ABL KINASE DOMAIN MUTATIONS AS A MECHANISM OF RESISTANCE TO TYROSINE KINASE INHIBITORS IN PHILADELPHIA-POSITIVE LEUKEMIAS: BIOLOGICAL, CLINICAL AND PROGNOSTIC RELEVANCE

INTRODUCTION

The Philadelphia chromosome: the molecular signature of chronic myeloid leukemia (CML) and of some acute lymphoblastic leukemias (ALL)

Chronic myelogenous leukemia (CML) is a rare disease worldwide. However, a tremendous stream of basic and clinical advances over the last 45 years have kept CML at the forefront of scientific attention.

The incidence of CML is approximately 1 to 2/100000.[1] It accounts for 15-20% of all cases of leukemia in adults.[1] The clinical hallmarks of CML are leukocytosis, a left shift in the differential count, and splenomegaly. If not effectively treated, CML follows a triphasic clinical course with an initial *chronic phase* (CP) that has an average duration of 5 years. During the chronic phase, there is gross expansion of the myeloid cell compartment, but the cells still retain the capacity to differentiate and function normally. Symptoms in this phase are generally mild and many patients are asymptomatic, being often diagnosed by routine blood sampling. Then the disease typically progresses to *accelerated phase* (AP), characterized by the appearance of more immature cells in the blood, frequent constitutional symptoms, and a less favorable response to therapy. The diagnostic criteria for accelerated phase are not universal, reflecting that disease progression from chronic to accelerated phase is a continuous process rather than a single step. Although the duration of accelerated phase varies from weeks to years, the disease inexorably progresses to the final stage of *blast crisis* (BC), that can be either *myeloid* or *lymphoid* in phenotype, where immature cells dominate and survival is measured in weeks to months. However, not all patients follow this triphasic course; some progress from the chronic phase directly to the blast phase and others die of complications in the chronic or accelerated phase.

CML was first described as a distinct clinical entity in 1845 by the pathologists Bennet, Craigie, and Virchow. In 1960, a major clue to the cause of CML was provided by Nowell & Hungerford's landmark discovery of the *Philadelphia (Ph) chromosome*, a minute acrocentric chromosome in the bone marrow cells of patients with CML (Figure 1).[2] This discovery was the first demonstration of a chromosomal rearrangement being consistently linked to a specific malignancy and sparked searches for associations of

additional chromosomal aberrations with other forms of cancer. In 1973, the Ph chromosome was recognized by Rowley and colleagues to result from the t(9;22)(q34;q11) reciprocal chromosomal translocation.[3] By the mid-1980s, it became apparent that the t(9;22) translocation resulted in the juxtaposition, on the Ph chromosome, of the Abelson *(ABL)* proto-oncogene, located on the long arm of chromosome 9, to a gene of unknown function on the long arm of chromosome 22, which was called *BCR* for Breakpoint Cluster Region, since DNA breaks occurred in a relatively small genomic region.[4] A new association of the Ph chromosome with B-cell *acute lymphoblastic leukemia* (B-ALL) was also discovered.[5] Advances in chromosome mapping and molecular biology enabled the specific B-ALL Ph chromosome gene product with its chromosome breakpoints and mRNA sequence to be analyzed and compared with that of CML samples. Initial studies in Ph-positive (Ph+) B-ALL showed that a smaller 7.0 kb mRNA, as opposed to a CML Ph chromosome 8.5 kb mRNA product, was formed.[6,7] Furthermore, the Bcr-Abl protein product in B-ALL samples was 185/190-kD (henceforth referred to as $p185^{BCR-ABL}$) as opposed to the 210-kD Bcr-Abl protein product (henceforth referred to as $p210^{BCR-ABL}$) in CML samples.[7,8] The difference in the Ph chromosome gene product in B-ALL versus CML were due to differences within the *BCR* breakpoints.[9] The translocation giving rise to the Ph chromosome in B-ALL was localized within the minor breakpoint cluster region (*m-bcr*) in *BCR*,[10-12] whereas in CML, the translocation site was within the major breakpoint cluster region (*M-bcr*) in *BCR*.[4] Further studies showed a high but not absolute correlation between the $p210^{BCR-ABL}$ form and CML, and between $p185^{BCR-ABL}$ and B-ALL, questioning whether specific forms of Bcr-Abl may play a role in the aetiology of each leukaemia. With a frequency of 20–30%, patients harbouring the Ph chromosome constitute the largest cytogenetically defined subgroup among adult ALL.[13]

The Bcr-Abl fusion protein: the pathogenetic principle of CML and Ph+ ALL

Of paramount importance was the discovery that the protein derived from the chimeric *BCR-ABL* gene had *tyrosine kinase* (TK) activity that was deregulated compared with normal Abl and correlated with its ability to transform cells to a malignant phenotype.[14] *In vitro* culture systems demonstrated that *BCR-ABL* can transform immature hematopoietic cells, some fibroblast cell lines, and hematopoietic cell lines rendering them growth factor independent.[15-17] In addition, several groups reported that a CML-like disease could be induced in mice transplanted with bone marrow infected with a *BCR-ABL* retrovirus.[18-20] This proved the point that *BCR-ABL* is the causative agent and not just a marker of the disease.

Both Bcr and Abl are multidomain proteins (reviewed in [21]; Figure 2). The physiological function of Bcr is not well understood. The 160-kd Bcr protein is ubiquitously expressed. Several structural motifs can be delineated (Figure 2). The first N-terminal exon encodes a serine-threonine kinase. The only substrate of this kinase identified so far is Bap-1, a member of the 14-3-3 family of proteins. A coiled-coil domain at the N-terminus of Bcr allows dimer formation in vivo. The center of the molecule contains a region with dbl-like and pleckstrin-homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors, which in turn may activate transcription factors such as NF-κB. The C-terminus has GTPase activity for Rac, a small GTPase of the Ras superfamily that regulates actin polymerization and the activity of an NADPH oxidase in phagocytic cells. In addition, Bcr can be phosphorylated on several tyrosine residues, especially tyrosine 177, which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway. Although these data argue for a role of Bcr in signal transduction, their true biologic relevance remains to be determined. The fact that *BCR* knockout mice are viable and the fact that an increased oxidative burst in neutrophils is thus far the only recognized defect probably reflect the redundancy of signaling pathways.

The *ABL* gene is the human homologue of the v-*abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV), and encodes a nonreceptor tyrosine kinase. Human Abl is an ubiquitously expressed 145-kd protein with 2 isoforms arising from alternative

splicing of the first exon. Several structural domains can be defined within the protein (Figure 2). Three SRC homology domains (SH1-SH3) are located toward the NH_2 terminus. The SH1 domain carries the tyrosine kinase function, whereas the SH2 and SH3 domains allow for interaction with other proteins. Proline-rich sequences in the center of the molecule can, in turn, interact with SH3 domains of other proteins, such as Crk. Toward the 3' end, nuclear localization signals and the DNA-binding and actin-binding motifs are found. Several fairly diverse functions have been attributed to Abl. The normal Abl protein is implicated in a wide range of cellular processes, including regulation of cell growth and survival, oxidative stress and DNA-damage responses, actin dynamics and cell migration, transmission of information about the cellular environment through integrin signaling. To this purpose, Abl interacts with a large variety of cellular proteins – including signalling adaptors, other kinases, phosphatases, cell-cycle regulators, transcription factors and cytoskeletal proteins. Overall, it appears that the Abl protein serves a complex role as a cellular module that integrates signals from various extracellular and intracellular sources and that influences decisions in regard to cell cycle and apoptosis.

Whereas the native c-Abl shuttles between the nucleus and the cytoplasm and has tightly regulated kinase activity, the Bcr-Abl counterpart is exclusively found in the cytoplasm and shows aberrant kinase activity. Under physiologic conditions, the SH3 domain and the more 5' regions of Abl protein appear to play a critical role in kinase inhibition and both cis- and trans-acting mechanisms have been proposed to mediate this inhibition. Loss of 5' regions of Abl, together with fusion of Bcr sequences encompassing the oligomerization domain, abrogate the physiologic suppression of the kinase (Figure 3).

Two major mechanisms have been implicated in the malignant transformation by Bcr-Abl, namely a) altered adhesion to stroma cells and extracellular matrix, and b) constitutively active mitogenic signaling and reduced apoptosis.

a) Altered adhesion properties - CML progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix. In this scenario, adhesion to stroma negatively regulates cell proliferation, and CML cells escape this regulation by virtue of their perturbed adhesion properties. Interferon-α (IFN-α), an active therapeutic agent in CML, appears to reverse the adhesion defect. β-

integrins play an important role in the interaction between stroma and progenitor cells, and CML cells have been found to express an adhesion-inhibitory variant of β1 integrin that is not found in normal progenitors. On binding to their receptors, integrins are capable of initiating normal signal transduction from outside to inside; it is thus conceivable that the transfer of signals that normally inhibit proliferation is impaired in CML cells. Because Abl has been implicated in the intracellular transduction of such signals, this process may be further disturbed by the presence of a large pool of Bcr-Abl protein in the cytoplasm. Furthermore, Crkl, one of the most prominent downstream tyrosine-phosphorylated proteins in *BCR-ABL*-transformed cells, is involved in the regulation of cellular motility and in integrin-mediated cell adhesion by association with other proteins such as paxillin and the focal adhesion kinase Fak.

b) Activation of mitogenic signaling and inhibition of apoptosis – multiple signals initiated by Bcr-Abl have proliferative and anti-apoptotic effects that are frequently difficult to separate, in line with the concept that a proliferative signal leads to apoptosis unless it is counterbalanced by an anti-apoptotic signal. Thus, Bcr-Abl may shift the balance toward the inhibition of apoptosis while simultaneously providing a proliferative stimulus. Several cellular cascades activated by Bcr-Abl are involved. They include:

    - Ras and the MAP kinase pathway: autophosphorylation of Bcr tyrosine 177 provides a docking site for the adapter molecule Grb-2. Grb-2, after binding to the Sos protein, stabilizes Ras in its active, GTP-bound form. Two other adapter molecules which are known to be substrates of Bcr-Abl-mediated phosphorylation, Shc and Crkl, can also activate Ras. Circumstantial evidence that Ras activation is important for the pathogenesis of Ph+ leukemias comes from the observation that activating mutations are uncommon, even in advanced phases of the disease, unlike in most other tumors. This implies that the Ras pathway is constitutively active, and no further activating mutations are required. Activation of Ras and subsequent recruitment of the serine-threonine kinase Raf to the cell membrane initiates a signaling cascade through the

serine-threonine kinases Mek1/Mek2 and Erk, which ultimately leads to the activation of gene transcription.

- Stat pathway: Bcr-Abl can directly phosphorylate Stat1 and Stat5 transcription factors. Although Stat5 has pleiotropic physiologic functions, its effect in *BCR-ABL*-transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of Bcl-xL.
- PI3K pathway: Bcr-Abl forms multimeric complexes with phosphatidyl-inositol-3-kinase (PI3K), Cbl, and the adapter molecules Crk and Crkl, in which PI3K is activated. The next relevant substrate in this cascade appears to be the serine-threonine kinase Akt. This kinase had previously been implicated in anti-apoptotic signaling. A recent report placed Akt in the downstream cascade of the IL-3 receptor and identified the pro-apoptotic protein Bad as a key substrate of Akt. Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins such as Bcl_XL and it is trapped by cytoplasmic 14-3-3 proteins. Altogether this indicates that Bcr-Abl might be able to mimic the physiologic IL-3 survival signal in a PI3K-dependent manner. Ship and Ship-2, 2 inositol phosphatases with somewhat different specificities, are activated in response to growth factor signals and by Bcr-Abl. Thus, Bcr-Abl appears to have a profound effect on phosphoinositol metabolism, which might again shift the balance to a pattern similar to physiologic growth factor stimulation.

A detailed overview of the cellular molecules involved in Bcr-Abl transforming signaling is shown in Figure 4.

The Bcr-Abl inhibitor imatinib mesylate: the first example of targeted molecular therapy

The only known *cure* for CML is *allogeneic stem cell transplantation* in the chronic phase of the disease. This therapy has demonstrated long term disease free survival of 50–80% after 3–10 years of follow-up.[22,23] Unfortunately, this treatment is not an option for the majority of patients due to the lack of an HLA-matched related donor or the risk of transplantation-related mortality. Introduced at the beginning of the 80s, *IFN-α*, alone or in combination with cytarabine, has been the standard treatment for patients unable to undergo allogeneic stem cell transplantation until the introduction of tyrosine kinase inhibitors (TKIs). While this therapy is able to induce cytogenetic remissions in up to 50% of CML patients when treated in CP, it is associated with a range of adverse effects.[24-29]

The considerable wealth of knowledge on the molecular and cell biology of CML gathered over the past twenty years of research created the essential platform for molecular targeted therapies to be engineered. It soon became clear that the Bcr-Abl oncoprotein itself is the ideal target, since (a) it has a central role in CML pathogenesis, and (b) it is not expressed by normal cells. Furthermore, the dissection of the signal transduction pathways affected by the deregulated kinase activity of Bcr-Abl provided information on additional or alternative signalling steps that could be interrupted in an attempt to block the leukemogenic process.

The first breakthrough in the treatment of CML has been the development of imatinib mesylate. Imatinib mesylate, originally designated signal transduction inhibitor 571 (STI571), arose from a time-consuming process of random screening of large numbers of compounds created using the structure of the ATP-binding site. Imatinib is a 2-phenyl-amino-pyrimidine and it emerged as one of the most potent substances inhibiting the Abl protein. It also inhibits other kinases, predominantly those related to platelet-derived growth factor receptors and c-Kit. The catalytic domains of eukaryotic serine/threonine and tyrosine kinases have a highly conserved bilobed structure. The NH_2-terminal lobe (N-lobe) contains a β-sheet and a conserved α-helix (helix C), whereas the COOH-lobe (C-lobe) is helical. In the interface between the two lobes there is a cleft where a series of highly conserved residues form the ATP binding and

catalytic sites.[30] The activation state of kinases is dependent on the position of the activation loop, a portion of the C-lobe, which in Abl comprises amino acid residues 381–402. In active kinases, the activation-loop is in an "open" conformation, because it swings away from the catalytic center of the kinase. The three NH2-terminal residues of the activation-loop (amino acids 381–383) contain a strictly conserved DFG (aspartate-phenylalanine-glycine) motif, which is crucial for catalytic activity. Aspartate 381 is able to bind Mg^{2+}, which in turn coordinates the phosphate groups of ATP. The COOH-terminal portion of the activation-loop serves as a platform for substrate binding. Although the conformation of the activation loop is highly conserved in kinases when they are in the active, open conformation, there are considerable differences between their inactive (closed) conformations. Kinases are activated by phosphorylation of key serine/threonine or tyrosine residues within the activation-loop. In the case of Abl, tyrosine 393 is phosphorylated and points away from the center of the kinase, allowing substrates to bind. In the inactive state of Abl, tyrosine 393 is unphosphorylated and points toward the center of the kinase, mimicking a substrate by forming a hydrogen bond with asparagines 363. In this conformation, the mouth of the kinase is occluded, preventing substrate binding.[30,31]

Co-crystal structure analysis revealed that imatinib selectively binds to a distorted inactive conformation of the Abl kinase domain (KD) through an induced-fit mechanism.[31-33] Remarkably, the potency of imatinib towards constitutively active Bcr-Abl implies a dynamic equilibrium from which imatinib can trap the deregulated Bcr-Abl oncoprotein when it transits through its inactive conformation (Figure 5). This mode of binding is potentially an Achilles' heel for imatinib compared with other small-molecule kinase inhibitors that are effective against the catalytically active kinase conformation.

Functionally, imatinib acts by revoking the effects of the Bcr-Abl oncoprotein through inhibition of Bcr-Abl autophosphorylation and substrate phosphorylation, thus blocking proliferation and inducing apoptosis.[34-36] Its favourable oral bioavailability profile and lack of significant toxicity in animal models led to the design of large phase I and II trials to test its safety and efficacy in humans (Table 1). Thus, in the spring of 1998, a phase I clinical trial was initiated in the United States in which patients with CML in CP who had failed IFN-α were treated with imatinib in increasing doses, from 25 mg/d up

to 1000 mg/d. Adverse side effects from imatinib were minimal, with the most common being nausea, myalgias, edema, and diarrhea. In fact, a maximum tolerated dose could not be identified as a result of these studies, despite a trend for a higher frequency of grade 3 to 4 adverse events at doses of 750 mg/d or higher. Complete hematologic responses (CHR) were seen in 53 of 54 patients who were treated with imatinib in doses of 300 mg/d or more. Of these 54 patients, cytogenetic responses occurred in 29. Seventeen patients achieved major cytogenetic responses (MCgR) and 7 had a complete cytogenetic remission (CCgR). These results were truly remarkable for this group of patients. For this reason, a second phase I trial was performed in 58 patients with myeloid (38 patients) or lymphoid (20 patients, including Ph+ ALL) BC of CML. These patients received doses ranging from 300 to 1000 mg/d. Fifty-five percent (21/38) of the patients with myeloid BC demonstrated responses; 4 of these 21 patients had a CHR. Seventy percent (14/20) of the patients with lymphoid BC or Ph+ ALL had a response, including 4 who had a CHR. Seven patients with myeloid BC were continuing to receive treatment at the time of the report. All but one of the patients with lymphoid BC or Ph+ ALL who responded had relapsed. Subsequently, three large, multinational phase II studies were initiated in late-CP, AP and myBC patients (Table 1). Imatinib was administered at a dose of 400 mg/d in late-CP patients and at doses of 400 or 600 mg/d in AP and BC patients. The results of these studies indicated that the rate of both hematologic and cytogenetic responses increased as the treatment was started earlier in the course of the disease. In the advanced phase studies, a dose of 600 mg/d was superior to 400 mg/d in terms of response rates and time to progression. Importantly, landmark analyses indicated that the achievement of a hematologic and/or cytogenetic response was associated with improved survival and progression-free survival. Adverse events were again mild in the setting of CP patients. Neutropenias and thrombocytopenias were more common in patients with advanced disease, suggesting that hematologic toxicity may be related more to an underlying compromised bone marrow reserve rather than to the drug itself.

