr-Abl, we used an inducible system where coexpression can be tested within the same stable cell lines. Thus, we expressed the deletion mutants in the TonB210 cell line, in which native Bcr-Abl expression is tetracycline-inducible.[125] The TonB210 cells were infected with Δ248-274- and Δ184-274-MIGR1 retrovirus and sorted for GFP-positivity. TonB210 cells stably expressing Δ248-274 and Δ184-274 became IL-3 independent upon addition of Doxycycline (Dox) in the cell culture medium to induce p210 Bcr-Abl (Figure 4.4A). Likewise, Bcr-Abl autophosphorylation was detected only following p210 induction (Figure 4.4B). Autophosphorylation increased with expression of Δ248-274 and Δ184-274, consistent with increased transphosphorylation of the mutants by native Bcr-Abl. Collectively, the data from these cell lines also shows that the deletion mutants do not exhibit a dominant-negative effect versus native Bcr-Abl with respect to Bcr-Abl activation and its ability to confer IL-3 independence.

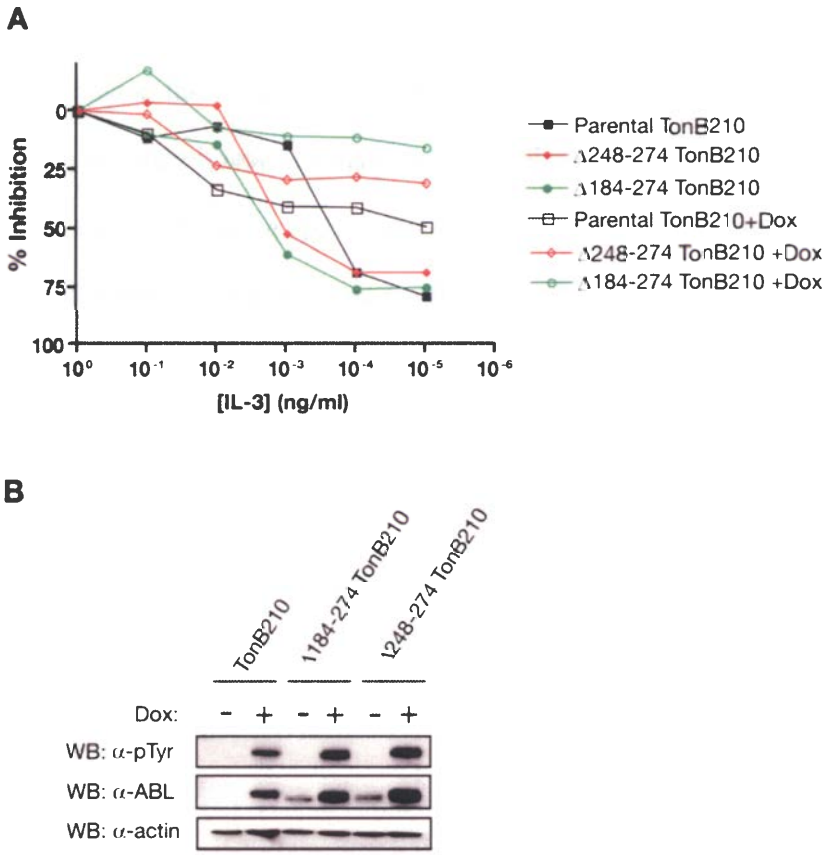

Figure 4.4. Deletion mutant coexpression in TonB210 cells with inducible p210 Bcr-Abl. (A) TonB210 cells stably expressing the Δ248-274 and Δ184-274 mutants were tested for IL-3 dependence with or without 48 hours Dox treatment. In tetracycline-free media the Δ248-274 and Δ184-274 mutants had IL-3 dependence, as did the parental TonB210 cells (open symbols). Treatment of Dox to induce native Bcr-Abl coexpression resulted in IL-3 independence (filled symbols). (B) Immunoblotting was performed for Bcr-Abl autophosphorylation and expression from cells grown with or without Dox addition for 48 hours. Deletion mutant expression was observed without autophosphorylation in tetracycline free media. Induction of native Bcr-Abl was observed in the presence of Dox and resulted in autophosphorylation. The graphs represent the means of two experiments.

4.4.4 Coexpression of P-loop deletion mutants in Ba/F3-p210 cells increases sensitivity to imatinib.

To test whether the deletion mutants may affect imatinib sensitivity, the Δ248-274 and Δ184-274 mutant Ba/F3-p210 cell lines were grown in the absence of IL-3 and tested for their sensitivity to imatinib in cell proliferation assays. Unexpectedly, coexpression led

to an increase in imatinib sensitivity that was reproducible in multiple experiments (Figure 4.5A-B). Furthermore, the increase in imatinib sensitivity was consistently highest in the lines expressing the highest levels of deletion mutant Bcr-Abl. As predicted, expression of an additional copy of native p210 Bcr-Abl reduced imatinib sensitivity. Thus, coexpression of deletion mutants consistently results in increased imatinib sensitivity, although the total level of Bcr-Abl phosphorylation is increased with their expression. This observation indicates that there is a dominant-negative effect of deletion mutant coexpression on the native Bcr-Abl with respect to imatinib sensitivity, but not with respect to transformation capacity.

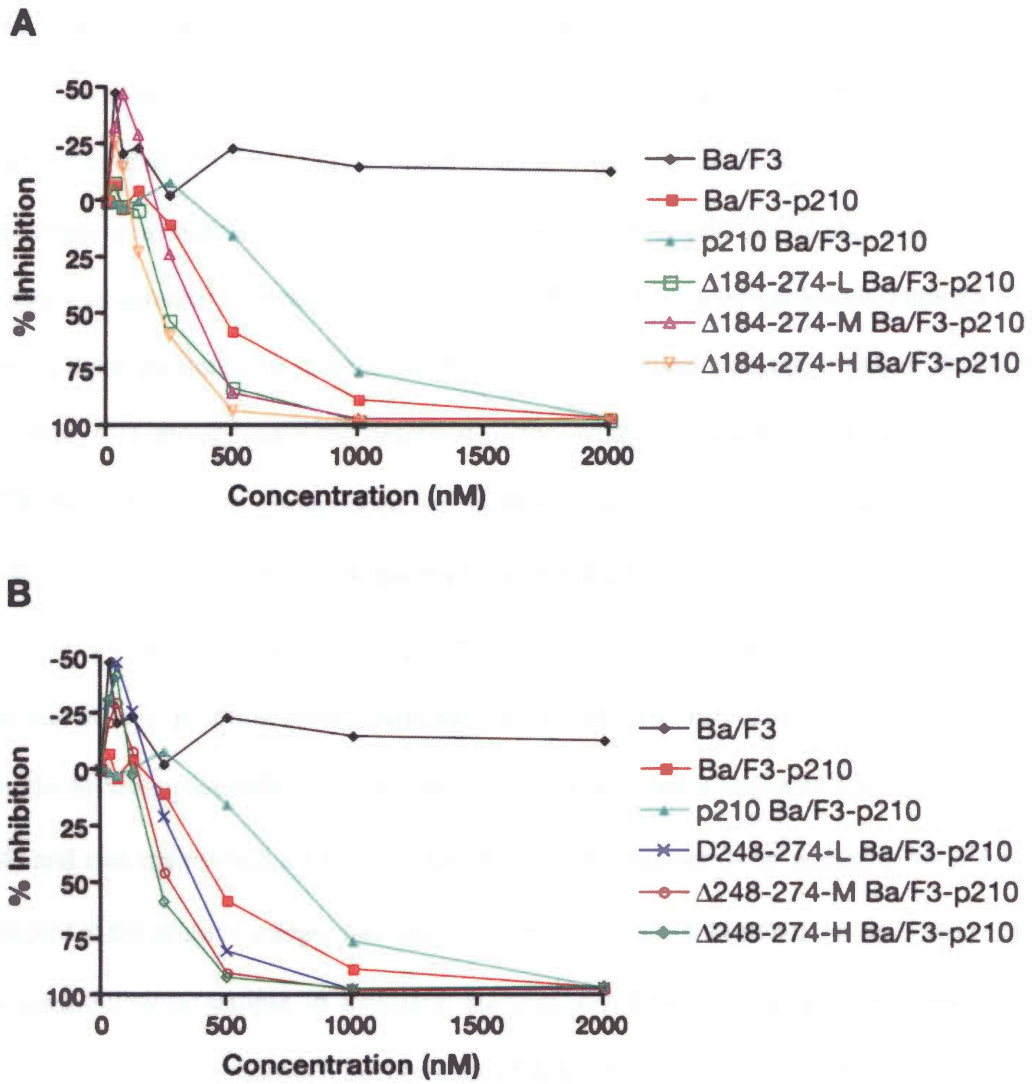

Figure 4.5. Coexpression of deletion mutants with p210 Bcr-Abl increases the sensitivity of cells to imatinib. Δ248-274 (A) and Δ184-274 (B) mutant Ba/F3-p210 cell lines maintained in the absence of growth factor were tested for sensitivity to imatinib in cell proliferation assays. Ba/F3-p210 cells infected with another p210 copy displayed decreased sensitivity to imatinib, but deletion mutant expression did not have this effect. L, M and H Δ248-274 showed an increase in imatinib sensitivity compared to Ba/F3-p210 cells that paralleled the levels of mutant proteins. For Δ184-274 cells, the deletion mutant expression also increased imatinib sensitivity, but the effect of the expression level was less clear. The graphs represent the means of two experiments.

4.4.5 Follow-up analysis of patients with detection of a P-loop deletion mutation.

To assess possible clinical implications in the eight patients expressing the Bcr-Abl deletion mutants the relevant laboratory values were collected and mutation analysis performed on available follow-up samples. In two patients (patients #2 and 7) the deletion mutant was detected coincidently with a rise in BCR-ABL transcript levels (Figure 4.5A). One of these patients also had the L248V mutation. In three patients, a rise in *BCR-ABL* occurred 3-21 months after the detection of the deletion (patients #1, 4, and 5). Figure 4.5B shows follow-up cytogenetics and mutation analysis. Of the two patients with the L248V and Δ248-274 mutations, patient #1 relapsed 25 months later; no follow-up data is available for patient #3. Of the five patients with the Δ184-274 mutant, two have also had a kinase domain point mutation found and lack cytogenetic response at 19 (#5) and 0 (#7) months follow-up. Another patient with Δ184-274 obtained a major cytogenetic response (#4) and two maintained a CCR (#2 and 8). Of note, patient #4 in whom Δ184-274 was detected at the original sample had instead detection of Δ363-444 OOF (exon 7 loss) at the 6-month follow-up sample. In summary, the clinical follow-up in these patients reveals no consistent association between the presence of deletion mutants and relapse.