Taken together, these results established imatinib as a safe and effective therapy for all stages of CML and were the basis for the initial marketing approval by the Food and Drug Administration (FDA) on May, 2001, i.e., after less than 3 years after the start of the first phase I study. On the same month, imatinib made the cover of *Time* as “the

magic bullet" to cure cancer. These encouraging results led to a large randomized trial comparing first-line therapy with imatinib at 400 mg/d versus standard IFN-α in combination with low-dose cytarabine in patients with newly diagnosed CP CML – the International Randomized Trial of Interferon and STI571 (IRIS). One thousand one hundred and six patients were enrolled – 553 in each arm. Crossover to the alternative therapy was allowed in case of failure to achieve a CHR at 6 months, failure to achieve a MCgR at 12 months, loss of response, intolerance. Indeed, 359 (65%) patients in the IFN-α plus cytarabine arm crossed over to imatinib (as against 14 [2.5%] patients in the imatinib arm), mainly because of intolerance, making the comparison between the two treatments formally impossible. The initial report of this study,[37] with a median follow-up of 19 months, showed that the estimated rate of MCgR at 18 months was 87% in the imatinib arm and 35% in the IFN-α plus cytarabine arm (P<.001). CCgR was achieved in 76% of patients who were given imatinib, as against 15% of patients who were given IFN-α plus cytarabine (P<.001). The five-year-updated results of this trial have recently been published,[38] confirming stability of responses to imatinib (with the quality of hematologic, cytogenetic and molecular responses even improved over time) and decreasing incidence of serious adverse events. At 60 months, for patients treated with imatinib the estimated CHR rate is 98%, the MCgR rate is 85%, the CCgR rate is 92%. The estimated rate of progression to AP/BC has decreased from 1.5% and 2.8% in the first and second year of treatment, respectively, to 1.6%, 0.9%, 0.6% in the third, fourth and fifth year, respectively.

Resistance to imatinib: incidence and mechanisms

Shortly after the encouraging results of the first clinical trials were announced there came the first of reports of resistant cases, which represented the proof of principle that the Bcr-Abl-positive clone may evolve to evade Bcr-Abl inhibition.

Primary resistance to imatinib, defined as an inability to achieve landmark response, is comprised of the 2% of patients who fail to achieve hematologic response (HR) and 8-13% who fail to achieve MCgR or CCgR using early CP CML treated with imatinib at diagnosis as a benchmark.[38] Secondary resistance includes patients who achieve but subsequently lose relevant response – loss of cytogenetic or hematologic response and progression from chronic to advanced-stage disease. Again per the benchmark IRIS trial of CP patients with primary imatinib therapy,[38] the rate of all progression events, including cytogenetic and hematologic relapse within CP and transformation to advanced phase, is 18% after a median of 5 years. However, such events appear to be most evident in the first 3 years of treatment in the IRIS trial, where progression to advanced phases of disease averaged 2% per year and progression within CP 5% per year; year 4 then showed diminished rates and in year 5 both risks are less than 1%. For CP patients with intolerance or failure during prior IFN-α, early, significant response to imatinib predicts for maximal protection from progression; after 5 years of follow-up, 69% of patients overall have remained free of progression; however, those achieving CCgR or MCgR by 3 months are 94% and 87% free from progression to AP/BC, respectively, versus only 55% for the remaining patients.[39] The relapse rate in this cohort appeared to be fixed at approximately 7% per year.[39] Lastly, for patients with advanced disease treated with imatinib as salvage therapy, rates of resistance and relapsing disease is dramatically higher, occurring in 75% or more of AP patients and 95% of myeloid BC patients.[40]

Early investigations in advanced phase CML cases who had relapsed on imatinib therapy had first indicated either *BCR-ABL* gene amplification or a mutation at residue 315 in the Abl KD as the determinants of Bcr-Abl reactivation within the leukemic clone. On binding, the hydroxyl group of threonine 315 of Bcr-Abl, the so-called 'gatekeeper' residue, forms a hydrogen bond with imatinib, and the side chain present at position 315 also sterically controls the binding of the inhibitor to hydrophobic regions

adjacent to the ATP-binding site.[30,31] The substitution of threonine with a bulkier and more hydrophobic isoleucine was shown to eliminate this hydrogen bond required for high-affinity inhibitor binding and to create a steric hindrance interfering with imatinib placement within the ATP-binding site.[30,41] Importantly, although threonine 315 is essential for imatinib, this is not the case for ATP binding, which does not depend on the accessibility of the same hydrophobic cavity and is therefore not affected by the incorporation of a bulky isoleucine side chain. The individual structural requirements of ATP and imatinib mean that the catalytic activity, and therefore the tumour-promoting function, is preserved in the imatinib-insensitive T315I mutant. A strikingly identical amino acid substitution was later shown to occur at homologous positions in the KD of c-kit (T670I) and PDGFRα (T674I) kinases in imatinib-resistant gastrointestinal stromal tumors and hypereosinophilic syndromes, respectively,[42,43] further highlighting the central role of this highly-conserved 'gatekeeper' threonine in controlling the accessibility of the ATP-binding pocket to inhibitors.

The vast number of mutation reports since published[33,44-50] has led to an exponential increase in the number and type of amino acid substitutions found in CML patients who either lost or did not achieve response to imatinib treatment (a comprehensive list may be found in Table 2). c-Abl has 3 Src homology domains, including SH3, which is a negative regulator of kinase activity; SH2, which binds peptides that contain phosphotyrosine; and SH1 – the KD – which encodes for catalytic function. At the NH_2-terminal end of the Abl kinase is a highly conserved nucleotide phosphate binding domain for adenosine triphosphate, which is known as the P-loop. At the COOH-terminal end of the molecule is a flexible activation loop that is essential for the control of catalytic activity and changes conformation depending on whether the molecule is in the inactive or active state. Between these 2 loops is the catalytic site of the molecule, which resides in a cleft where imatinib and other small molecule tyrosine kinase inhibitors bind. Abl shifts between an inactive or a closed conformation and a catalytically active or open conformation, and this shift from an inactive to active state appears to be regulated by the kinase itself in a process known as ''autoinhibition.'' In the inactive state, the activation loop just described is folded inward toward the catalytic site, and in the active state, this activation loop flips away from the catalytic region. Thus, it can act as a support for substrate binding. Mutations disrupt imatinib binding by

affecting critical residues implicated in direct contact with the inhibitor or preventing Bcr-Abl from effectively adopting the specific inactive conformation which imatinib binds.

The majority of these Bcr-Abl mutant forms are now well characterized in terms of the extent to which they confer insensitivity to imatinib (Table 2).[33,44,51] Indeed, while some mutations (T315I and mutations falling within the P-loop region, i.e., G250E, Y253F/H and E255K/V) confer a highly resistant phenotype, other are associated with a relatively modest increase in imatinib IC_{50} and might therefore be overcome by a dose increase.

Controversy remains over the point in time when such mutations are acquired. In anecdoctical reports, the same mutant clone observed at relapse was retrospectively traced back to the sample archived prior to imatinib start,[45,52] speaking to the theory of outgrowth with selection pressure. However, this is not a universal finding – a study on a large, unselected cohort of imatinib-naïve patients has recently demonstrated that in some cases different mutant clones may observed before therapy and at relapse.[53] Beyond the "signature" of a KD mutation (the particular amino acid substitution and kinase region being predictive), the "fitness" of mutant clones – their ability to sustain long-term proliferation with a relative advantage over Ph-negative clones or wild type Bcr-Abl – is most relevant to risk. While it is accepted that expansion of a Ph+ CML clone bearing an Abl KD mutation may be associated with resistance to imatinib, the fact that mutations may be identified prior to imatinib exposure and do not strictly correlate with clinical resistance suggests a role for additional mechanisms to trigger outgrowth of mutants, or that genesis of mutant clones reflects greater genetic instability. Cytogenetic clonal evolution has indeed been linked to mutation detection prior to imatinib. screening for mutations prior to imatinib and for those with stable minimal residual disease are worthless because they may yield misleading information.

Similarly, rare Ph+ cells harbouring mutations have been detected in 12 to 38% of patients who are in stable CCgR on imatinib.[54,55] However, in some cases they turned out to be only a transient finding – the mutated clone did not outgrow and did not lead to clinical relapse. In other cases, the patient actually relapsed, but with evidence of a different mutated clone. The cases in which outgrowth of the mutated clone and subsequent relapse were indeed observed were all cases in which a significant rise in *BCR-ABL* transcript levels had accompanied detection of the mutation. Therefore,

monitoring responding patients with supersensitive techniques for the emergence of KD mutations is worthless, too, unless a significant rise in *BCR-ABL* transcript levels is observed.

Besides Abl KD mutations, other mechanisms of resistance exist or have been hypothesized on the basis of *in vitro* observations in cell lines selected for imatinib resistance (Figure 6). Drug efflux, for example, has often been implicated in resistance to chemotherapeutics. In one study, investigators reported increases in the multidrug resistance (ABCB1; MDR1) gene that encodes the P-glycoprotein (Pgp) in a subclone of a resistant human leukemia cell line (Lama-84R) derived by growing the cells in increasing concentrations of imatinib.[21] Increased expression of Pgp could potentially reduce or even deplete intracellular levels of imatinib, thereby reactivating Bcr-Abl signaling. While some *in vitro* models have supported this finding, others have demonstrated that overexpression of Pgp in K562, a human CML cell line, does not confer resistance to imatinib.[56-59] More recently, another multidrug transporter, ABCG2 (BCRP), has been implicated in imatinib transport and resistance.[56,60] Reduced drug influx has also been hypothesized to play a role in imatinib resistance. Lower baseline values of the mRNA encoding the drug transporter hOCT1[61] have recently been reported in CP patients with primary cytogenetic resistance to imatinib.[62] Other studies showed that imatinib may be sequestered in the plasma by drug-binding proteins. The plasma protein alpha-1 acid glycoprotein (AGP) has been shown to bind imatinib at physiologic concentrations *in vitro* and block the ability of imatinib to inhibit Bcr-Abl kinase activity.[63] However, whether drug influx/efflux and/or plasma sequestration represent major mechanisms of resistance to imatinib in CML still remains controversial.

Bcr-Abl-independent mechanisms have also been hypothesized. In such a scenario, the leukemia cells no longer rely on Bcr-Abl for their proliferative drive, but rather rely on alternate oncogenic signalling pathways which allow the leukemic clone to bypass Bcr-Abl inhibition by imatinib. In an *in vitro* model of resistance, one group demonstrated that an imatinib-resistant K562 subline was sensitive to inhibition of the Src-family kinases Lyn and Hck, and that these kinases were up-regulated in patients who acquired resistance during the course of imatinib therapy.[64,65] These observations are supported by precedent finding that Src family kinases may operate downstream of Bcr-Abl in

CML. The same group extended these observations to continuous cell lines derived from imatinib-resistant patients,[66] but there is little current evidence that Src activation is responsible for true clinical resistance to imatinib in patients.

Therapeutic strategies to address imatinib-resistant disease

Despite the excellent clinical results obtained with the introduction of imatinib, it was soon recognized that there was still a need to improve on the treatment of Ph+ leukemias. Relapse of the disease due to the emergence of resistance to imatinib is a major problem. In addition, most patients who are in CCgR on imatinib still do harbour residual Bcr-Abl-positive cells – this is known as "residual disease" and can be detected by sensitive assays like nested reverse transcription-polymerase chain reaction (RT-PCR).

These two obstacles have fostered intensive efforts aiming at the development of alternative inhibitors or alternative inhibitory strategies. Several approaches can be envisioned and are currently being explored.

High-dose imatinib (800 mg/d) early in disease continues to be studied in comparison to standard dose, with randomized trials ongoing and data forthcoming; further update of previously published single center experiences now shows similar ultimate depth of response for both 400 and 800 mg dosing, yet increased rapidity of response and also potentially lower risk of progression for higher dose imatinib.[67] It still remains to be assessed whether increased toxicity of 800 mg dosing may be limiting.

More potent and specific inhibitors capable of binding Bcr-Abl with less stringent conformational requirements have been rationally designed and some of them have already advanced to clinical trials. They include dasatinib (BMS-354825), nilotinib (AMN-107) and bosutinib (SKI-606).

Dasatinib (Figure 7) is a thiazolylamino-pyrimidine emerged from a programme directed towards immunosuppressant drugs and, in addition to inhibiting the Src family kinases Fyn, Lck, Src and Yes, it potently inhibits Abl, c-Kit, PDGFRβ, EPHA2, HER1 and p38 MAP kinases.[68] Dasatinib exhibited ~300-fold higher potency than imatinib against Bcr-Abl and was shown to retain activity *in vitro* against most of the clinically relevant imatinib-resistant Bcr-Abl mutant forms, with the notable exception of the T315I.[69,70] Recent co-crystal studies have indicated that dasatinib, unlike imatinib, is able to recognize multiple conformations of Bcr-Abl.[71]

Nilotinib is a phenylamino-pyrimidine derivative structurally related to imatinib. It was rationally designed based upon the crystal structure of imatinib-Abl complexes together with medical chemistry paradigms for drug discovery. As a Bcr-Abl kinase inhibitor,

AMN107 is in the range of 20- to 30-fold more potent than imatinib and is highly selective for Bcr-Abl (rank order of potency Bcr-Abl>PDGFR>c-Kit), whereas the latter is most potent against PDGFR kinases (rank order of potency PDGFR>c-Kit>Bcr-Abl). Nilotinib binds the inactive conformation of Bcr-Abl, as imatinib does, but with a less stringent requirement in the absolute shape and charge of the binding surface of the protein compared to imatinib. For this reason, it was found to maintain potency against both Bcr-Abl phosphorylation and cell viability in cell lines expressing several imatinib-resistant mutant forms, although, again like dasatinib, with the exception of T315I.[70]

Dasatinib and nilotinib were brought to clinical trials nearly simultaneously. Phase I trials in CML for both agents were recently reported simultaneously in paired articles.[72,73] Phase I studies for both agents included patients with resistant CP disease, with slightly different inclusion criteria (mainly allowance for imatinib intolerant patients [20% of the total] in the dasatinib study and patients with cytogenetic resistance only [i.e., still in CHR] in the nilotinib trial). The rate of CHR was identical for both at 92%, as was CCgR at 35%, with an additional 10% of patients on dasatinib achieving partial cytogenetic response, bringing the totals for MCgR to 45% for dasatinib and 35% for nilotinib. No dose-limiting toxicity was observed for dasatinib, with a range of 15-240 mg per day administered; for nilotinib, dosing at 600 mg BID was limiting, with associated liver (predominantly grade 3 indirect bilirubin and transaminase) and pancreatic enzyme elevations (including grade 2 pancreatitis), as well as one grade 3 subdural hematoma. Pleural effusions deemed therapy related were observed in 15 of 84 dasatinib treated patients overall in phase I and were treated with diuretics and/or drainage. Other higher-grade toxicity from dasatinib included edema, headache, and elevated transaminase levels. Myelosuppression was observed beyond the level seen with imatinib for both agents, and was more pronounced with dasatinib; however, comparison may be difficult due to the fact that patients with imatinib failure and intolerance may be at greater risk due to longer disease duration or other factors. Activity was seen for advanced phases of CML and Ph+ ALL with both agents in phase I. Phase II studies for dasatinib in all phases of CML and Ph+ ALL have been reported[74-76] and supported rapid FDA approval of the compound (named Sprycel) on 29/6/06 for both indications at the recommended dose of 70 mg BID. In the phase II trial of dasatinib in CP CML, 60% of patients required dose reductions over time for

toxicity and the median dose was closer to 100 mg per day; ongoing trials continue to explore dosing options for dasatinib, including varying total dose and QD versus BID dosing.[77,78] Early phase II data has been presented for nilotinib,[79-81] expanding experience with the 400 mg BID dosing. Results for both agents in CP remain impressive, with the majority of patients achieving sustained HR and approximately one-half MCgR and one-third CCgR. Advanced phase results show more limited salvage capability for both agents, particularly for the Ph+ acute leukemias, with early relapse common; in AP, with both agents, a subset of responders remains fairly durable, albeit with limited follow-up. A randomized trial of dasatinib (70 mg BID) versus imatinib 800 mg/d for patients with hematologic or cytogenetic resistance to lower dose of imatinib (400-600 mg/d)[82] reported early improvement in CCgR rate for dasatinib over high dose imatinib.

Bosutinib (SKI-606) is an anilino-quinolinecarbonitrile that, like dasatinib, belongs to the class of dual Src/Abl inihibitors.[83] Bosutinib proved to be an active inhibitor of Bcr-Abl in several CML cell lines and transfectants, with IC_{50} values in the low nanomolar range, 1 to 2 logs lower than those obtained with imatinib.[84,85] Bosutinib retained activity in imatinib-resistant cell lines displaying *BCR-ABL* gene amplification as well as in BaF3 cell expressing Y253F-, E255K and D276G-Bcr-Abl.[85] Again, the T315I was highly resistant. Phase I/II trials are ongoing.[86]

The lack of efficacy of these second-generation Bcr-Abl inhibitors, as well as of several additional compounds that have been sinthesized and tested pre-clinically (reviewed in [87]), against the T315I mutation represents a major concern. For T315I-positive patients, however, effective therapeutic approaches might soon be available. To expedite the identification and the availability of second-line strategies overcoming resistance induced by the T315I mutation, three approaches are successfully being pursued. The first is to design inhibitors binding regions of Bcr-Abl other than the ATP binding pocket. This is the case of ON012380, a substrate-competitive inhibitor which exhibited activity at low nanomolar concentrations against wild-type Bcr-Abl and all imatinib-resistant Bcr–Abl mutants, including the T315I, both in biochemical and in cellular assays.[88] Unfortunately, ON012380 has not yet entered clinical trials where it must prove its safety in use as well as its in vivo efficacy in achieving remission and preventing resistance. A second approach is to test targeted agents with a different mode

of action, i.e., molecules which target Bcr-Abl stability or a Bcr-Abl downstream signal transducer, alone or in combination with imatinib (or another Bcr-Abl kinase inhibitor). Very recently, the histone-deacetylase inhibitor LBH589 has been documented to deplete Bcr-Abl and induce growth arrest and apoptosis in cells expressing T315I-Bcr-Abl, both when administered alone and, even more effectively, when administered in combination with nilotinib.[89] Safety and tolerability of the orally-available LBH589B are being assessed in a phase I trial in patients with advanced solid tumors and cutaneous T cell lymphomas,[90] and phase II trials in CML are being planned. Similar encouraging results have been obtained with another histone-deacetylase inhibitor, vorinostat (suberoylanilide hydroxamic acid, SAHA), alone or in combination with dasatinib.[91] Combinations of vorinostat with various conventional agents are currently being evaluated in several malignant conditions (http://www.clinicaltrials.gov). By inhibiting histone deacetylase 6 and inducing acetylation of heat shock protein 90 (hsp90), LBH589 and vorinostat have been shown to attenuate the ATP-binding and chaperone function of hsp90.[92] This leads to polyubiquitylation, proteasomal degradation and depletion of hsp90-client proteins, including Bcr-Abl itself and its downstream effectors c-Raf and AKT. Moreover, vorinostat and LBH589 are known to induce apoptosis in human leukemia cells via a general mechanism of accumulation of pro-apoptotic proteins (Bax, Bim) coupled with depletion of anti-apoptotic factors (Bcl-2, Bcl-XL, survivin), which may enhance the effects of Bcr-Abl inhibitors.[93]

A third, intriguing approach is to explore the possibility of whether molecules that have been developed as inhibitors for other protein kinases and are already undergoing clinical trials, might include the T315I-Bcr-Abl among their "off-targets". One such screening has recently disclosed that the p38 inhibitor BIRB-796 and the aurora kinase inhibitor MK-0457 (VX-680) are both capable of binding T315I-Bcr-Abl.[94] BIRB-796, currently being evaluated in clinical trials for inflammatory bowel disease, binds T315I-Bcr-Abl with good affinity (K_d=40nM), but has significantly weaker affinity for wild-type and other imatinib-resistant forms of Abl (K_d values >1μM). MK-0457 (VX-680) is able to bind both wild-type and mutated Bcr-Abl and has been reported to inhibit T315I-Bcr-Abl at low micromolar concentrations in primary patient cells.[95] Additionally, recent co-crystal studies have shown that this Y-shaped molecule engages the Abl KD in such a way that a close encounter with the gatekeeper residue is avoided,

explaining why the compound is able to accommodate the substitution of threonine with isoleucine without any significant decrease in binding affinity.[95] MK-0457 is currently undergoing a phase I trial in leukemias, including advanced phase CML and Ph+ ALL, and encouraging responses in patients harbouring the T315I mutation have been reported.[96,97] A phase II trial in the specific setting of T315I-positive Ph+ leukemias is forthcoming.