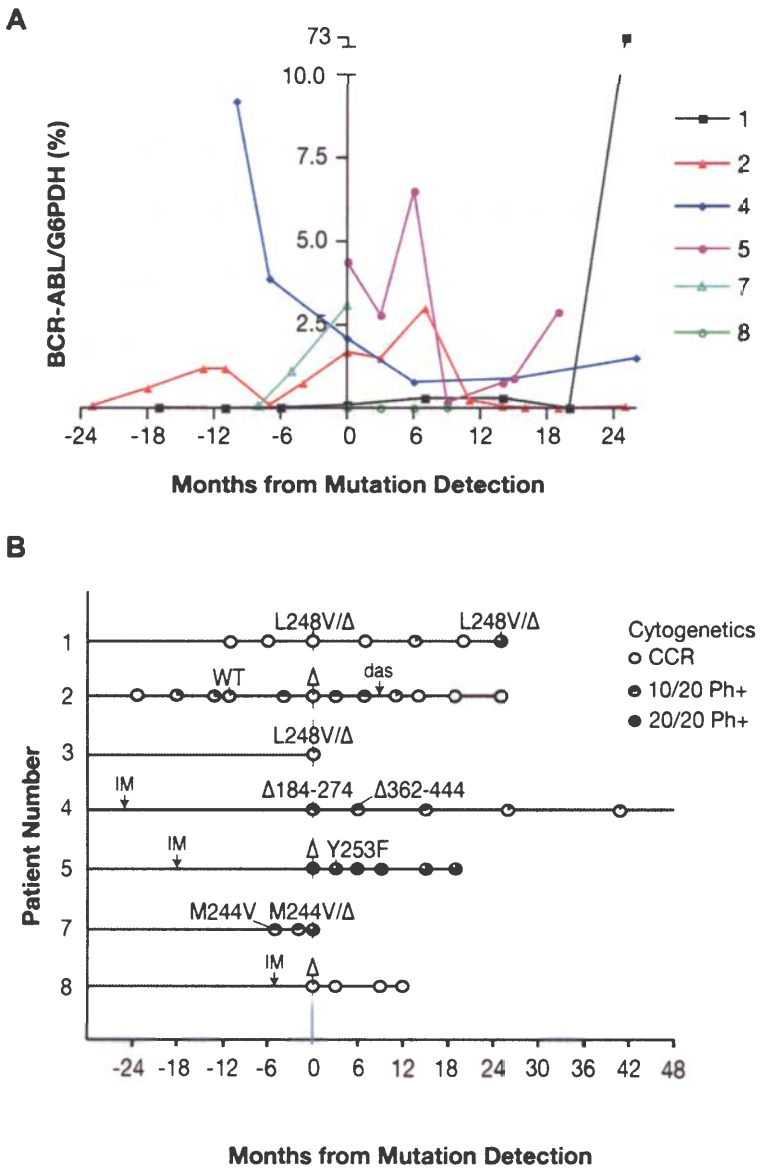

Figure 4.6. Clinical data for patients with a P-loop deletion mutation. (A) Available qRT-PCR data for BCR-ABL in patients with a P-loop deletion mutation was graphed for the period of 24 months before and after the original detection of a mutant. The qRT-PCR data is not graphed for patient #3 to due the long time period between samples. (B) Clinical timelines are shown to indicate the cytogenetic and mutation analysis before and after the index sample with deletion mutation detection. All patients were taking imatinib during the entire period shown, unless noted at imatinib (IM) start or switch to dasatinib (das). Due to a cryptic rearrangement in patient #1 not apparent on cytogenetics, FISH percentages are indicated instead.

4.5 Discussion

Point mutations in the kinase domain of Bcr-Abl are a common and extensively studied mechanism of acquired resistance to imatinib. In contrast, deletion mutations have only recently been described, their incidence is unknown and no functional analysis has been carried out. We detected BCR-ABL deletion mutations in 14 of 101 patients tested by direct sequencing of PCR products. The Δ248-274 mutant exclusively collocates with the L248V mutation. This is thought to be due to introduction of an alternative splice site by the CTG>GTG substitution at codon 248.[120] However, in our series the most common deletion mutation was Δ184-274, which eliminates the entire *ABL* exon 4. The lack of detection of this mutation previously is likely to be due the use of forward sequencing primers localized 3' of residue 184, which would detect wild type, but not the Δ184-274 transcript. For example, our standard protocol for the sequencing the kinase domain used a forward primer beginning at residue 211.[104] The mechanism of exon 4 loss is likely to be due to alternative splicing, although it is unclear why this occurs in some patients to a detectable level but not others. Another possibility is that intronic mutations influence splicing in these patients. Unfortunately, this would be difficult to investigate due to the sizes of the *ABL* introns and the resulting distance of the intron/exon junctions from the breakpoint (>200Kb).[126]