It will be interesting to assess whether more potent Bcr-Abl inhibitors administered first-line might prove valuable in sinking the pool of residual leukemic cells from which mutant clones may emerge. Clinical trials are being planned in order to evaluate this premise. In addition, combination of inhibitors with a non-overlapping spectrum of resistance mutations or, even better, with non-overlapping mode of action, have been predicted to be particularly promising[98] and their assessment in clinical trials is also warranted. These strategies could be particularly suitable for the treatment of Ph+ ALL, where high genomic instability rapidly drives the emergence and selection of T315I and other highly resistant Abl KD mutations.

Tables

Study	Indication	Patients (n)	Enrollment period
Phase I studies			
Druker et al[99]	Late-CP	83	Jun 1998-May 2000
Druker et al[100]	BC, ALL	58	Apr 1999-Mar 2000
Phase II studies			
Sawyers et al[101]	Myeloid BC	260	Aug 1999-Jun 2000
Talpaz et al[102]	AP	235	Aug 1999-Mar 2000
Kantarjian et al[103]	Late-CP	532	Dec 1999-May 2000
Ottmann et al[104]	Lymphoid BC, ALL	56	Sep1999-May 2000
Phase III study			
IRIS[38]	Early-CP	1106	Jun 2000-Jun 2001
Expanded access program			
Protocol 113	Late-CP	4131	May 2000-Sep 2002
Protocol 114	AP	3093	May 2000-Sep 2002
Protocol 115	BC, ALL	1068	May 2000-Sep 2002

Table 1 – Outline of the clinical development program of imatinib in Ph+ leukemias.

	Bcr-Abl	imatinib IC_{50} (nM)	
		Biochemical	Cellular
	Wild-type	300	260-500
	M244V	380	2000
P-loop	L248V	n.a.	1500
P-loop	G250E	1000	1350-3900
P-loop	Q252H	n.a.	1200-2800
P-loop	Y253F*	>5000	3475
P-loop	Y253H*	>5000	>10000
P-loop	E255K	2800	4400-8400
P-loop	E255V	>5000	>5000
	D276G	n.a.	1500
	T277A	n.a.	n.a.
	F311L	775	480
	F311I	n.a.	n.a.
	T315I*	>5000	>10000
	F317L*	900	810-1500
	M343T	n.a.	n.a.
Catalytic domain	M351T	820	930
Catalytic domain	M351V	n.a.	n.a.
Catalytic domain	E355D	n.a.	n.a.
Catalytic domain	E355G	n.a.	400
Catalytic domain	F359V*	4700	1200
Activation loop	V379I	800	1630
Activation loop	A380T*	340	2450
Activation loop	F382L	n.a.	n.a.
Activation loop	L387M	1500	1000
Activation loop	L387F	n.a.	1100
Activation loop	H396P	340-800	850-4200
Activation loop	H396R	1950	1750
	S417Y	n.a.	n.a.
	E459K	n.a.	n.a.
	F486S	1230	2800

Table 2 – IC_{50} values of Bcr-Abl mutations observed in patients who are resistant to imatinib. Shaded boxes highlight residues belonging to the P-loop, catalytic domain and activation loop, as indicated. Residues marked with an asterisk (*) represent imatinib contact sites. IC_{50} is the concentration that inhibits by 50% the biochemical kinase activity of Bcr-Abl and suppresses by 50% the growth of Ph+ cell lines. n.a., not available.

Figures

Figure 1 – Representative karyotype from a CML patient showing the Philadelphia chromosome. The t(9;22) chromosomal translocation results in the formation of a shortened chromosome 22 (the Philadelphia chromosome) and a longer chromosome 9.

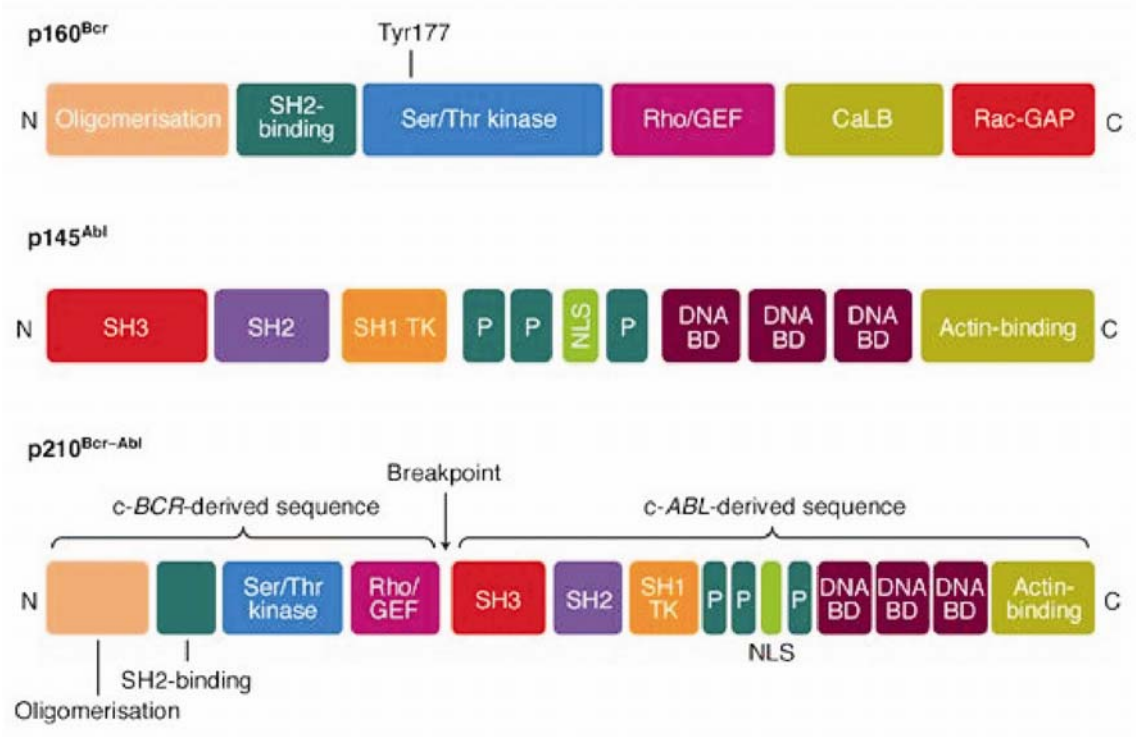


Figure 2 – Structure of the c-Bcr, c-Abl and Bcr-Abl proteins. c-Bcr comprises an oligomerization domain, a domain thought to mediate binding to SH2-domain-containing proteins, a serine/threonine kinase domain, a region with homology to Rho guanine-nucleotide-exchange factor (Rho-GEF), a region thought to facilitate calcium-dependent lipid binding (CaLB) and a region showing homology to Rac GTPase activating protein (Rac-GAP). The main phosphorylation site of Bcr (Tyr 177) is indicated. c-Abl comprises an SH3 and SH2 domain, an SH1 tyrosine kinase domain, several proline-rich domains (P), a nuclear localization signal (NLS), several DNA-binding domains (DNA BD) and an actin-binding domain. The Bcr-Abl fusion protein comprises the first four domains of c-Bcr and all the c-Abl domains except the N-terminal SH3 domain.

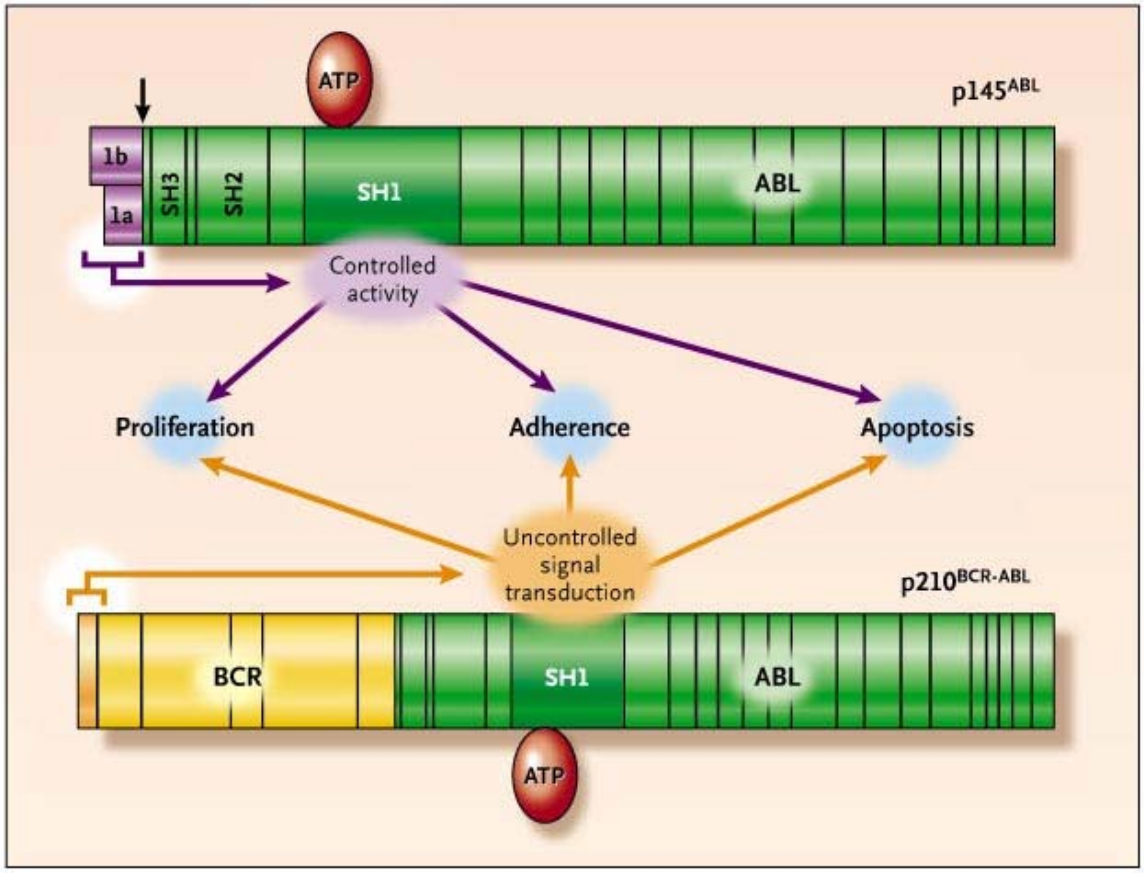


Figure 3 – Mechanisms responsible for Bcr-Abl-induced malignant transformation in Ph+ cells. As a consequence of the t(9;22) translocation, the regulatory regions at the NH_2-terminus of c-Abl are lost and replaced by the oligomerization domain of c-Bcr. This induces constitutive dimerization and autophosphorylation of Bcr-Abl, whose uncontrolled activity is responsible for alterations in the physiological processes regulated by c-Abl – proliferation, apoptosis and adherence to marrow stroma.

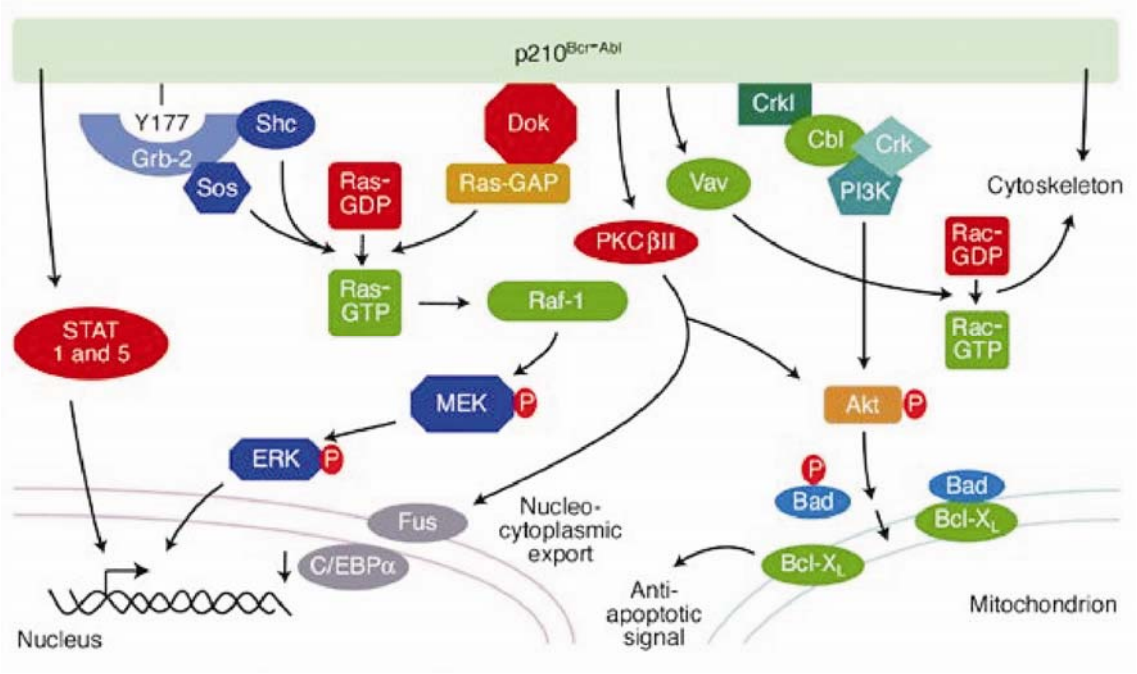


Figure 4 – Some of the cellular events triggered by Bcr-Abl kinase activity. Ras activation in Bcr–Abl-expressing cells is mediated by Bcr–Abl interaction with the adaptor signalling molecules Grb2, Shc, Sos and Dok. The sum of these interactions results in the favouring of Ras in its active GTP-bound form. This in turn leads to the activation of Raf-1 serine/threonine kinase activity with the subsequent activation of the MEK pathway (resulting in promitotic transcriptional regulation) and the PI3K (phosphoinositide 3-kinase) pathway (by phosphorylation of Akt, which leads, via Bad phosphorylation and dissociation from Bcl-X_L, to an anti-apoptotic signal mediated by Bcl-X_L). Interestingly, Bcr–Abl-dependent activation of the PI3K pathway has also been shown to be mediated by Bcr–Abl interaction with the adaptor molecule Crkl. The guanine-nucleotide-exchange factor Vav is known to interact with and act as a phosphorylation substrate of Bcr–Abl. This activated Vav subsequently favours the existence of Rac in its active GTP-bound state, leading to its effects on cytoskeletal reorganisation. The activation of STAT1 and 5 by phosphorylation has been shown in Bcr–Abl-expressing cells, although the mediators of this activation remain unclear. STAT1 and 5 phosphorylation results in their translocation to the nucleus, where they activate transcription. Bcr–Abl inhibits the proteasomal degradation of Fus by a protein kinase C βII (PKCβII) mechanism. Fus regulates nucleocytoplasmic export and RNA processing, thereby altering myeloid transcription factor (C/EBPα) expression, with subsequent effects on myeloid transcriptional regulation.

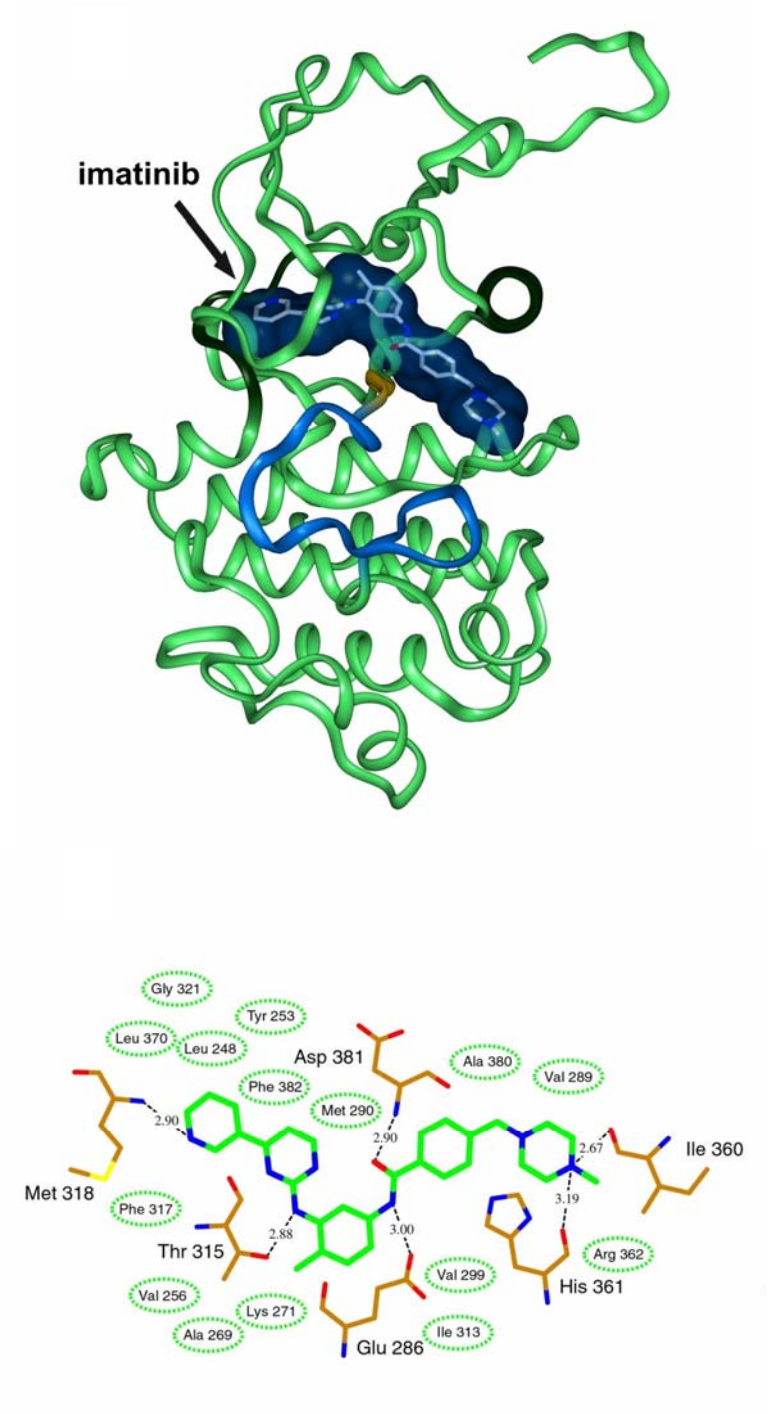


Figure 5 – The Abl kinase domain in complex with imatinib. Upper panel, overview. The activation loop is highlighted in blue. Lower panel, schematic diagram of the interactions between imatinib and Bcr-Abl. Nitrogen atoms are coloured blue, oxygen atoms are coloured red, sulphur atoms are coloured yellow, protein carbon atoms are coloured brown, imatinib carbon atoms are coloured green. Hydrogen-bonds are indicated with dotted lines along with their distances, and residues making van der Waals interactions with the inhibitors are circled with dotted lines.

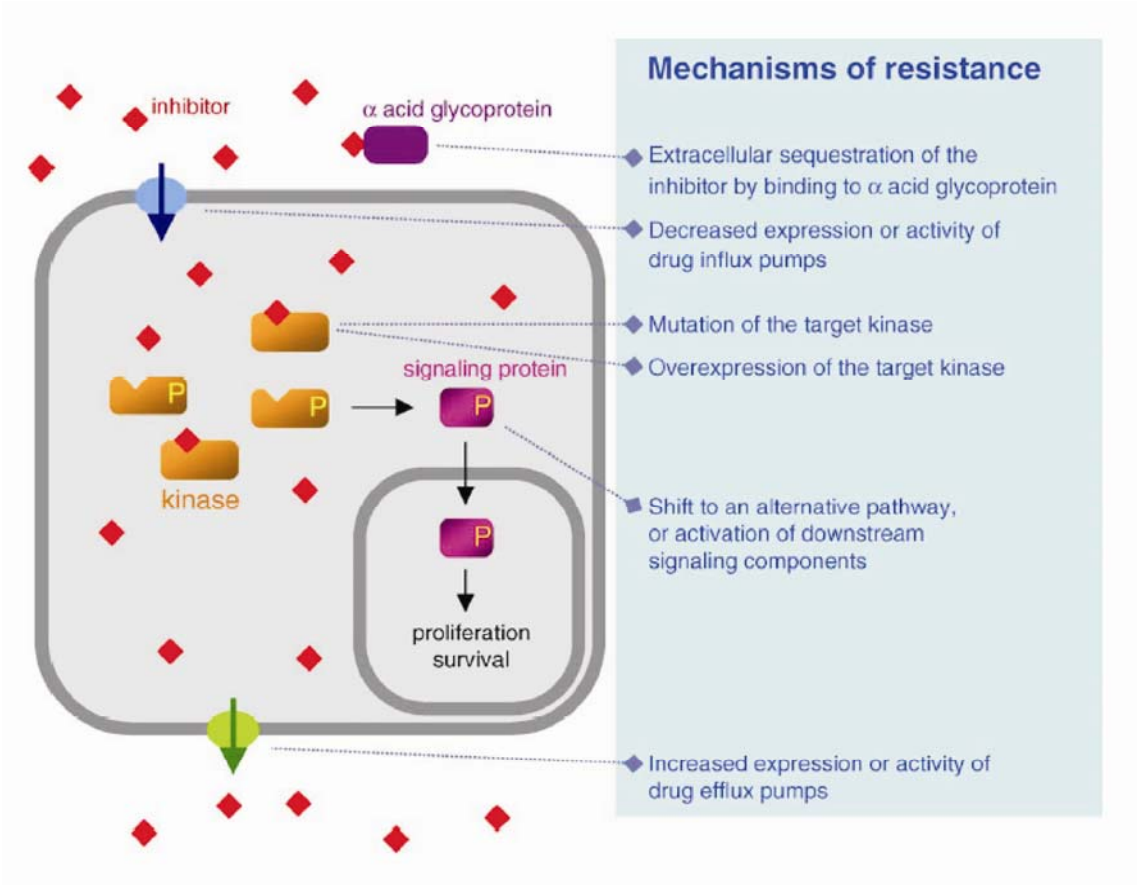


Figure 6 – Overview of different mechanisms of resistance that have been observed or hypothesized in Ph+ leukemias. Schematic representation of a cell, the oncogenic kinase and the downstream signaling pathways. P indicates a phosphorylated protein. The concentration of the small molecule inhibitor inside the cell is dependent on its influx and efflux rates. The different sites at which resistance can originate are indicated.

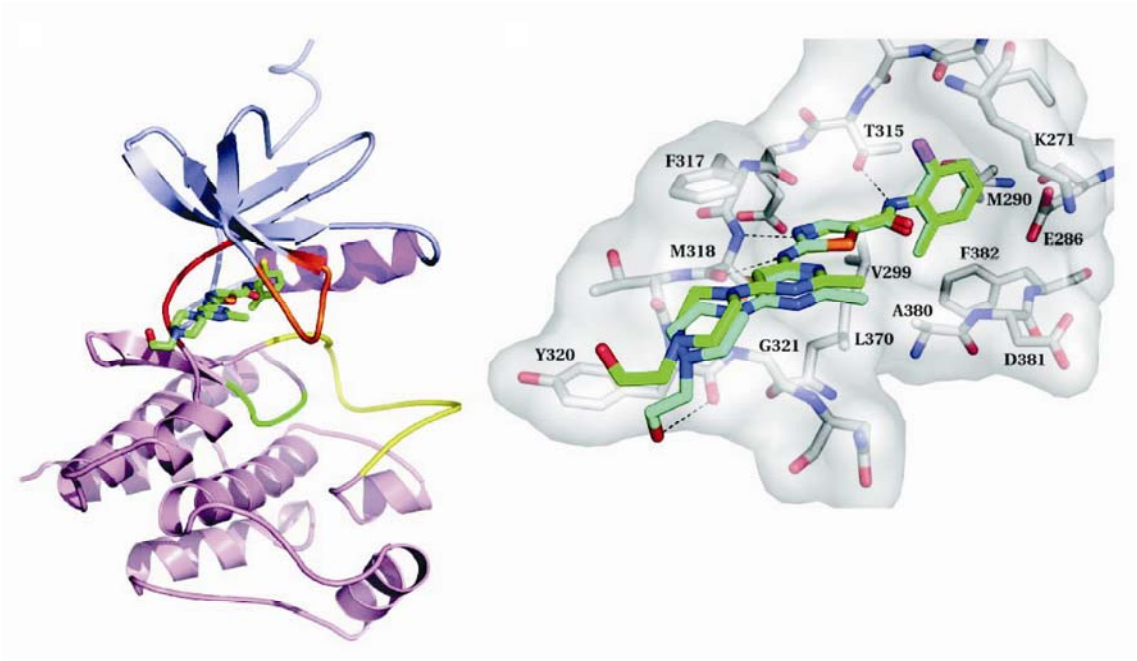


Figure 7 – Three-dimensional structure of the Abl kinase domain in complex with dasatinib. Left panel, overview. Stick, dasatinib; green, carbon atoms. Blue, NH2-terminal lobe of Abl kinase; orange, P-loop; magenta, helix a-C; red, hinge region; pink, COOH-terminal lobe; green, catalytic loop; yellow, activation loop. Right panel, cut-away detailed view of dasatinib and nearby residues in the ATP-binding site. Green and blue, inhibitor molecules found in the two asymmetric units; gray, one protein structure with surface of residues displayed. Dashed lines, hydrogen bonds.

AIMS

Imatinib mesylate is a remarkably effective inhibitor of the oncogenic Bcr-Abl tyrosine kinase. In some cases, however, the leukemic clone may evolve to evade Bcr-Abl inhibition. Point mutations in the Abl KD are frequently observed in patients who relapse on imatinib therapy. These mutations disrupt imatinib binding since they affect critical residues implicated in direct contact with the inhibitor or prevent Bcr-Abl from effectively adopting the specific inactive conformation which imatinib binds. While some mutations are associated with a moderate degree of resistance and may therefore be overcome by a dose escalation of imatinib, other confer a highly resistant phenotype. In such cases, imatinib is no longer effective and alternative treatment strategies, such as allogeneic stem cell transplant or a second-generation tyrosine kinase inhibitor, have to be considered. In patients with either suboptimal response or evidence of resistance to imatinib, the knowledge of the presence, as well as of the type of mutation is therefore a useful guide to a rational therapeutic reassessment. During the three year research period, studies were conducted in order to

a) set up and validate a sensitive, high-throughput denaturing-high performance liquid chromatography (D-HPLC)-based method for mutation screening of Ph+ patients who have a suboptimal response or evidence to resistance to imatinib;

b) investigate the frequency and the clinical significance of Abl KD mutations in a large series of Ph+ patients resistant to imatinib;

c) assess which pre-existent or emerging Abl KD mutations may be challenging for the clinical efficacy of the second-generation tyrosine kinase inhibitor dasatinib in imatinib-resistant Ph+ patients.

PART I:

SET UP AND VALIDATION OF A NOVEL, SENSITIVE AND HIGH-THROUGHPUT D-HPLC-BASED ASSAY FOR DETECTION OF ABL KD MUTATIONS IN PH+ LEUKEMIA PATIENTS RESISTANT TO IMATINIB

Background

The encouraging results from trials of imatinib mesylate for the treatment of patients with CML have established it as the new standard of care for the disease. Imatinib is a potent and selective inhibitor of Bcr-Abl tyrosine kinase, which is known to be deregulated in as many as 95% of CML patients, as well as in 20-30% of Ph+ ALL patients. Despite high rates of hematologic and cytogenetic responses, primary refractoriness and acquired resistance have been observed. Point mutations within the KD of the *BCR-ABL* gene are emerging as the most frequent mechanism for reactivation of kinase activity within the leukemic clone.[33,41,44-50] A large number of mutations have been reported in association with the resistant phenotype, and most of them are well characterized in terms of the ability and degree to which they induce resistance.[33,44,51] While some mutations (i.e., Y253F/H, E255K/V, T315I) confer a true resistant phenotype and suggest withdrawal of imatinib in favor of alternative therapeutic strategies, others (i.e., M244V, F311L, F359V) may be overcome by dose-escalation. Thus, in order to optimize therapeutic response, not only the presence of a mutation but also the actual amino-acid change should be investigated in patients displaying hematologic or cytogenetic resistance to imatinib. A recent study[50] has suggested that a) in late-CP and AP patients treated with imatinib, mutations can be detected by direct sequencing prior to clinical evidence of resistance, thus predicting the subsequent course of the disease; and, b) that mutations in the nucleotide-binding loop of the kinase region (P-loop) are associated with a particularly poor prognosis.

For these reasons, routine mutation testing of CML and Ph+ ALL patients who have a non-optimal response to imatinib is being introduced in order to assure a more rational therapeutic management. Sequencing has been widely used to screen for Abl KD mutations in published studies,[33,41,44-50] but it is expensive and time-consuming,

especially when subcloning of polymerase chain reaction (PCR) products is performed. Several alternative methods have been reported, such as allele-specific oligonucleotide PCR (ASO-PCR),[45] restriction fragment length polymorphisms (RFLP)-based assays[44,105] and peptide nucleic acid (PNA)-based clamping techniques.[106] They are more sensitive but seem not to be suitable for large-scale screening of the entire spectrum of mutations reported to date. Here we present a novel, rapid and straightforward method for monitoring and detection of leukemic cells containing imatinib resistance-associated mutations based on PCR amplification and subsequent screening of PCR products by denaturing-high performance liquid chromatography (D-HPLC).

Patients and methods

Patients and samples. The study was retrospectively performed on bone marrow or peripheral blood samples obtained from 15 patients with CML and 15 patients with Ph+ ALL who had evidence of resistance to imatinib. Resistance was primary (failure to achieve a CCgR after 12 months of treatment) in 5 cases and acquired (loss of CCgR, loss of HR or progression to AP/BC) in the remaining 25 cases. All patients provided informed consent for participation in this study. Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation and then stored at –80°C in guanidinium thiocyanate until use.

Positive and negative controls. Cell lines carrying the Y253F, E255K, T315I and M351T Abl mutations (kindly provided by Michael Deininger and Brian Druker, Oregon Health and Science University, Portland, OR) were used as positive controls. G250E- and H396R-mutated amplicons from two patients were subcloned into a pCR2.1-TA vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA) and used as positive controls, as well. A sample from a CML patient known to be wild-type for Abl mutations was used as negative control.

RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR). Total cellular RNA was extracted from mononuclear cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was spectrophotometrically quantified by 260 nm absorbance and its integrity was assessed by electrophoresis on ethidium bromide-stained 2% agarose gel. One μg of total cellular RNA was reverse transcribed to cDNA in 50 μL final volume using 25 μM of random hexamer primers (Applied Biosystems, Foster City, CA) and 200U of M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Experimental design of D-HPLC analysis. All samples were analyzed using D-HPLC Wave 3500HT DNA Fragment Analysis System (Transgenomic Ltd, Cramlington, UK). To increase the sensitivity and specificity of *ABL* KD amplification we set up a nested-PCR approach. The first round of amplification was done using 100 ng of cDNA and the following primers: either Fwd-BCR-p190 (positioned on exon 1 on *BCR* mRNA) or

Fwd-BCR-p210 (positioned on exons 12/13 on *BCR* mRNA), and Rev-ABL (positioned on exon 10 on *ABL* mRNA) (Table 1, Figure 1). This procedure ensured that the normal, non-rearranged *ABL* transcript was not analyzed. An initial denaturation step of 5' at 95° was followed by amplification for 25 cycles (denaturation: 30" at 95°C; annealing: 1' at 60°C; extension: 2' 30" at 72°C) and final extension for 7' at 72°C. Reamplification of a 1 μL aliquot was then performed using three internal primer pairs: Fwd-ABL-A/Rev-ABL-A, Fwd-ABL-B/Rev-ABL-B and Fwd-ABL-C/Rev-ABL-C (Table 1). In this way, the entire *ABL* KD was divided into three partially overlapping fragments of optimal length for D-HPLC analysis, i.e., ABL-A (393 bp, codons 206-335), ABL-B (482 bp, codons 262-421) and ABL-C (465 bp, codons 371-524)(Figure 1). For the second rounds of amplification, the following PCR conditions were used: initial denaturation step of 5' at 95°C; amplification for 35 cycles (denaturation: 30' at 95°C; annealing: 40" at 60°C; extension: 50" at 72°C); final extension for 7" at 72°C. All PCR experiments were performed in 50 μL final volume containing 1.5 U of Optimase Polymerase (Transgenomic), 10X reaction buffer (Transgenomic), 200 μM of each dNTP (Invitrogen), 1.5 mM $MgSO_4$ (Transgenomic), and 0.5 μM of each primer. Specificity and efficiency of the amplification reactions were checked by electrophoresis of a 5 μL aliquot on ethidium bromide-stained 2% agarose gel. Using Wavemaker software, Version 4.1.40 (Transgenomic), the melting curves were calculated to select optimal elution temperatures and gradient conditions to resolve heteroduplexes in the three fragments to be screened. Base changes located in various positions along a fragment are affected by the melting characteristics of the surrounding nucleotides. For example, GC-rich regions of an amplicon lose helicity at higher temperatures than AT-rich domains. The melting characteristics of a fragment therefore determine the temperature(s) at which the base change will resolve using D-HPLC. Wavemaker software identifies melting subdomains with respect to the whole fragment sequence and predicts melting temperatures to maximize the resolution of heteroduplex and homoduplex peaks within each subdomain – which usually requires an helical fraction ranging between 70% and 85%. Principles of D-HPLC-based mutation screening are illustrated in Figure 2. Aliquots of 8 μL crude PCR products, preheated for 10' at 96°C and then gradually reannealed for 10' at room temperature to allow heteroduplex formation, were eluted into a DNASep HT column (Transgenomic) at the

following temperatures: 61.3°C - 62.4°C - 63.5°C for ABL-A; 60.3°C - 61.1°C - 61.9°C for ABL-B; 60.2°C - 60.9°C - 61.2°C for ABL-C. DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mM triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate (1.5 mL/min). Each elution took approximately 3 minutes. Gradient was formed by mixing buffer A (0.1mM TEAA; Transgenomic) and buffer B (0.1mM TEAA, 25% acetonitrile; Transgenomic). Eluted amplicons were detected by 260 nm UV absorbance. A wild-type sample was used as a negative control. The chromatogram from each tested patient was overlaid with the wild-type profile, and samples with extra peak(s) were scored as positive. To ensure that mutations present in ≥90% of Bcr-Abl-positive cells could not escape D-HPLC detection, for all samples studied a mixture of a wild-type negative control and patient PCR products in a 1:1 ratio was also run.

Direct sequencing. Direct sequencing was done in parallel on all the samples using an ABI PRISM 3730 (Applied Biosystems). A 20 μL-aliquot of the same PCR product analysed by D-HPLC was purified (Qiaquick PCR Purification Kit, Qiagen) and sequenced using Big Dye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems) according to manufacturer's instructions. Removal of unincorporated dideoxy-nucleotides was done with the Dye-Ex 2.0 Spin Kit (Qiagen). Sequences were compared to the wild-type sequence using BLAST [*ABL* accession number, X16416]. Sequence analysis was routinely performed on both strands for each fragment.