Both P-loop deletion mutants are intrinsically kinase inactive and fail to confer growth factor independence. Therefore the prediction is that exclusive expression of deletion mutant protein in a CML cell will eliminate Bcr-Abl activity and with it the competitive advantage over normal hematopoiesis. Although we did not analyze mutant and native *BCR-ABL* co-expression in single cells, our data is consistent with this

assumption for most patients. In four patients, the proportion of deletion mutant sequence was apparently 100% by direct sequencing; the meaning of this observation is currently unknown. With coexpression, one might predict that a kinase inactive allele may exhibit a dominant-negative effect on the active allele. However, we did not find evidence for this in terms of growth properties and signaling activity, at least in Ba/F3 cells. It is possible though that in primary cells with lower overall Bcr-Abl expression dominant-negative effects on cell growth may be operational. Unexpectedly, coexpression of deletion mutant Bcr-Abl reproducibly increased imatinib sensitivity. This represents a dominant-negative effect with respect to the native protein's sensitivity to inhibition by imatinib. The underlying mechanism of this imatinib sensitization remains elusive. One possibility is that heterodimers or heterotetramers may stabilize an inactive Bcr-Abl conformation that is able to bind imatinib.[26] In the case of L248V/Δ248-274, the high intrinsic imatinib resistance of the point mutant may overcome any handicap imposed by the deletion mutant. However, the tendency of this mutation to favor the generation of Δ248-274 alleles and the additional effects on imatinib sensitivity may attenuate the biological phenotype of this mutant, which is highly imatinib resistant in vitro.[119]

The discovery of the Δ184-274 mutation extents an emerging body of work suggesting that alternative splicing in Bcr-Abl is more common than previously thought. A recent report described alternatively spliced *BCR-ABL* transcripts joining *BCR* to either *ABL* exon 4 or 5, which results in out of frame translation of *ABL*.[122] These events were shown to occur in 80% of newly diagnosed CML cases tested with gel extraction and sequencing of the smaller bands.[122] This high frequency of alternatively spliced products is similar to preliminary work by another group.[121] We believe the lower frequency of

detection of these transcripts in our patients is due to (1) our practice of sequencing the entire PCR product (where small transcripts must compete with wild type to show on the chromatogram) and (2) the make-up of our cohort being patients on imatinib, rather than newly diagnosed. Thus, the majority of CML patients are likely to express Bcr-Abl kinase dead fusions at varying levels along with the wild type protein. The consequences of coexpressing Δ184-274 and Δ248-274 mutants with native Bcr-Abl in our work is likely to extrapolate to the other alternatively spliced products as well.

Our data show that deletion mutations of Bcr-Abl are relatively frequent in patients on imatinib therapy. Given that automated sequencing is used for mutation detection in most labs, it is likely that many deletion mutations are interpreted as high background sequencing traces and therefore escape diagnosis. Both the Δ184-274 and Δ248-274 mutants are kinase inactive, confirming structure-based predictions. The fact that the Δ248-274 mutant arises from a point mutation that confers a high level of imatinib resistance, while at the same time increasing imatinib sensitivity when co-expressed with native Bcr-Abl suggests that the biological effects on the response of patients to drug must be complex. It is also possible that patients prone to express kinase inactive Bcr-Abl due to exon loss may have better response to imatinib. Thus, larger studies will be required to determine the prognostic impact of deletion mutations.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

Continued investigation of mutations that occur in CML patients during imatinib therapy provides a better understanding of the dynamic nature of the disease. Prior to our work, a paradigm had been established that patients who relapse during imatinib treatment frequently harbor drug resistant mutations in the Bcr-Abl kinase domain. However, we investigated whether these mutations are detectable prior to relapse in patients with a complete cytogenetic response. Although a subset of these patients have detectable kinase domain mutations, they do not always relapse and may not have continuous mutation detection over time. Thus, the dynamics of Bcr-Abl mutant clones is complex, as they do not always expand to become the dominant clone. This suggests other factors at work in the growth and selection of CML cells, but the alternative mechanism(s) remain unknown at this time.

More extensive sequencing of *BCR-ABL* revealed two additional phenomena. The first observation was that some patients harbor mutations in *BCR-ABL* outside the kinase domain. Although nine mutations were discovered within the SH3-SH2 domains, only one displayed substantial resistance to imatinib when expressed in the Ba/F3 cell line. Thus, on rare occasion imatinib-resistant mutations occur in the SH3-SH2 domain of Bcr-Abl that may cause relapse. The occurrence of several mutations that were not drug resistant reinforces the point made above that mutations occur in patients with a CCR that likely play no role in relapse. Also, several of these mutations were detected in combination with imatinib-resistant kinase domain mutations, suggesting that the SH3-SH2 mutations were likely passengers.

A second observation through sequencing *BCR-ABL* was that some patients harbor deletion mutations. This observation was made repeatedly by our laboratory and others [120-122] and prompted us to perform a systematic analysis. Direct sequencing of *BCR-ABL* PCR products revealed that deletion mutations most frequently reflect missplicing that either involves *ABL* exon 4 (Δ184-274 and Δ248-274), or produces out of frame *ABL* translation with premature stop codons from loss of exons 2 and 3 (Δ27-183) or exon 7 (Δ362-444). Each of these deletions would be predicted to produce kinase dead Bcr-Abl proteins. Characterization of deletion mutants involving the P-loop confirmed them to lack kinase activity. Coexpression of kinase inactive proteins is intriguing because they may hetero-dimerize or –tetramerize with the native Bcr-Abl through the Bcr coiled-coil domain. These complexes might be anticipated to disrupt the transphosphorylation that is induced by oligomerization, one of the mechanisms believed to be responsible for the deregulated tyrosine kinase activity of Bcr-Abl. In our experiments with the coexpression of deletion mutants and native Bcr-Abl we found signaling and growth factor independence were not effected. Although a dominant-negative effect was not observed in the oncogenic properties of Bcr-Abl, a dominant-negative effect was observed with an increased sensitivity to imatinib. This finding raises the question of whether high levels of missplicing in a subset of patients may correlate with deeper imatinib response and better prognosis.