Cloning. In the two cases scored positive by D-HPLC but in whom direct sequencing failed to evidence any nucleotide change, the PCR fragment was cloned into a pCR2.1-TA vector (TOPO TA Cloning Kit; Invitrogen). Twenty independent clones were then harvested and sequenced in both directions.

Results

Set up of the D-HPLC assay. The entire KD spans seven exons of the *ABL* gene (exon 4 through 10). Therefore, we decided to perform mutation analysis at the RNA level. A nested RT-PCR protocol was set up to ensure high sensitivity and to avoid co-amplification of the normal, non-translocated *ABL* allele. For the first amplification round, two alternative forward primers had to be designed in order to allow amplification of both p190- and p210-positive cases. In the second round of PCR, three sets of primers were designed so as to amplify three adjacent, partially overlapping fragments of optimal length for D-HPLC analysis (393bp, 482 and 465 bp, respectively, the recommended length being between 200 and 500 bp; Figure 1). Having established the DNA fragment sequences to be studied, we subsequently determined the melting temperatures to be used for D-HPLC screening. WAVEmaker software (version 4.1.40; Transgenomic) allowed to visualize the position of melting subdomains with respect to the whole fragment sequence and to predict melting temperatures to maximize the resolution of heteroduplex and homoduplex peaks within each subdomain. Predicted temperatures were then assessed for their actual ability to resolve homoduplex and heteroduplex peaks by eluting fragments already known to harbour some clinically relevant mutations and slight adjustments were made to maximize resolution (Figure 3). The elution conditions finally established proved able to allow the detection of all the mutations tested. In Figure 4, representative D-HPLC chromatograms are illustrated. Wild-type PCR products show a single peak because only homoduplexes are present. By comparison, the mutant PCR products show additional peaks, corresponding to heteroduplex species, which is more or less resolved depending on the percentage of Bcr-Abl-positive cells which harbour the mutation and on the type and position of nucleotide substitution within the amplified fragment.

Sensitivity of the D-HPLC assay. To assess sensitivity, which has been reported to be variable in a range of 1-10% depending on the sequence and length of the fragments to be analysed,[107-109] we prepared limiting dilution experiments by mixing wild type and mutant (G250E, Y253F, E255K, T315I, M351T and H396R) plasmids or cell lines at the following ratios: 50%:50%; 60%:40%; 70%:30%; 75%:25%; 80%:20%; 83%:17%; 85%:15%; 88%:12%; 90%:10%; 93%:7%; 95%:5%; 98%:2%; 99%:1%. Representative

results for the H396R mutation are shown in Figure 5: the peak corresponding to the heteroduplex fraction was still clearly detectable when the mutant represented 10% of the total, but a shoulder on the leading edge of the homoduplex peak was still visible when the mutant represented 7% and even 5% of the total. For all the mutations tested, the lower detection limit of D-HPLC was at least 10%. Sensitivity of direct sequencing is shown for comparison in Figure 6.

Parallel D-HPLC and sequencing analysis of CML and Ph+ ALL patient samples. Characteristics of patients and results of D-HPLC and sequence analyses are reported in Table 2. In 20 out of 30 (67%) patients, D-HPLC analysis showed an abnormal elution profile suggesting the presence of a sequence variation. Sequence analysis confirmed the presence of a point mutation in all but two cases (patients no. 27 and 28 in Table 2). In order to rule out the possibility that D-HPLC had yielded false positive results, cloning was then performed. Sequencing of twenty independent clones for each sample revealed that a mutation was actually present at low levels (3/20 and 2/20 clones; Table 2). Figure 7 shows D-HPLC and direct sequencing outputs for one of these two cases (patient no. 28). Conversely, all the samples scored as wild-type by D-HPLC did not show evidence of mutations by direct sequencing. In most of the cases, additional peak(s) were detected at all three melting temperatures routinely used for D-HPLC screening. However, depending on the mutation and on its position within the PCR fragment, one of the temperatures was usually most helpful in highlighting additional peak(s) clearly resolved from the homoduplex peak, the other two temperatures yielding only smaller modifications of the elution profile. In rare cases, the additional peak(s) were detected at one temperature only. These findings suggest that, at least in our experience, elution at all three temperatures established with the help of WAVEmaker software is helpful in order to establish or rule out the presence of a mutation.

Discussion

Since point mutations affecting critical sites within the Abl KD seem to be one of the most frequent mechanisms of resistance (with frequencies ranging from 26 to 90% depending on the phase of the disease), a mutation screening of patients who have suboptimal or no response to imatinib treatment should routinely be performed in order to help decision-making on dose-escalation or alternative treatment options.

So far, sequencing of PCR products (either subcloned or not) has been widely employed to search for known and unknown Abl KD variants in CML and Ph+ ALL patients. A major limitation of sequencing, however, is the low level of sensitivity. Several authors have reported that direct sequencing of *ABL* PCR products generally reaches a sensitivity of 20%.[44,48] Subcloning of PCR products followed by sequencing has a higher sensitivity, the lower detection limit depending on the number of clones tested. By cloning and sequencing of 108 clones per sample (thus reaching a lower detection limit <1%) Hofmann et al.[52] reported that rare cells bearing the E255K mutation could retrospectively be detected prior to imatinib administration in two advanced Ph+ ALL patients. The mutation was found in a single clone from each patient. Despite being sensitive enough to detect the presence of a point mutation in pre-treatment samples, such a method is expensive, cumbersome and time-consuming, and its application for screening of large series of patients is unlikely.

A PCR-SSCP screening method has also been described by the same author.[52] The method was tested in a cohort of Ph+ ALL patients previously analysed by direct sequencing.[49] Although PCR-SSCP analysis can generally find conformational changes of DNA with a minimum detection level of about 2% to 5%, it proved to be inferior to sequencing since there were two imatinib-resistant samples known to have the E255K mutation that did not show any shifted band.

Alternative methods, allowing the detection of specific Abl mutations, have been set up. They include PCR-RFLP or modifications,[44,105] ASO-PCR[45] and PNA-based PCR clamping techniques.[106] With conventional RFLP,[44] a maximum sensitivity of 5% can be reached, but the assay is limited to a very small number of Abl mutations (Y253F/H, E255K/V, T315I, M351T) for whom the nucleotide change determines the abolishment or creation of a restriction site. Recently, an enhanced PCR-RFLP method for T315I and Y253F mutations has also been described.[105] It relies on the artificial introduction

of a TaqI restriction site in the mutant, but not in the wild-type sequences, by using mismatched, allele-specific primers. This procedure is meant to rescue only mutation-containing molecules, thus enhancing the sensitivity of the downstream detection methods. This approach proved to be very sensitive, being able to reliably detecting artificially-generated mutated amplicons in the presence of 10^3-fold excess of wild-type molecules. Another extremely sensitive technique for detection of specific point mutations is ASO-PCR. In a paper by Roche-Lestienne et al,[45] ASO-PCR with a sensitivity of 1:10000 (10^{-4}) was developed to retrospectively trace the presence of F311L, T315I and M351T mutations in 5 mutated patients out of 24 screened at the time of resistance by direct sequencing. In all the patients, the mutations turned out to be already detectable in the samples collected prior to Imatinib initiation. More recently, Kreuzer et al[106] developed a PNA-based PCR clamping technique with 1:500 (0.2%) sensitivity for Y253H, E255K and T315I mutations. With this method, the E255K mutation was again retrospectively detected at diagnosis in a single AP-CML patient who never achieved either cytogenetic or hematological remission.

Despite the fact that the methods cited above proved to be highly sensitive when employed for longitudinal screening of resistant patients known to have a specific point mutation, some drawbacks exist. ASO-PCR can easily produce false-positive results because a single, erroneous primer hybridisation may lead to the generation of artificial templates. Moreover, it is not suitable for widespread testing since a panel of more than 30 specific primers should be employed in order to screen for all the mutations so far reported. The latter disadvantage applies also to PNA-based PCR clamping techniques, that rely on expensive sets of primers and fluorescent probes for detection of point mutations. More importantly, all these techniques can not be used for further identification of new point mutations occurring in the KD of Abl protein.

Here we describe a novel method, which may play an important role as primary screening tool. To the best of our knowledge, this is the first application of D-HPLC for screening of Abl point mutations in CML or Ph+ ALL patients. D-HPLC is a reverse-phase ion pair HPLC specifically developed for detection of DNA sequence variations such as point mutations, small insertions and deletions.[110] Under conditions of partial heat denaturation within a linear acetonitrile gradient, heteroduplexes that form in PCR samples having internal sequence variations display reduced column retention time with

respect to their homoduplex counterparts (Figure 2). The elution profiles for such samples are distinct from those having a homozygous sequence, making the identification of samples harbouring polymorphisms or mutations a straightforward procedure. After D-HPLC analysis, only those samples showing an abnormal elution profile are subjected to sequencing in order to determine the precise sequence abnormality. Of note, D-HPLC instrument may be coupled to a fragment collector, which allows automated fragmentation and purification of the eluted PCR product corresponding to the mutated peak, thus increasing the sensitivity of the downstream steps required to determine the precise sequence abnormality.[109,111] Despite our instrument is at present not equipped with a fragment collector, data from the literature suggest that, in case of low-level mosaicism, mutations which can not be characterised by direct sequencing of unfractionated PCR products are successfully resolved after the selective collection of the low-level, mutant heteroduplex peaks.

Our results in a panel of 30 patients analysed in parallel by D-HPLC and direct sequencing showed that D-HPLC is a reliable method for pre-screening of PCR products. All the samples showing an abnormal elution profile turned out to have a nucleotide change and vice-versa. With respect to sequencing, the method described herein is much faster – in contrast with the very cumbersome evaluation of sequence data, the evaluation of results by D-HPLC is quite effortless because the investigator has to discriminate only between single and multiple peaks in the elution profiles. Moreover, PCR-generated products do not need any further manipulation before analysis. They are directly loaded onto the D-HPLC column and up to 192 samples can be screened in a single run. By using a microtiterplate autosampler, the mutational screening can be almost totally automated. Another major advantage is the fact that D-HPLC analysis is less expensive than sequencing.[112]

Our preliminary experiments with serial dilutions of wild-type and mutant *ABL* PCR products at different ratios showed that D-HPLC reaches a lower detection limit between 5 and 10% for G250E, E255K, Y253F, T315I, M351T and H396R mutations (Figure 4). Such a lower detection limit is in agreement with what has been reported in the literature.[107-109] Although we have not checked the sensitivity of our method for the whole spectrum of mutations reported in the literature, the six mutations tested so far are not only the most frequently found in Ph+ patients, but also those which show a

dramatic increase of IC_{50} (drug concentration required to inhibit proliferation by 50%) to values which can not be reached simply by escalating the dose of imatinib – thus indicating the need for an alternative treatment option. The lower detection limit of direct sequencing ranges between 20 and 30% (Figure 5). The higher level of sensitivity of D-HPLC with respect to sequencing was further testified by two cases of our series. In these cases, D-HPLC had clearly shown evidence of a sequence variation, whereas subsequent direct sequencing had failed to identify any amino acid substitution. However, cloning and sequencing of ten independent clones confirmed that a mutation was indeed present at a low level. Sensitivity of D-HPLC is an important advantage since a timely detection of mutated clones emerging in resistant patients may allow for a more efficient therapeutic intervention.

In conclusion, the D-HPLC-based method herein described is rapid, sensitive and reliable, and may play an important role as a primary screening tool for the detection of resistance-associated mutations in CML and Ph+ ALL patients.

Tables

Primer name	5’ to 3’ sequence	Position
Fwd-BCR p210	GAGCAGCAGAAGAAGTGTTTCAGA	*BCR*, exons 12/13
Fwd-BCR p190	CAACAGTCCTTCGACAGCAG	*BCR*, exon 1
Rev-ABL	CTTGGAGTGAGGCATCTCAG	*ABL*, exon 10
Fwd-ABL-A	CATCATTCAACGGTGGCCGACGG	*ABL*, exon 4
Rev-ABL-A	GTTGCACTCCCTCAGGTAGTC	*ABL*, exon 6
Fwd-ABL-B	GAAGAAATACAGCCTGACGGTG	*ABL*, exon 4
Rev-ABL-B	CGTCGGACTTGATGGAGAA	*ABL*, exon 7
Fwd-ABL-C	TGGTAGGGGAGAACCACTTG	*ABL*, exon 7
Rev-ABL-C	CCTGCAGCAAGGTACTCACA	*ABL*, exon 10

Table 1 – Sequences of primers used for the amplification of *ABL* KD.

No	Disease type	Type of resistance	Months on IM	Bcr-Abl type	D-HPLC result	Sequencing result	Base change	Amino acid substitution
1	ALL	Acquired	12	p190	MUT	MUT	ATG>ACG	M351T
2	ALL	Acquired	6	p210	MUT	MUT	CAG>CAT	Q252H
							GAG>AAG	E255K
							GAC>GGC	D276G
3	ALL	Acquired	8	p190	MUT	MUT	GAG>AAG	E255K
4	ALL	Acquired	22	p190	MUT	MUT	ATG>GTG	M244V
							CTG>GTG	L248V
5	ALL	Acquired	9	p190/p210	WT	WT	-	-
6	ALL	Acquired	2	p210	MUT	MUT	ACT>ATT	T315I
7	ALL	Acquired	9	p190	MUT	MUT	TAC>CAC	Y253H
8	ALL	Acquired	7	p210	MUT	MUT	TTC>GTC	F359V
9	ALL	Acquired	5	p190	WT	WT	-	-
10	ALL	Acquired	14	p210	MUT	MUT	TAC>CAC	Y253H
11	ALL	Acquired	9	p210	WT	WT	-	-
12	ALL	Acquired	7	p190	MUT	MUT	GAG>AAG	E255K
13	ALL	Acquired	4	p190	MUT	MUT	ACT>ATT	T315I
							ATG>ACG	M351T
							TTG>ATG	L387M
14	ALL	Acquired	11	p210	MUT	MUT	GAG>AAG	E255K
							ACT>ATT	T315I
15	ALL	Acquired	3	p190	MUT	MUT	ACT>ATT	T315I
16	CML	Primary	13	p210	WT	WT	-	-
17	CML	Primary	15	p210	WT	WT	-	-
18	CML	Acquired	24	p210	MUT	MUT	ATG>ACG	M351T
19	CML	Acquired	15	p210	WT	WT	-	-
20	CML	Primary	13	p210	WT	WT	-	-
21	CML	Primary	12	p210	WT	WT	-	-
22	CML	Acquired	19	p210	MUT	MUT	GAG>GGG	E355G
23	CML	Acquired	18	p210	MUT	MUT	TTC>TTA	F317L
24	CML	Acquired	5	p210	WT	WT	-	-
25	CML	Acquired	3	p190	MUT	MUT	TAC>CAC	Y253H
26	CML	Acquired	23	p210	WT	WT	-	-
27	CML	Acquired	13	p210	MUT	WT	(TAC>CAC)	(Y253H, 3/20)
28	CML	Acquired	12	p210	MUT	WT	(ACT>ATT)	(T315I, 2/20)
29	CML	Acquired	33	p210	MUT	MUT	CAT>CGT	H396R,
							GAG>AAG	E459K
30	CML	Primary	13	p210	MUT	MUT	TTG>ATG	G250E

Table 2 – Results of D-HPLC and direct sequencing analyses. In 20 patients, D-HPLC analysis showed an abnormal elution profile compatible with the presence of one or more sequence variations (MUT). Sequence analysis confirmed the presence of a point mutation in all but two cases. In these cases, cloning was performed to increase sensitivity of sequencing. Results of cloning are indicated in parentheses, together with the number of positive clones out of the total clones analyzed. Conversely, all the samples scored as wild-type (WT) by D-HPLC did not show evidence of mutations by direct sequencing. Abbreviations: IM, imatinib.

Figures

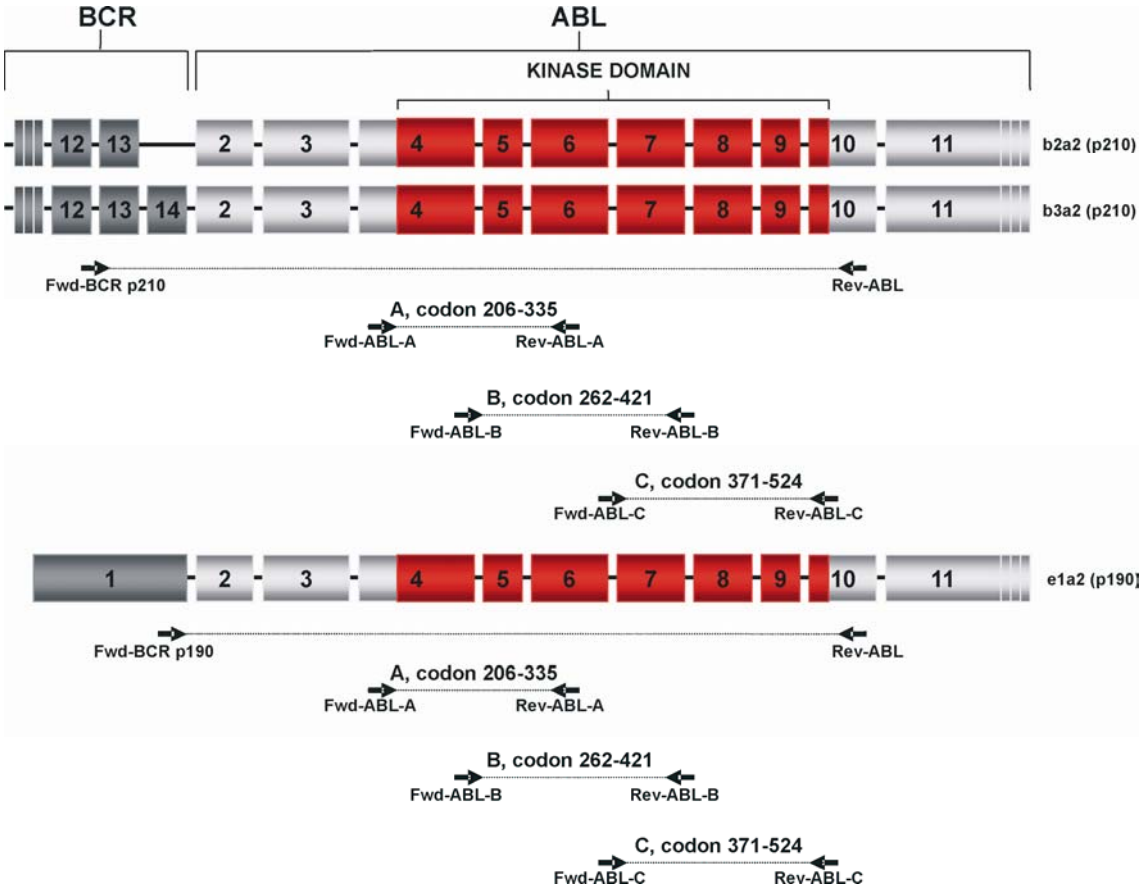


Figure 1 – KD amplification strategy. Fusion proteins encoded by *BCR-ABL* vary in size due to differences in breakpoint positions and alternative splicing patterns in the *BCR* gene. In most CML and a minority of Ph+ ALL patients, the hybrid transcript displays either a b2a2 or a b3a2 junction, resulting from the juxtaposition of *BCR* exons 13 or 14, respectively, to *ABL* exon 2. In both cases, the protein product is $p210^{BCR\text{-}ABL}$. In the remaining CML and in most Ph+ ALL patients, the fusion gene displays the e1a2 junction, i.e. the juxtaposition of *BCR* exon 1 to *ABL* exon 2. This transcript encodes the $p190^{BCR\text{-}ABL}$ protein. In the first amplification round we used a common reverse primer located on *ABL* exon 10 and two alternative forward primers, located either on *BCR* exon 1 (for e1a2-positive patients) or on *BCR* exons 12/13 (for both b2a2- and b3a2-positive patients). For all patients, the second round of amplification was then performed using three primers pairs, in order to subdivide the entire *ABL* KD into three partially overlapping fragments (A, B and C) of optimal length for D-HPLC analysis.

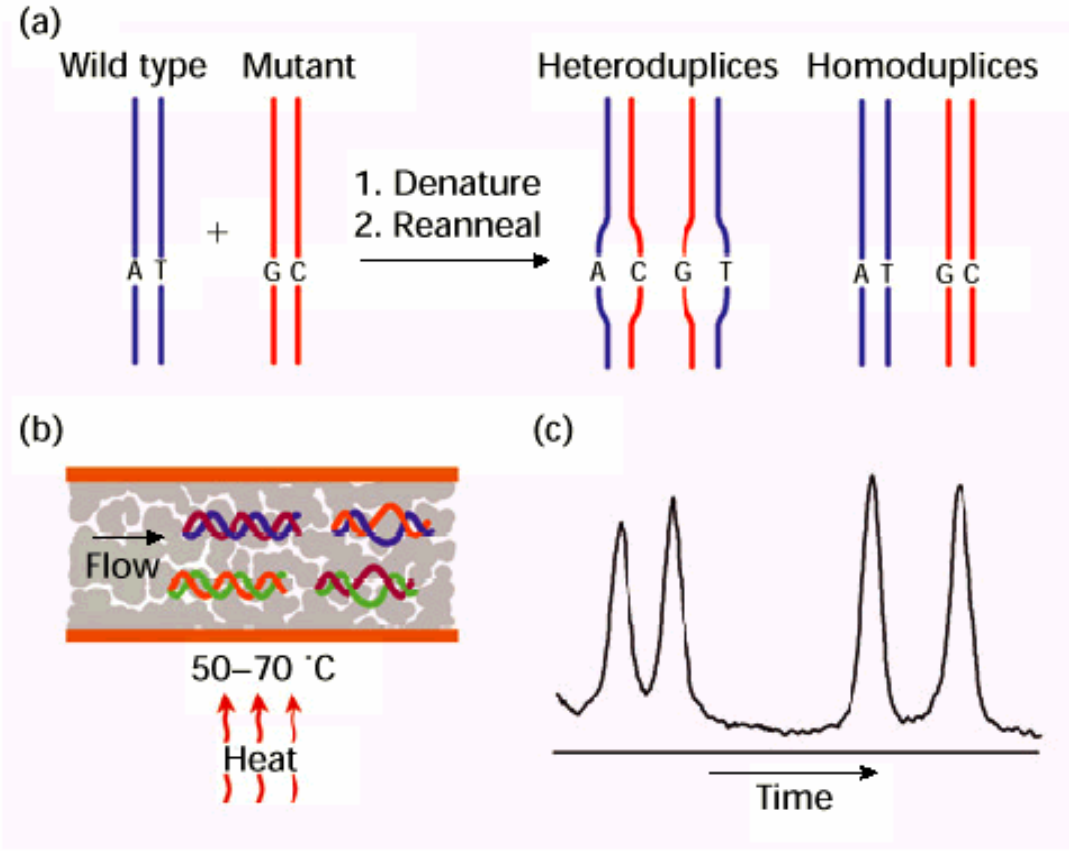


Figure 2 – Principles of D-HPLC-based mutation screening. Amplified fragments are denatured at 96°C for 10 min before they are allowed to reanneal by gradually lowering the temperature. In the presence of a mutation, this will generate both homoduplexes (from the reannealing of two wild-type or two mutated DNA strands) and heteroduplexes (from the reannealing of a wild-type and a mutant DNA strand).On the basis of the GC content of the fragment to be analyzed, column temperature is set to maintain partial denaturation. The thermally less stable heteroduplexes denature more extensively and consequently, have a shorter column retention time. Depending on several factors, including size of fragments, influence of nearest neighbor on the stability of both matched and mismatched base pairs and column temperature, the four species can be resolved completely or only in part.

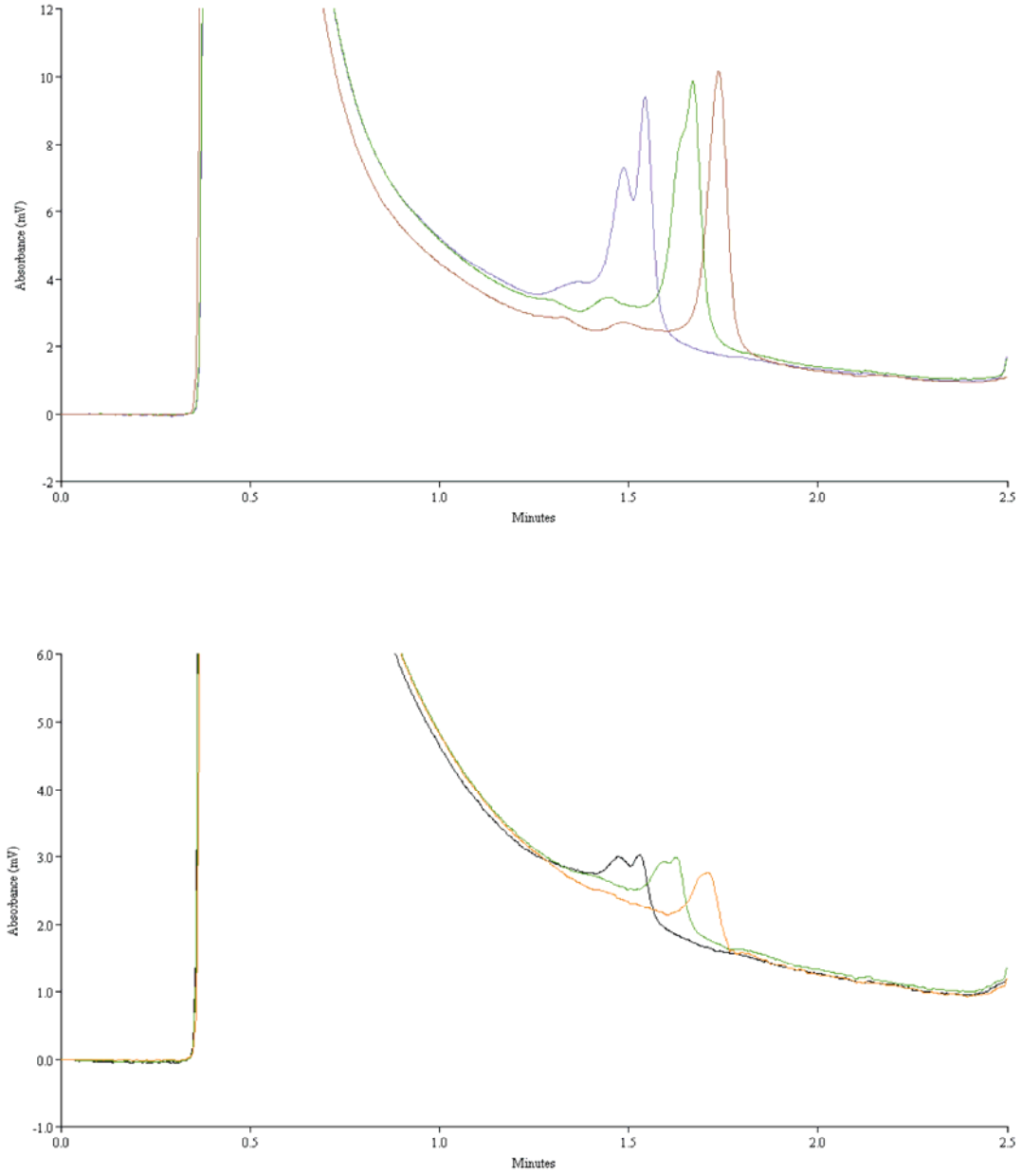
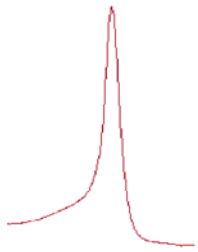
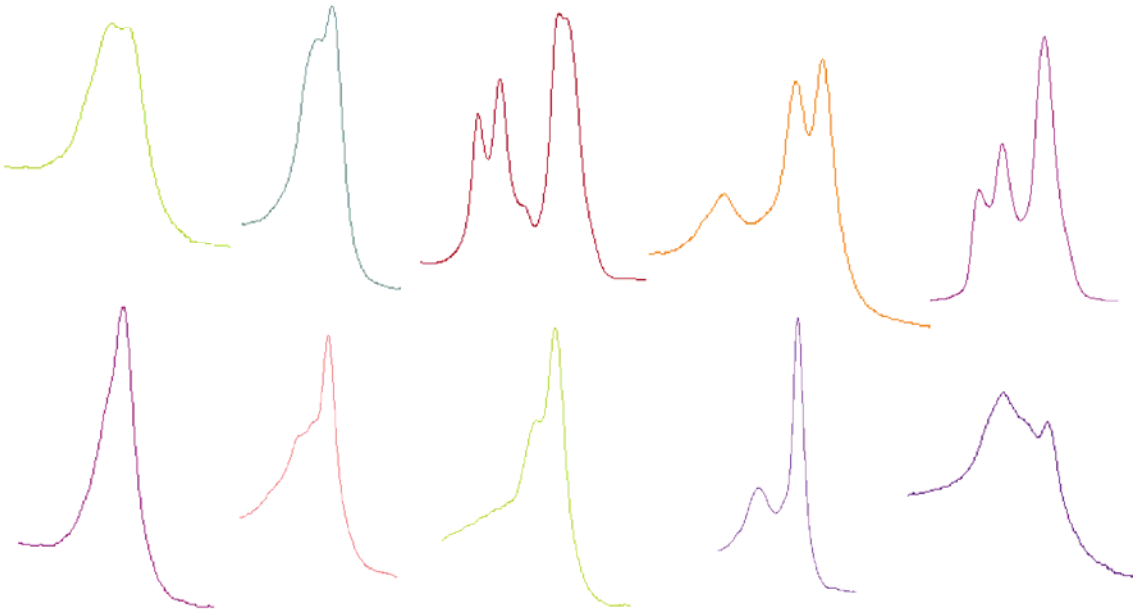


Figure 3 – Examples of how elution temperature may influence resolution of homoduplex and heteroduplex peaks. Upper panel, elution profiles of a *BCR-ABL* fragment known to be positive for the Y253F mutation at 62.8°C (right), 63.1°C (middle), 63.5°C (left). Lower panel, elution profiles of a *BCR-ABL* fragment known to be positive for the G250E mutation at 62.8°C (right), 63.1°C (middle), 63.5°C (left).



Elution profile of a wild-type sample



Elution profiles of samples harbouring mutations

Figure 4 – Examples of D-HPLC outputs for wild-type and mutated samples. In the case of a wild-type sample, the elution profile will consist of a single peak because only homoduplexes are formed. In the case of a mutated sample, we may observe elution profiles with multiple peaks, or just the separation of the homoduplex and heteroduplex species, or even only a small shoulder on the leading edge of the homoduplex peak. This is influenced both by the percentage of Bcr-Abl-positive cells which harbour the mutation and by the type and position of nucleotide substitution within the amplified fragment.

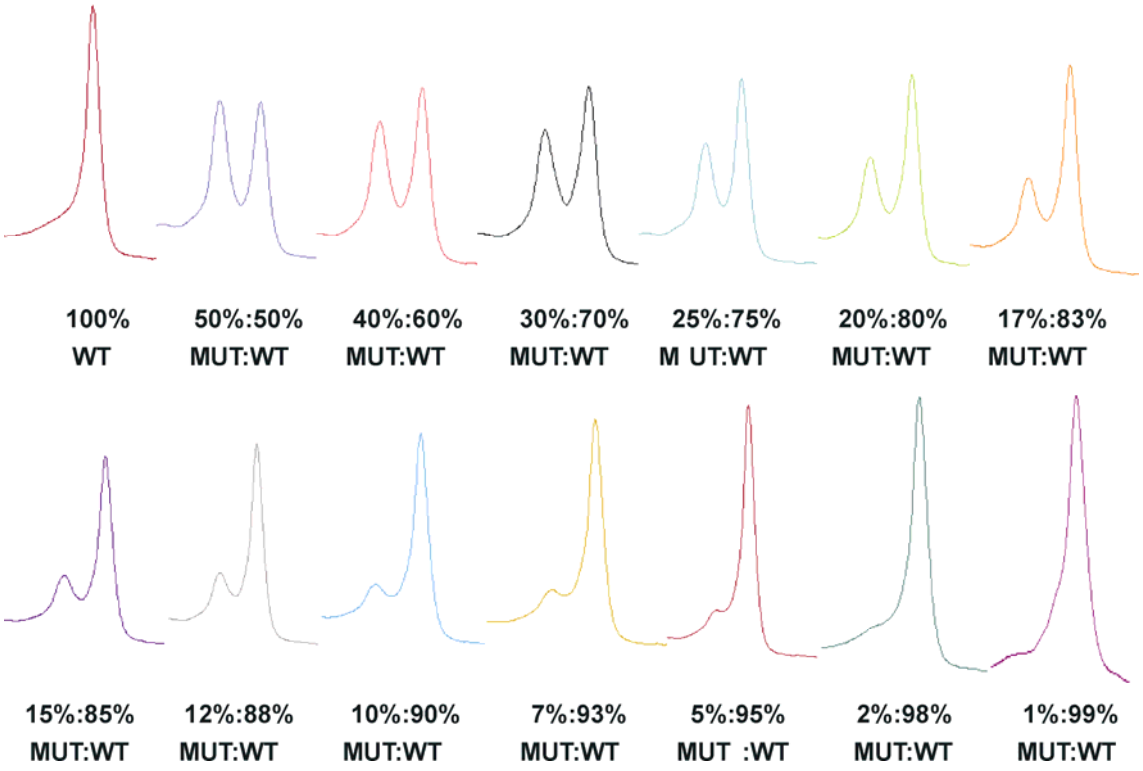


Figure 5 – Lower detection limit of D-HPLC analysis. Two plasmids containing a wild-type and a H396R-positive amplicon were mixed at different ratios, as indicated. By D-HPLC, with a standard UV detector, the additional peak corresponding to the heteroduplex fraction is clearly visible when the mutated clone is at a ratio of 7-10%, but a shoulder on the leading edge of the homoduplex peak is still evident even at a 5% ratio.

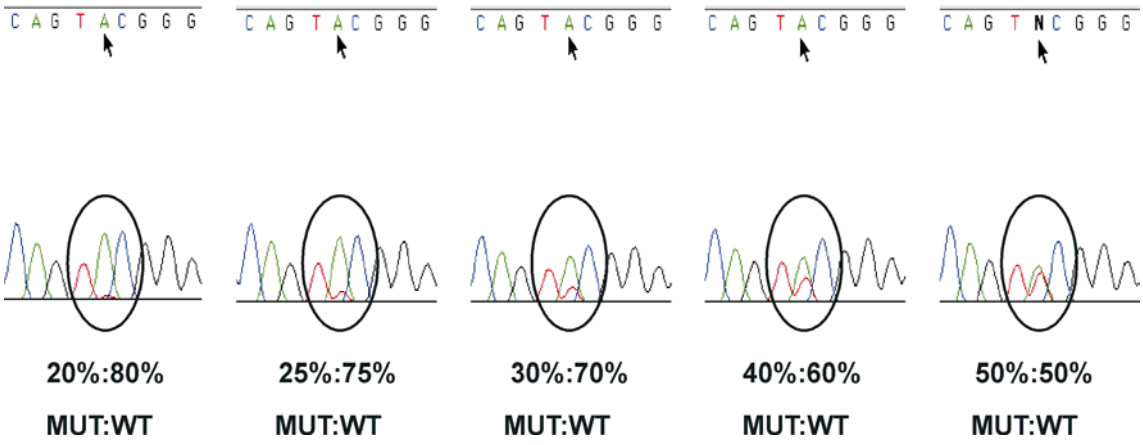


Figure 6 – Lower detection limit of direct sequencing. Amplicons from wild-type and Y253F-positive Ba/F3 cells were mixed at different ratios, as indicated. By direct sequencing, the red peak (T; mutated) is still clearly detectable under the green peak (A; wild-type) at a ratio of 30%, less clearly detectable at 25% and hardly distinguishable from the background noise at 20%.