5.2 Kinase domain mutations: Future directions

The presence in some patients with a CCR of Bcr-Abl kinase domain mutations without associated relapse in is surprising. Although we cannot rule out these mutations as false positives, the presence of kinase domain mutants subsisting at low levels has also

been shown by another laboratory using pyrosequencing.[127] It will be important to monitor these patients over an extended time period to see if mutations detected previously ever reemerge and cause relapse. The underlying mechanism may lie in the growth characteristics of Bcr-Abl-positive hematopoietic stem cells (HSCs). It is possible that kinase domain mutant and Bcr-Abl expressing HSCs coexist in the bone marrow. In this scenario, the HSC that enters the cell cycle and produces progeny may vary over time, allowing for transient mutation detection in patients with a CCR. An alternative hypothesis is that mutations may occur in a progenitor cell population that is not capable of self-renewal and thus dies out over time. This is supported by our observation in CCR patients that four of eight patients with detection of a kinase domain mutation had a concomitant rise of *BCR-ABL* transcript levels that subsequently decreased in three of the patients.

The questions raised about the cellular compartment dynamics of Bcr-Abl-positive clones highlight an important area for further research. The mechanism whereby Bcr-Abl-positive stem cells survive during imatinib treatment is unknown. It may be possible to elucidate the characteristics of these cells by fluorescence activated cell sorting (FACS) of patient samples. However, there are two significant barriers to overcome with this approach. First, during imatinib treatment the depth of response in most patients makes the target cells extremely rare. As a result, newly diagnosed (imatinib naïve) patient samples are desirable, but difficult to obtain. Second, there is currently no specific marker for Bcr-Abl that could allow for separation of Bcr-Abl-positive HSCs by FACS. Unfortunately, experience has demonstrated it is extremely difficult to raise Bcr-Abl junction specific antibodies. Other indirect ways of separating Bcr-Abl-positive cells may exist. For

example, Abl-Bcr, the reciprocal fusion protein to Bcr-Abl, was postulated to be a possible marker. However, Abl-Bcr expression was not detectable in Ph-positive cell lines (data not shown). Progress in finding such a marker would represent an important step in facilitating the study of Bcr-Abl dynamics in patients.

5.3 SH3-SH2 domain mutations: Future directions

The identification of the T212R imatinib-resistant mutation in the SH2 domain of Bcr-Abl has both clinical and biological implications. Clinically, inclusion of the SH3-SH2 domains in BCR-ABL sequencing studies would better establish the incidence of mutations in patients. It will also be important to characterize any novel mutations through cell line expression, rather than assuming them to be imatinib-resistant. Based on the intermediate degree of resistance of T212R, mutations in the SH3-SH2 domains will likely be very sensitive to nilotinib and dasatinib. Thus, detection of a characterized imatinib-resistant SH3-SH2 domain mutation in a patient with increasing *BCR-ABL* transcript levels may be grounds for changing therapy to one of the second-generation inhibitors.

The mechanism of resistance in the T212R mutant has proven elusive, but its further investigation may reveal new information about the Bcr-Abl tyrosine kinase. We hypothesized that the T212R mutant is drug resistant via a propensity to activate kinase activity, based on its location in the autoinhibitory apparatus. After investigating this possibility, however, it is clear that the relationship between kinase activity and imatinib-resistance is not straightforward. A common assumption in the field of CML research is that mutations that enhance kinase activity are resistant because imatinib binds only the inactive conformation of the Abl kinase domain.[26] However, the conformational changes that lead to activation of the c-Abl kinase are complex (Figure 5.1). Mutations that

increase Abl kinase activity may do so at either of the steps along its path to activation, and thus may not influence resistance in the same way. Also, the extent to which the SH3-SH2 autoinhibition functions in Bcr-Abl remains unclear. It was previously thought that this autoinhibition is preserved to some degree. This was based on the observation that transformation potency lost in coiled-coil domain mutants was rescued by Abl activating mutations in SH3 or the SH2-kinase linker.[56] However, our work with several such Abl activating mutations introduced into Bcr-Abl revealed no effect on imatinib sensitivity. Although this suggests that the SH3-SH2 domain autoinhibition of the kinase domain is lost in Bcr-Abl, the definitive test will be to look at the activation kinetics of these mutants in purified full-length Bcr-Abl.