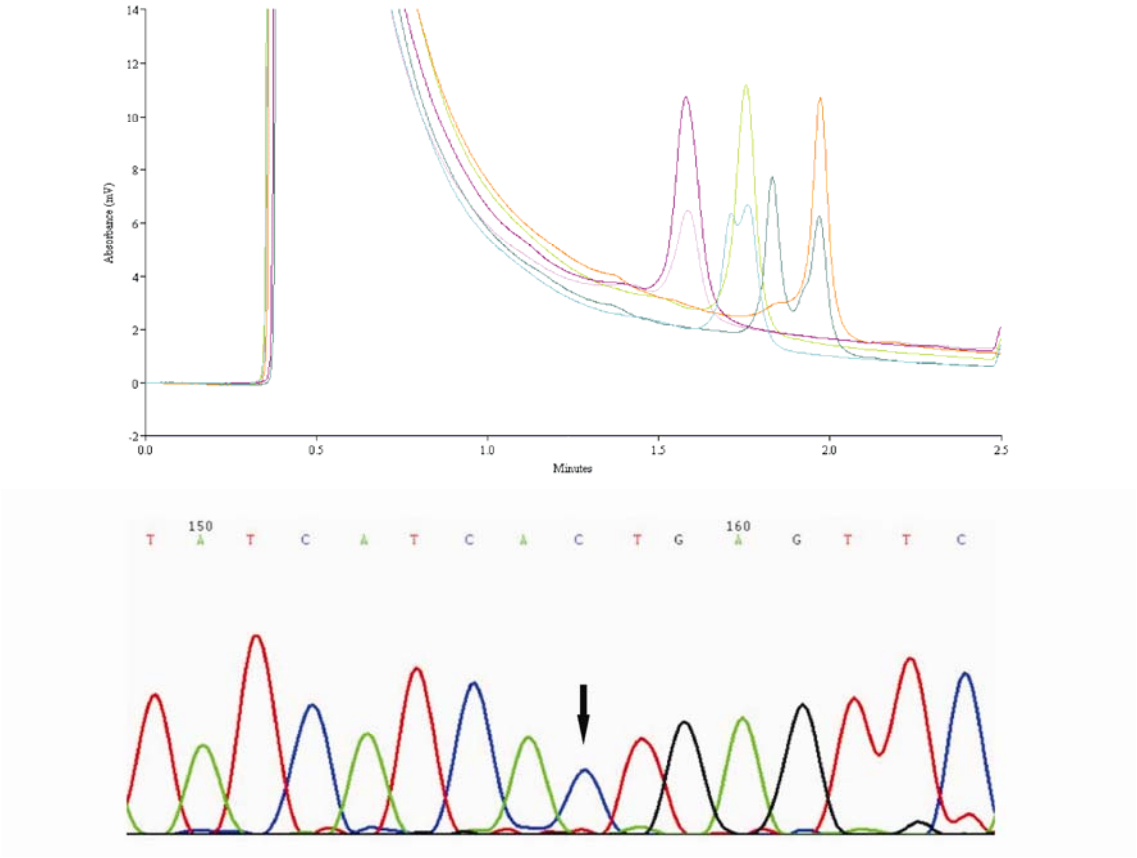


Figure 7 – Example of sensitive Abl KD mutation detection by D-HPLC. Upper panel, elution profiles for fragment B of patient no. 28, overlaid with those of a wild-type control for comparison. D-HPLC showed unambiguous evidence of a sequence variation both at the second (61.1°C) and at the third elution temperature (61.9°C). Mutation later turned out to be a T315I (AC<u>T</u>>A<u>T</u>T). Lower panel, direct sequencing output for the same sample. Only the wild-type peak ("C"; indicated by the vertical arrow) could be detected.

PART II:

CONTRIBUTION OF ABL KD MUTATIONS TO IMATINIB RESISTANCE IN DIFFERENT SUBSETS OF PH+ LEUKEMIA PATIENTS

Background

Despite the striking efficacy of imatinib mesylate for the treatment of Ph+ leukemias, resistance is observed in a proportion of patients, especially those with Ph+ ALL or advanced stage CML. Through the contribution of several research groups, the past four years have brought us considerable knowledge on the molecular mechanisms underlying resistance to imatinib (reviewed in [32]). Reactivation of Bcr-Abl tyrosine kinase activity within the leukemic clone is known to be most commonly associated with the emergence of point mutations in the Abl KD that impair imatinib binding without affecting ATP binding or kinase activity.[113,114] To date, more than forty different amino acid substitutions have been reported,[33,41,44-50,115,116] and most of them have already been characterized in terms of the extent to which they abrogate sensitivity to imatinib.[33,44,46,51] However, very few studies have been published on large and homogeneous series of resistant patients, and this has made it difficult to establish to what extent mutations do account for or at least contribute to resistance in its different clinical manifestations or in distinct disease categories.

To shed further light on this issue, we have collected and analyzed for the presence of Abl KD mutations samples from 370 CML or Ph+ ALL patients treated with imatinib at multiple centers of the GIMEMA Working Party on CML, who had clinical evidence of hematologic or cytogenetic resistance.

Patients and Methods

Patients and definitions. Between January 2004 and November 2005, bone marrow and/or peripheral blood samples from 370 CML or Ph+ ALL patients at the time of first evidence of resistance to imatinib (according to the definitions given below) were collected from multiple centers of the GIMEMA Working Party on CML and analyzed at our Institution for the presence of Abl KD mutations. All the patients were receiving imatinib at the standard doses of 400-600 mg/d. Informed consent for participation to this study was provided according to the Declaration of Helsinki. CP, AP and BC were defined as in recent studies.[101,102,117,118] Clinical features of lymphoid BC resembled Ph+ ALL and therefore these diseases were analyzed together. Primary hematologic resistance was defined as failure to achieve and sustain any HR for at least four weeks during the first three months of imatinib therapy. Primary cytogenetic resistance was defined as failure to achieve and sustain a CCgR (100% Ph-negative metaphases, based on the evaluation of a minimum of 20 marrow cells) for at least four weeks during the first 12 months of imatinib therapy. Acquired resistance was defined either as loss of CCgR, or loss of HR, or progression to AP or BC. Sensitivity and reliability of mutation detection is very dependent on the quality and integrity of RNA.[50] Given that no bedside RNA stabilization was done before shipment of blood or bone marrow to our Institution, samples were first of all assessed for the level of *BCR-ABL* and *ABL* transcripts [119,120] and were subjected to mutation screening only if the RNA obtained from the sample contained a measurable level of *BCR-ABL* transcript and the *ABL* control gene level indicated a nondegraded RNA. Samples from 73 (20%) patients were therefore discarded because of inadequate RNA quality, which was influenced neither by patient clinical features nor by type of resistance, but depended only on shipment conditions and time to delivery. Two hundred and ninety-seven patients were therefore evaluable for the aims of this study. Median age at imatinib start was 49 years (range, 17-70). Median time between diagnosis and imatinib start was 32 months (range, 0-160). Median duration of imatinib was 25 months (range, 4-42). Classification of patients in terms of disease phase and type of resistance is shown in Table 1.

RNA extraction and RT-PCR. Total cellular RNA was extracted from leukocytes and reverse transcribed as previously described.

D-HPLC analysis. Scanning of the Abl KD for the presence of mutations was performed as previously described. Briefly, after a first amplification of a fragment spanning both the *BCR-ABL* breakpoint and the *ABL* KD, three overlapping amplicons covering the KD (amino acids 206-335, 262-421 and 371-524) were generated by nested polymerase chain reaction and screened for the presence of sequence variations by D-HPLC (WAVE 3500-HT; Transgenomic, Cramlington, UK). Sensitivity of the assay ranged between 5 and 10% (data not shown). To ensure that mutations present in ≥90% of Bcr-Abl-positive cells could not escape D-HPLC detection, for all samples studied a mixture of wild-type and patient PCR products in a 1:1 ratio was also run.

Direct sequencing. Direct sequencing of D-HPLC-positive cases was done on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA), as previously described. Sensitivity of the method was 20-25% (data not shown).

Statistical analysis. Fisher's exact test was used to test for differences in mutation frequency among categories of patients. Analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

D-HPLC and sequence analyses for Abl KD mutations. D-HPLC analysis showed evidence of one or more sequence variations in 127/297 (43%) patients. Subsequent direct sequencing failed to detect any nucleotide substitution in 18 patients (4 CP patients treated with imatinib frontline, 12 CP patients post-IFN failure, 1 AP patient and 1 Ph+ ALL), but in all cases presence of a mutation became evident when sequence analysis was repeated on a second sample taken after one to two months from D-HPLC analysis. In 8 patients (2 Ph+ ALL, 2myBC, 2 lyBC, 1 AP and 1 CP post-IFN-α failure) multiple mutations simultaneously occurred so that, overall, 135 mutations were detected. Mutations mapped to 17 codons, the most frequent ones being E255K/V (21 patients, 17%), Y253F/H (17 patients, 13%), T315I (15 patients, 12%), M351T (14 patients, 11%), F359V/I (14 patients, 11%), M244V (13 patients, 10%), G250E (13 patients, 10%). Distribution and relative frequency of KD mutations found in our study are shown in Figure 1. Mutations falling within the 'P-loop' region (codons 248 through 255) were found in 58 patients (46% of all mutated patients).

Frequency and distribution of mutations according to disease phase at the time of analysis. Mutations were found in 54/198 (27%) patients in first CP (6/44 (14%) treated with imatinib frontline, 48/154 (31%) treated with imatinib after IFN-α failure), 11/21 (52%) AP patients, 24/32 (75%) myBC patients and 38/46 (83%) lyBC/Ph+ ALL patients (CP vs. AP, P=.02; AP vs. BC, P=.02; CP vs. BC, P<.0001) (Table 2). When we examined position and relative frequency of mutations by disease phase, we noticed a trend towards the preferential association of P-loop and T315I mutations and advanced stages of disease (Figure 2).

Frequency and distribution of mutations according to type of resistance. Mutations were associated in 45/152 (30%) patients with primary resistance (8/18 hematologic and 37/134 cytogenetic) and in 82/145 (57%) patients with acquired resistance (12/52 patients who lost CCgR, 18/33 patients who lost HR, 52/60 patients who progressed to AP/BC) (primary vs. acquired, P<.0001) (Table 3). Primary or acquired resistance did not seem to significantly differ in terms of type or relative frequency of mutations

responsible for (Figure 3), but 40 out of 52 mutated patients who had progressed to AP/BC harboured P-loop or T315I mutations (data not shown).

Discussion

Aim of the present study was to investigate the presence of Abl KD mutations in a large series of Ph+, imatinib-resistant cases, in order to assess to what extent mutations do account for or at least contribute to resistance in its different clinical manifestations or in distinct disease categories. Of 297 CML and Ph+ ALL patients with adequate RNA quality for *BCR-ABL* PCR amplification and D-HPLC/sequencing analyses, 127 (43%) showed evidence of one or more KD mutations. Such an incidence is somewhat lower than expected, but this is mainly due to the high degree of variability in mutation frequencies we observed among different subsets of patients. Indeed, when we separately considered the mutation frequencies by disease phase (Table 2) we could observe that in CP patients the contribution of KD mutations to the resistant phenotype was much lower than in AP and BC patients, with lymphoid BC and Ph+ ALL patients being the ones with the greatest likelihood of harbouring one or even multiple mutations. Similarly, mutation incidence in patients with primary resistance was much lower than in patients with acquired resistance (Table 3). The relatively low overall frequency of mutation in our series with respect to other published studies may therefore be explained by the predominance of CP patients (67%) as well as by the relatively high number of patients with primary resistance (51%).

The difference in mutation incidence between disease phases leads to several considerations. On one hand, it points towards advanced phase CML and Ph+ ALL cases as patients at high-risk for the emergence of resistance-associated mutant clones. There are currently no published data supporting the evidence that a systematic screening allowing for an early detection of emergent KD mutations is more beneficial than examining *ABL* sequences only in case overt resistance to imatinib is observed. However, our data suggest that at least in the setting of advanced phase CML or Ph+ ALL patients it might be worth assessing – ideally in the context of a prospective study – whether a regular mutation monitoring may assist clinicians in treatment optimization.

On the other hand, the evidence that in CP patients mutations account for approximately a quarter of resistant cases only highlights the need to find out which is/are the actual predominant mechanism(s) of resistance acting in this setting which now gathers the overwhelming majority of CML patients on imatinib therapy. *BCR-ABL* gene amplification and additional chromosomal aberrations are also known to be

associated with imatinib resistance,[41,44] but they seem to play a role mainly in advanced CML phases. Point mutations in Bcr or Abl regions other than the KD have been hypothesized based on results of an in vitro saturation mutagenesis screening for mutations conferring resistance to imatinib[121] and on the assumption that any amino acid substitution favoring the active conformation of Bcr-Abl (to whom imatinib is unable to bind) may confer resistance, but have never been described in patients as yet. Significantly lower expression of the drug transporter hOCT1, involved in imatinib influx, has recently been reported in some CP patients with primary cytogenetic resistance[62] and is an issue which deserves further elucidation. Among CP patients, however, mutation incidence in those who had received imatinib after IFN-α failure was approximately twice as high as in those who had received imatinib as first-line therapy (31% as against 14%). Such an intriguing difference between early- and late-CP supports the hypothesis that mutations tend to accumulate during the natural course of the disease as a result of a progressively increasing genetic instability and are therefore a feature of CML clinical deterioration and not necessarily a phenomenon observed only against a background of imatinib exposure. This would fit with the recent observation that KD mutations may be detected in a substantial fraction of imatinib-naïve patients with advanced phase CML.[53]

Even though the codons affected by mutations were seventeen and the relative frequencies of each single amino acid substitution were consequently low, we could observe that P-loop and T315I mutations were more recurrently found in advanced stage CML and Ph+ ALL patients (Figure 2). Even more importantly, in most cases there seemed to be a close association between the emergence of these mutant clones and progression of patients from CP to AP or BC. The P-loop (amino acids 248 through 256) is a highly conserved region responsible for ATP phosphate binding.[31,122] Amino acid 315 is the so-called 'gatekeeper' residue – the hydroxyl group of threonine 315 forms a hydrogen bond with imatinib, and the side chain also sterically controls the binding of the inhibitor to the ATP-binding site. Substitution of threonine with a bulkier and more hydrophobic isoleucine abolishes the hydrogen bond and determines a steric clash that renders the active site inaccessible not only to imatinib but also to most second-generation inhibitors.[123] Among several mutants, the G250E, Q252H, Y253F/H, E255K/V, and T315I are the ones which displayed the highest IC_{50} values in

biochemical and cellular assays.[51] But the virtually complete insensitivity to imatinib conferred by such mutations is likely not to be the only explanation for such a particularly aggressive leukemic phenotype. It has actually been hypothesized that the ones above may be gain-of-function mutants, characterized by a greater transforming potency with respect to other mutant forms or to wild-type Bcr-Abl, at least under the selective pressure of imatinib.[47,124]

While it is rather well established that mutations are the main cause of resistance in relapsing patients, few and contrasting data are currently available about the incidence of mutations in patients with primary resistance to imatinib.[44,49,125] Some authors even hypothesized that resistance mechanisms other than Abl KD mutations may underlie lack of response to imatinib. To the best of our knowledge, our is the first study investigating the contribution of Abl KD mutations to primary resistance in a large series of patients. We show here that mutations can be found also in patients with primary resistance, even though at a much lower frequency, and that there is no difference between primary and acquired resistance in terms of the identity of amino acid substitutions responsible for. But some 70% of patients with primary resistance do not have evidence of KD mutations. Again, since the contribution of KD mutations to resistance in this setting of patients is modest, additional work is needed to find out whether one or more yet unidentified mechanism(s) exist.

According to recently published guidelines on CML management,[118] mutation analysis of patients treated with imatinib is suggested in case there is evidence of inadequate response or any sign of loss of response. The knowledge of whether a KD mutation is present, as well as of the type of mutation, may contribute to a timely and rational therapeutic management, especially now that the armamentarium against CML and Ph+ ALL is about to include second-generation inhibitors like dasatinib (BMS-354825)[72] and nilotinib (AMN-107).[73] For those patients harbouring mutations which are known to confer only moderate resistance to imatinib, dose-escalation may be beneficial. For those who have evidence of mutations conferring total insensitivity to imatinib, allogeneic transplant if feasible or alternative inhibitors have to be considered. Our data support the notions that a) mutation analysis should be performed both in case of imatinib failure and in case of loss of hematologic or cytogenetic response; b) the subsets of advanced phase CML and Ph+ ALL patients are to be considered a high-risk

group; c) the occurrence of P-loop, or T315I mutations in patients treated with imatinib should trigger a rational reconsideration of the therapeutic strategy.

Tables

	All	Primary resistance (%)	Acquired resistance (%)
Total no.	297	152 (51)	145 (49)
CP	198	116 (59)	82 (41)
- IM frontline	44	28 (64)	16 (36)
- IM after IFN-α failure	154	107 (69)	47 (31)
AP	21	5 (24)	16 (76)
myBC	32	5 (16)	27 (84)
lyBC/Ph+ ALL	46	7 (15)	39 (85)

Table 1 – Classification of the patient population under study.

	All	Mutated (%)
Total no.	297	127 (43)
CP	198	54 (27)
- IM frontline	44	6 (14)
- IM after IFN-α failure	154	48 (31)
AP	21	11 (52)
myBC	32	24 (75)
lyBC/Ph+ ALL	46	38 (83)

Table 2 – Frequency of mutations according to disease phase.

	All	Mutated (%)
Total no.	297	127 (43)
Primary resistance	152	45 (30)
- hematologic	18	8 (44)
- cytogenetic	134	37 (28)
Acquired resistance	145	82 (57)
- loss of CCgR	52	12 (23)
- loss of HR	33	18 (55)
- progression to AP/BC	60	52 (87)

Table 3 – Frequency of mutations according to type of resistance.

Figures

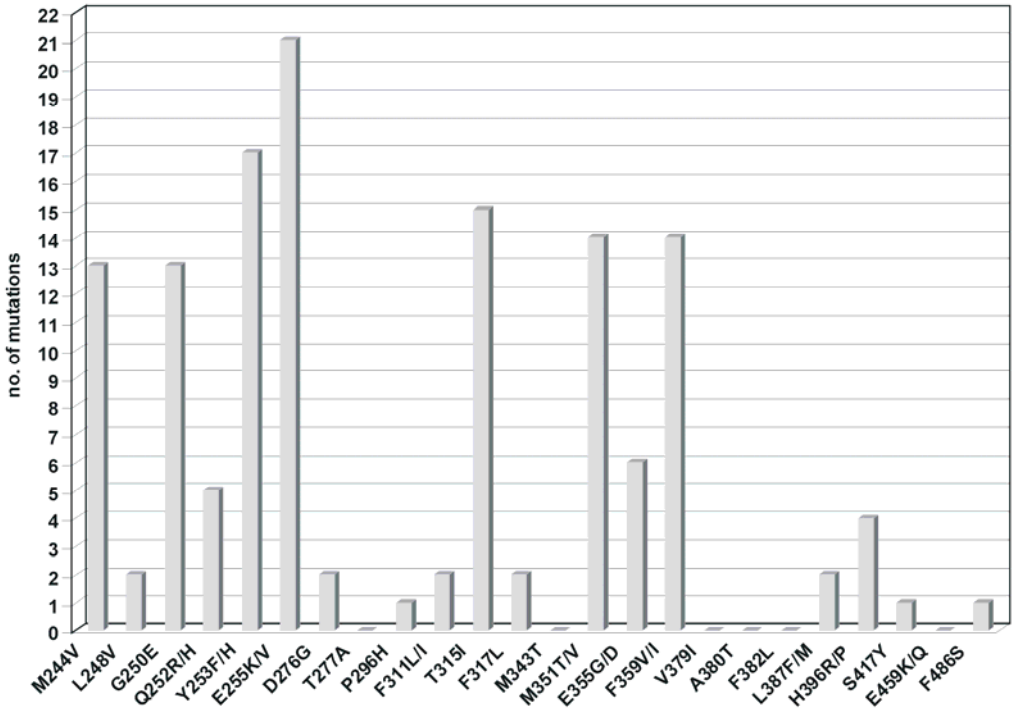


Figure 1 – Distribution and relative frequency of Abl KD mutations found in our study.