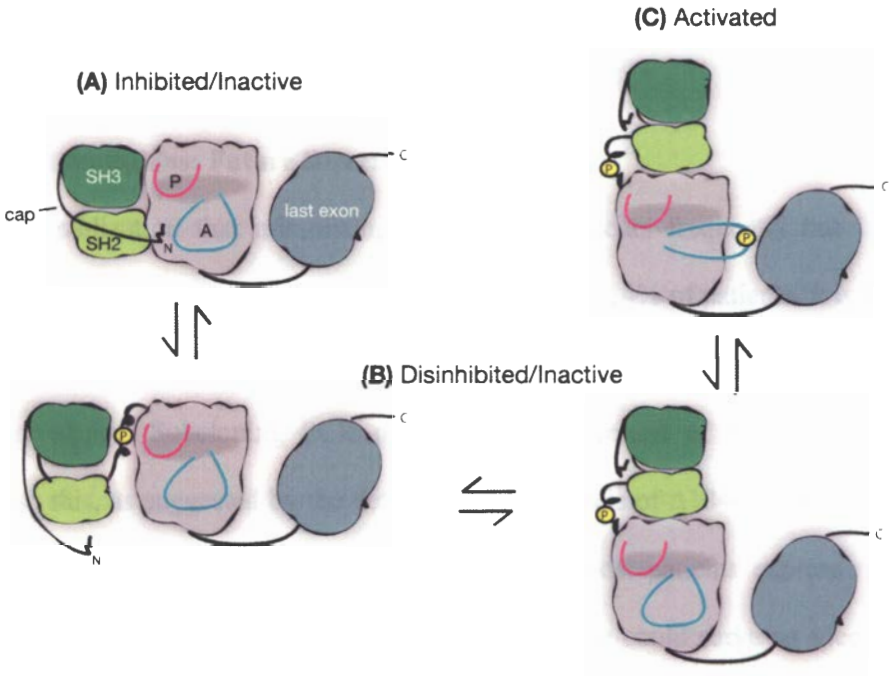

Figure 5.1. Conformational changes in the process of c-Abl activation. (A) In the autoinhibited conformation, the Cap, SH3 and SH2 domains restrict activity of the kinase domain (light gray). (B) Upon phosphorylation at Y226 in the linker between the SH2 and kinase domain the autoinhibition is relieved through disruption of SH3-linker-kinase domain interaction. Subsequently, the SH3-SH2 domains change position drastically and SH2 forms a new interaction with the N-lobe of the kinase domain.[117] (C) The disengagement of the autoinhibited state greatly facilitates full activation of kinase activity with phosphorylation and flipping of the activation loop. Note: although inefficient, the activation loop can assume the flipped conformation when the kinase is in an autoinhibited state.[128]

It remains possible that the T212R mutant stabilizes the disinhibited conformation by stabilizing interaction of SH2 with the N-lobe of the kinase domain. This possibility makes the T212R mutant an attractive candidate for structural investigation. It may be possible that interaction of T212R with the kinase domain could facilitate crystallization, as this conformation has only been shape reconstructed after small angle x-ray diffraction (SAXS).[117] This disinhibited conformation was isolated in this study by using an Abl

construct with the PP mutations and deletion of the Cap domain for activation. It is possible that T212R could further stabilize this conformation to allow for crystal structure determination. Thus, collaboration has been initiated with John Kuriyan's laboratory (UC Berkely) with the goal of crystallizing the T212R mutant.

5.4 Deletion mutations: Future Directions

The realization that missplicing of *BCR-ABL* occurs frequently has interesting ramifications. Although we detected deletion mutations in 14% of patients, it is likely that a more sensitive approach, such as that used by Volpe and colleagues, would detect a higher prevalence. Subcloning PCR products from a cohort of CML patients could accomplish this, as suggested by the detection of low levels of Δ184-274 in patients with the L248V and Δ248-274 mutations in our study. If most patients express misspliced BCR-ABL mutants at some level, it is possible that these could serve as specific tumor antigens. Volpe et al have explored this with the Δ27-183 (OOF) mutant with the long-term goal of developing a tumor specific vaccine for CML.[122] They found the Δ27-183 OOF protein product was detectable in Ph-positive cell lines with immunohistochemistry and that Δ27-183 OOF specific T cells could be detected in CML patients. In addition, deletion mutants could feasibly serve as markers for Bcr-Abl positive cells for the purpose discussed previously.

The finding that deletion mutant coexpression with native Bcr-Abl increases imatinib sensitivity may have clinical importance. Our experiments imply that the deletion mutants influence imatinib sensitivity in a "dose" dependent manner. Thus, a prospective study with a more sensitive strategy for deletion mutant detection would ideally follow these patients over time to better determine the amount of fluctuation that occurs in the

levels of these mutations. Determining how this phenomenon correlates with imatinib response and prognosis would be important for such a study.

5.5 Conclusion

CML is a relatively simple model for malignancy, with leukemic cells in every patient being dependent on Bcr-Abl for proliferation and survival. As such, it is an important model disease for the study of the intricacies of oncogene dependence. In this dissertation, the *BCR-ABL* transcript has been investigated for abnormalities that arise during targeted therapy with imatinib. Thus, RT-PCR with sequencing of *BCR-ABL* has revealed several processes at play during imatinib treatment. Kinase domain mutations in Bcr-Abl were a known mechanism of imatinib resistance; so we decided to examine this in less studied disease state of CCR. Following this, mutations were characterized outside the kinase domain, where they have not yet been described in CML patients. Lastly, the observation of deletion mutants of Bcr-Abl during imatinib treatment was investigated by characterizing the consequence of coexpressing these mutants with the goal of modeling their effect in patients. It is my hope that these studies will contribute to the understanding of CML during imatinib treatment.

APPENDIX

LEUKEMIA STEM CELLS IN CHRONIC MYELOID LEUKEMIA

Leukemia has been an important area of study in the evolution of the cancer stem cell hypothesis. It has long been postulated that normal hematopoietic stem cells (HSCs) are the most likely targets for leukemic transformation, for unlike their progeny, they have unlimited lifetimes during which to accrue the necessary mutations in oncogenes and tumor suppressors. To test this hypothesis, investigators have asked whether a population of leukemic stem cells (LSCs) exist that possess the key properties of (1) self-renewal and (2) pluripotency that separate stem cells from other types of cells in the body. Analogous to HSCs, leukemic stem cells (LSCs) would divide in a manner that preserves stem cell properties in one daughter cell. Thus, self-renewal occurs with one progeny cell destined for gradual differentiation and one remaining in an undifferentiated state. In addition, LSCs would have the ability to differentiate into all the necessary cell types that comprise the mature tissue, indicating they emerged through transformation of a HSC. In the case of the hematopoietic system, HSCs constantly regenerate the many blood lineages from a hierarchy that branches at its earliest point into myeloid and lymphoid lineages. Early experiments investigated whether leukemias, like CML, involved both myeloid and lymphoid lineages and thus contained pluripotent cells analogous to HSCs. The goal of this short chapter is to review some key historical research on LSCs in CML showing they possess the characteristics of pluripotency and self-renewal. Also, recent advances in leukemia modifying the cancer stem cell hypothesis and possible therapeutic applications will be described.

Pluripotency was the first essential stem cell quality to be investigated for CML LSCs. To suggest that CML originates from the mutation of a pluripotent HSC it had to be shown that the disease included both myeloid and lymphoid lineages. This was

difficult to demonstrate because expansion of Philadelphia chromosome (Ph)-positive cells occurs overtly only among myeloid lineages. In 1977 Fialkow and colleagues addressed this question in classic clonality experiments monitoring the Glucose-6-phosphate dehydrogenase (G6PD) expression in CML patients.[11] G6PD is a X-linked gene with two alleles expressing variant isoenzymes A and B that are resolvable on gel electrophoresis. In heterozygous females, X-inactivation leads to random expression of either A or B in each cell lineage, normally producing mosaic expression. In contrast, the X-inactivation pattern of a malignant population originating from a single cell can be observed as expression of only a single isoenzyme in that population of cells. In this manner, Fialkow and colleagues demonstrated that a clonal population of bone marrow derived (B)-lymphocytes, but not thymus derived (T)-lymphocytes, exists in CML patients.[11] However, a drawback of these experiments was that the authors were unable to demonstrate that the clonal population harbored the Ph chromosome. In later work, the same group of researchers took a different approach to their hypothesis. They used the EBV virus, which has tropism for B-lymphocytes, to generate cell lines from a single CML patient heterozygous for G6PD.[129] These cell lines expressed one isotype of G6PD, immunoglobulin (confirming them to be lymphocytic) and by cytogenetics were shown to have the Ph chromosome. From this it was concluded that CML originated in a pluripotent HSC.

The second essential stem cell quality that received attention was self-renewal. Pioneering work in the hematopoietic system led to the development of assays for self-renewal, allowing for characterization of LSCs. The first approaches involved optimizing techniques for culture to measure proliferative capacity that allowed identification of the

cells capable of long term replating ability.[130] These techniques have had limited yield in CML research because of an unexplained inability to sustain Ph-positive cell growth in culture.[131] The limitations of the in vitro systems led to development of an in vivo system in which LSCs are assayed for ability to regenerate morphologically identical leukemias when serially transplanted in severe combined immunodeficiency (SCID) mice. In 1994 John Dick and coworkers produced a pivotal publication using this xenograft approach.[132] Using acute myeloid leukemia AML samples, they showed the LSCs had a characteristic cell surface marker pattern of $CD34^{+}CD38^{-}$ and lacked lineage markers.[132] These LSCs were found to occur at a rate of 1 per 250,000 cancer cells.[132] This study confirmed that cell markers for HSCs were also found on LSCs and that only this small subset of leukemic cells are able to self-renew. Four years later, John Dick and colleagues used the xenograft model with CML patient samples to show that the LSCs are enriched in the $CD34^{+}$ cell compartment.[133] However, further characterization of the cell surface markers on CML LSCs has been limited by unexplained difficulty for Ph-positive cells to repopulate transplanted mice.

Assays for self-renewal that isolate and characterize LSCs has allowed an increased level of sophistication in the experiments on this topic during the last decade. For example, an alternate hypothesis has been examined for LSCs which postulated that they could be derived from progenitor cells in certain circumstances. The question of whether progenitor cell compartments can regain the property of self-renewal as part of the malignant process has been addressed in the context of chronic phase CML transition to blast crisis, the advanced stage that is analogous to acute leukemia. Progenitor cells are rapidly dividing cells that are descendents of HSCs, but have lost the capability for

unlimited self-renewal and are responsible for producing differentiated cells in specific lineages. For example, common lymphocyte progenitors and common myeloid progenitors are the earliest descendents of the HSCs and give rise to progenitors with increasing degrees of lineage restriction. In 2004 Jamieson and colleagues investigated whether the progenitors act as the LSCs after transformation to blast crisis.[49] They found that the granulocyte-macrophage progenitors (GMPs) from blast crisis patients generated replatable colonies, unlike chronic phase patients.[49] Blast crisis GMPs also showed high nuclear expression of β-catenin, a marker for stem cell self-renewal.[49] These results suggest that BCR-ABL does not directly affect self-renewal, which is instead conferred by later mutations that occur in transformation to blast crisis.

It is notable that the Ph chromosome also occurs in acute lymphoblastic leukemia (ALL). In 2005, Castor and colleagues compared HSC involvement in ALL patients with p210 or p190 forms of BCR-ABL.[134] They found in p190 cases that the Ph chromosome was absent from $CD34^+CD38^-$ cells that lacked the B-cell marker CD19 and that this HSC compartment was normal in size and function.[134] Instead, there was an abnormal $CD34^+CD38^-$ cell compartment that expressed CD19 and harbored the Ph chromosome.[134] In contrast, in p210 cases the $CD34^+CD38^-CD19^-$ HSC compartment contained the Ph chromosome and was greatly expanded.[134] However, to complicate the interpretation of this result, it was only the $CD34^+CD38^-CD19^+$ cells that displayed the ability to repopulate leukemia in NOD-SCID mice.[134] The cellular consequences of p190 versus p210 expression are unclear. Although it has been suggested that p190 possesses greater kinase activity, the reason for the different results between these forms of BCR-ABL will require further study. It is possible that the ALL cases studied by Castor and

colleagues were in fact cases of CML that were diagnosed after they had already progressed to lymphoid blast crisis. If this is true, the data from the p210 cases may indicate that transition to a blastic phase resulted after secondary mutations occurred in the $CD19^+$ progenitors, consistent with the study by Jamieson and colleagues.

In the case of chronic phase CML, it is generally accepted that the HSC is the target for transformation. Whether this occurs in other types of leukemia is less clear. In comparison to other leukemias, cancer cells in CML are clearly more homogeneous as BCR-ABL is the sole genetic abnormality in chronic phase patients. Comparison of what we know from the study of BCR-ABL and CML to other types of leukemia caused by different oncogenes has yielded intriguing information. In general, two types of oncogenic processes occur commonly in leukemia, aberrant protein kinase activation and dominant-negative mutations of transcription factors.[135] BCR-ABL is a prototype of the former class. Common examples of the latter class include the fusion proteins involving the RUNX1 protein, a key component of the Core Binding Factor complex crucial for coordinating the ontogeny of the hematopoietic system. Investigation of the LSCs for these oncogene classes has elucidated different target cell compartments for transformation. Castor and colleagues also found the ETV6-RUNX1 fusion protein that occurs in ALL to arise in B-cell progenitors, apparently conferring self-renewal.[134] D. Gary Gilliland and coworkers extended this finding by comparing another oncogenic transcription factor fusion, MOZ-TIF2, to BCR-ABL.[136] Similar to the earlier work with MLL-ENL[137], MOZ-TIF2 transformed progenitor cells and displayed self-renewal both in vitro and in vivo, whereas BCR-ABL did not.[136] The results of these studies suggest a general difference in the cell compartment transformed by BCR-ABL compared to ETV-

RUNX1, MLL-ENL and MOZ-TIF2. This is likely related to the different mechanisms of oncogenesis. Whereas BCR-ABL activates proliferation by several signaling pathways, transcription factor fusion proteins directly block the process of differentiation. Apparently BCR-ABL cannot confer the ability to self-renew to a progenitor cell and transformation must occur in a HSC to cause disease. For acute leukemias that frequently possess transcription factor fusions, it will be important to verify these generalizations. Presumably, the secondary genetic abnormalities that occur in these diseases result in the more aggressive phenotype.

Research characterizing LSCs has led to an important fund of rapidly growing knowledge. For CML, it is well supported that chronic phase occurs through mutation of an HSC, but blast crisis results after further mutation conferring self-renewal on a progenitor cell. The ability for LSC derivation from a progenitor cell is now apparent for transcription factor fusions that commonly occur in acute leukemias. Yet how will the research on LSCs translate to new therapeutics? The development of new treatment strategies must improve upon classical chemotherapeutics that typically target the rapidly dividing cells that comprise the tumor's bulk, but often leave the LSCs responsible for tumor recurrence unharmed. If progenitor cells are the targets of transformation in some leukemias, it is possible that targeting their abnormally activated self-renewal program will result in an effective therapy. For example, Jamieson and colleagues identified activation of β-catenin signaling in GMPs during blast crisis CML.[49] Further investigation of this mechanism may yield treatment options for this aggressive disease phase. Even when the HSC has been transformed, it is possible that differences exist between the LSC and normal HSCs that can provide a therapeutic target. For this, CML

has provided an excellent model. Although BCR-ABL has been effectively targeted with imatinib therapy, it does not eliminate LSCs, as discussed in chapters 1 and 2. However, Richard Van Etten and colleagues recently been reported that CML LSCs are more reliant on CD44 than normal HSCs for interaction with their niche in the bone marrow.[138] Such results demonstrating differences between LSCs and HSCs hold promise for the development of better therapeutics for aggressive forms of leukemia and for eradicating the cells responsible for disease recurrence.

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