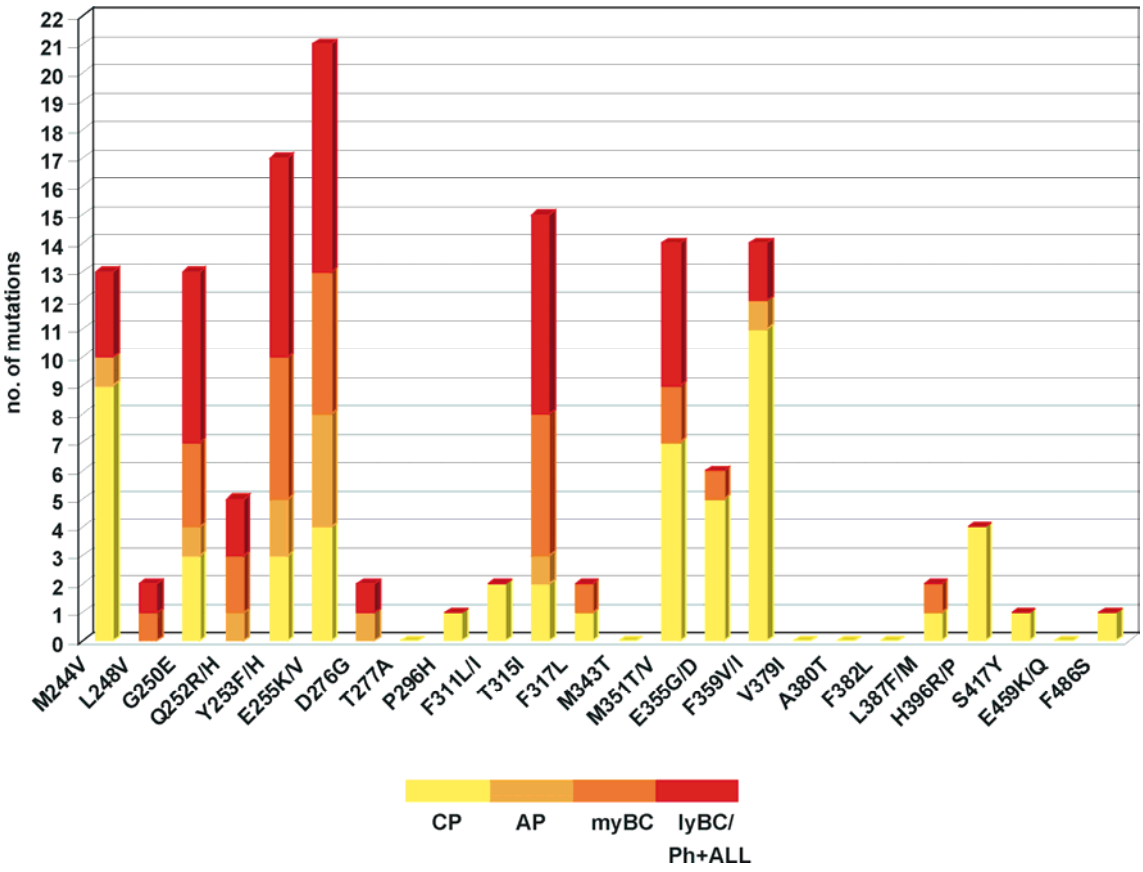


Figure 2 – Position and relative frequency of mutations according to disease phase.

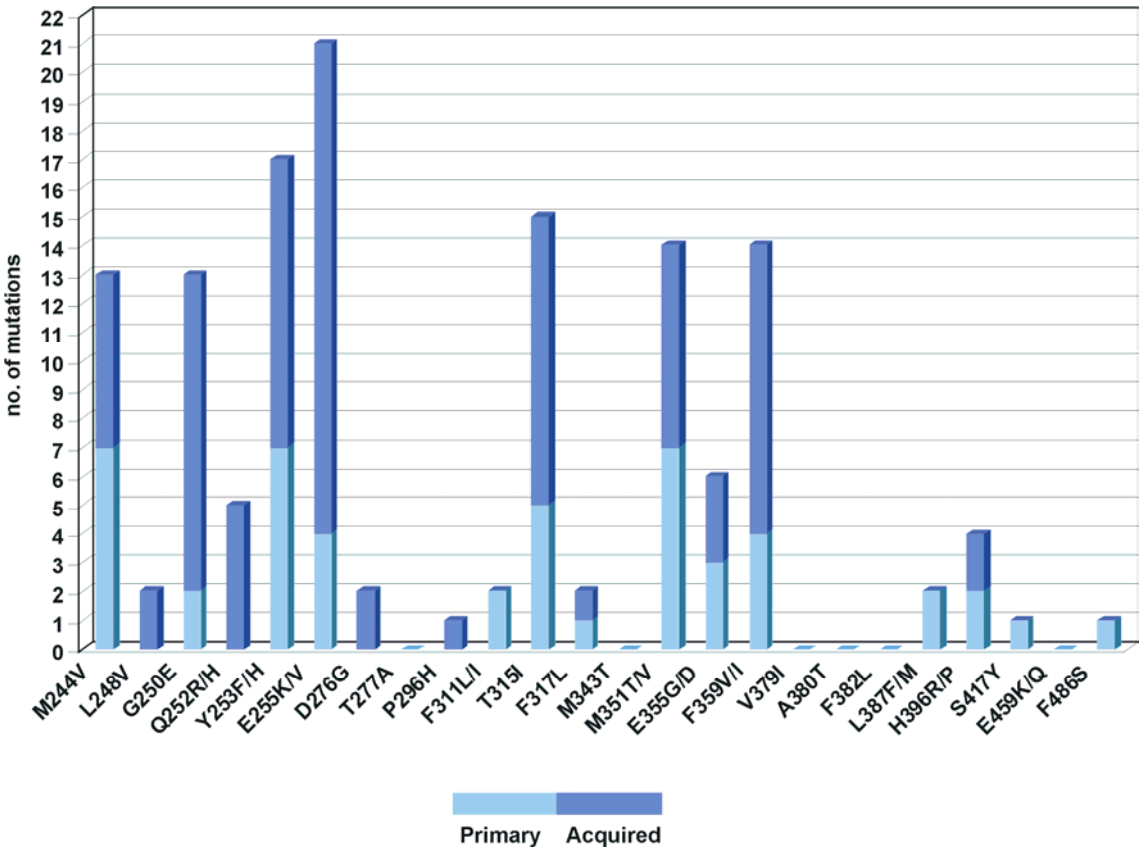


Figure 3 – Position and relative frequency of mutations according to type of resistance.

PART III:

ABL KD MUTATIONS ASSOCIATED WITH RESISTANCE TO DASATINIB IN PH+ LEUKEMIA PATIENTS

Background

The problem of resistance to imatinib in Ph+ leukemia patients has prompted the design of several second-generation Bcr-Abl inhibitors. One of these, dasatinib (BMS-354825), is now in clinical development.[72] *In vitro* assays[69,70] and crystallographic studies[71] have suggested that the less stringent conformational requirements for Bcr-Abl binding are likely to render dasatinib active against many of the KD mutants responsible for imatinib resistance. One remarkable exception appears to be the T315I, which has been shown to disrupt a hydrogen bond critical for dasatinib binding and to create a steric hindrance which interferes with the entrance of the inhibitor into the ATP-binding site.

In order to assess which pre-existent or emerging mutations are challenging for dasatinib clinical efficacy, we analyzed *BCR-ABL* KD sequences before and during treatment in Ph+ leukemia patients who failed to respond to or relapsed on dasatinib therapy.

Patients and methods

Patients and definitions. We have treated in the phase II program with dasatinib 70 mg twice daily a total of forty-five patients with CML (n=35) or Ph+ ALL (n=10) who were resistant to or intolerant of imatinib. Median age was 50 years (range, 18-74); median duration of CML was 32 months (range, 4-158); median duration of imatinib was 17 months (2-57). At the time of writing, with a median follow-up of 12 months (range, 1-19), twenty-one patients have had evidence either of primary or of acquired resistance, defined as failure to achieve any HR or loss of HR during treatment, respectively. All the patients provided written informed consent for participation in this study. Assessment of HR was done according to the criteria already described for the phase I study.[72] Assessment of cytogenetic response (CgR) was performed on 30 marrow metaphases with standard banding techniques.[126]

RNA extraction and RT-PCR. Total cellular RNA was extracted from leukocytes and reverse transcribed as previously described.

D-HPLC analysis. Mutation analysis of the *BCR-ABL* KD was performed as previously described. Briefly, after a first amplification of a fragment spanning both the *BCR-ABL* breakpoint and the *ABL* KD, three overlapping fragments covering the entire KD (amino acids 206 through 524) were generated by nested polymerase chain reaction and screened for the presence of sequence variations by D-HPLC (WAVE 3500-HT; Transgenomic, Cramlington, UK). Sensitivity of the assay ranged between 5 and 10% (data not shown). To ensure that mutations present in ≥90% of Bcr-Abl-positive cells could not escape D-HPLC detection, for all samples studied a mixture of wild-type and patient PCR products in a 1:1 ratio was also run.

Direct sequencing. In D-HPLC-positive cases, subsequent sequencing was performed on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA) to characterize the precise nucleotide substitution(s). Sensitivity of the method was 20-25% (data not shown).

Results

Abl KD mutations associated with primary resistance to dasatinib. Eight patients had primary resistance to dasatinib (patients no. 1-8 in Table 1 and Figure 1). In all these patients, a T315I or a F317L mutation was already detectable before the onset of treatment or became detectable after one month. The mutations persisted up to the time of disease progression (Figure 1), which occurred at a median of 1.5 months (range, 1-4) from dasatinib start despite the fact that in 5/8 cases (patients no. 1, 3, 4, 6 and 8; Figure 1) a dose increase to 90 or 100 mg twice a day was attempted.

ABL KD mutations associated with acquired resistance to dasatinib. Thirteen patients had acquired resistance to dasatinib (patients no. 9-21 in Table 2 and Figure 1). Relapse occurred after a median of 7 months (range, 3-15) from dasatinib start. Mutation analysis performed before the onset of treatment showed that five of these patients had a wild-type *BCR-ABL* sequence, while the remaining eight patients had evidence of various imatinib-resistant KD mutations (G250E, Y253H, E255K, D276G, M351T, L387M; Table 2 and Figure 1). At the time of relapse, however, most of the original mutant clones had disappeared whereas mutations either at codon 315 or at codon 317 had invariably emerged in 20/21 patients (Table 2 and Figure 1). Interestingly, in two of these cases relapse was associated with the selection of novel amino acid substitutions – a threonine to alanine change at codon 315 (T315A) and a phenylalanine to isoleucine change at codon 317 (F317I). In the remaining patient (no. 20 in Table 2 and Figure 1) a previously unreported K356R mutation was detected.

Discussion

These data complement *in vitro* observations[69,70] as well as structural studies,[71] confirming that the T315I mutation is highly resistant to dasatinib. In imatinib-resistant/intolerant CML or Ph+ ALL patients, the T315I accounted for dasatinib treatment failure in 13 of 21 cases. All the five patients (no. 2, 3, 5, 6 and 7 in Table 1 and Figure 1) who had already evidence of a T315I before the onset of dasatinib – at least by direct sequencing – failed to achieve any response. In the remaining eight patients, the T315I outgrew during treatment. In patients no. 1 and 8 it became detectable after only one month of therapy and no HR could be observed. In patients no. 9, 11, 12, 13, 14 and 16, the T315I emerged after the patient had achieved a HR and in most cases even a CgR, and invariably preceded or accompanied relapse. Interestingly, an additional patient (no. 17) was found to harbour a variant amino acid substitution at codon 315 (T315A) at the time of relapse. The T315A has never been reported in patients, but has recently been identified in an *in vitro* saturation mutagenesis screening for Bcr-Abl mutants conferring resistance to dasatinib.[127] Ba/F3 cells expressing the T315A-Bcr-Abl were shown to have a 90-fold higher IC_{50} with respect to those expressing wild-type Bcr-Abl when incubated with dasatinib.[127] Of note, the increase in imatinib IC_{50} for this mutant was only 2.4-fold, suggesting that in such a case resuming imatinib alone or in combination might prove effective. In our patient, rechallenging with imatinib at the dose of 800 mg/d was actually attempted after dasatinib discontinuation and resulted in a reduction of leukocytosis and a decrease of the proportion of T315A- and F317L-positive cells, but at the same time promoted the rapid selection of an additional G250E mutation. For this reason, and because of disease persistence, imatinib was withdrawn after seven weeks. The patient is now being treated with nilotinib with normalization of blood cell count, but the T315A and the G250E were still detectable after two months of treatment.

In six of 21 patients, lack of response or relapse were strikingly associated with the presence or the selection of a mutation at codon 317 (F317L in five cases, F317I in one case). Co-crystal studies have demonstrated that the aromatic ring in the side chain of phenylalanine 317 directly interacts with the pyrimidine and thiazole rings of dasatinib.[71] Accordingly, several amino acid substitutions affecting residue 317 were observed in the *in vitro* saturation mutagenesis screening for dasatinib-resistant mutants,

including both the imatinib-resistant F317L and presently unreported variants like F317V, and F317S, and the F317I found in our patient.[127] The F317L has been shown to induce a 9 to 13.5-fold increase of dasatinib IC_{50} in cellular assays.[69,127,128] In two patients, a dose increase from 70 to 90 or 100 mg twice daily was attempted but this did not succeed in eliminating the F317L/I-positive mutant clone.

Finally, in one patient of our series (no. 20) a K356R mutation became detectable by direct sequencing at the time of relapse. This mutation has never been reported, either *in vitro* or *in vivo*, in association with imatinib or dasatinib resistance. It is unclear whether in this patient the emergence of the K356R was the actual determinant of resistance, or some other mechanism might rather have intervened and ultimately determined the expansion of the Bcr-Abl-positive cells harbouring the mutation.

In some cases (patients no. 10, 11, 14, 16 and 17), pre-existing dasatinib-sensitive mutant clones (i.e., E255K, Y253H, D276G) were selectively eliminated, but soon replaced by a newly-emerged dasatinib-resistant T315I- or F317L-positive clone. In other cases (patients no. 8, 13, 18 and 21), the T315I- or F317L-positive cells were selected by dasatinib treatment as a subclone of the original, imatinib-resistant mutated clone, since both the original and the newly outgrown mutations were detected on the same allele (data not shown). Given that this was invariably observed in advanced-CML and Ph+ ALL cases, it can be hypothesized that in this clinical setting the high rate of genomic instability may foster the development over time of multiple mutations within the same or in different Bcr-Abl-positive sub-clones, which will then be selected or de-selected depending on the spectrum of sensitivity and resistance to the inhibitor employed.

At the time of relapse, resistance-associated mutations were clearly detected by direct sequencing since they always accounted for at least 50% of the Bcr-Abl-positive cells (Figure 1). However, the more sensitive D-HPLC analysis[116,129] could in some cases (patients 9, 11, 19, 20 and 21) detect the emergence of the mutations one to three months earlier than sequencing, thus proving a particularly valuable tool for monitoring imatinib-resistant patients treated with second-generation inhibitors.

The T315I is usually observed in approximately 15% of Ph+ leukemia patients who are resistant to imatinib,[130-132] but is likely to become the prevalent mutation in those who fail second-line treatment with dasatinib or other novel inhibitors for whom threonine

315 is a critical binding residue.[87] At present, there is only one compound (MK-0457, also known as VX-680) in clinical development in the field of leukemias that has been documented to be effective in CML and Ph+ ALL patients harbouring the T315I mutation,[96,97] since it has been shown not to require interaction with threonine 315 for efficient binding and inhibition in recent co-crystal studies.[95] Observation of a larger series of patients is required to assess whether the F317L will turn out to be another problematic mutant in dasatinib-treated patients.

Tables

Pt no.	Sex	Age	Disease phase or type	Reason for imatinib discontinuation	Mutation status before dasatinib start	Best HR on dasatinib	Best CgR on dasatinib	Months on dasatinib	Mutation status at progression
Primary resistance									
1	M	48	CML/AP	resistance	WT	NR	NR	4	**T315I**
2	F	61	CML/AP	resistance	T315I	NR	N.E.	1	**T315I**
3	F	33	CML/myBC	resistance	T315I	NR	N.E.	1	**T315I**
4	M	62	CML/myBC	resistance	F317L	NR	NR	3	**F317L**
5	F	38	CML/lyBC	resistance	T315I	NR	N.E.	1	**T315I**
6	F	21	Ph+ ALL	resistance	T315I, M351T, L387M	NR	N.E.	2	**T315I**, M351T, L387M
7	M	40	Ph+ ALL	resistance	T315I	NR	N.E.	1	**T315I**
8	M	37	Ph+ ALL	resistance	F359V	NR	N.E.	2	F359V, **T315I**

Table 1 – Patients with primary resistance to dasatinib: characteristics and response to dasatinib according to mutational status. Definitions of hematologic and cytogenetic response as reported in [72]. Abbreviations: Pt, patient; M, male; F, female; AP, accelerated phase; myBC, myeloid blast crisis; lyBC, lymphoid blast crisis; WT, wild-type; NR, no response; N.E., not evaluated.

Pt no.	Sex	Age	Disease phase or type	Reason for imatinib discontinuation	Mutation status before dasatinib start	Best HR on dasatinib	Best CgR on dasatinib	Months on dasatinib	Mutation status at progression
Acquired resistance									
9	M	35	CP	intolerance	WT	CHR	mCgR	15	**T315I**
10	F	60	CML/myBC	intolerance	G250E	NEL	NR	8	**F317L**
11	M	25	CML/lyBC	resistance	Y253H	CHR	CCgR	9	**T315I**
12	M	27	CML/lyBC	resistance	WT	CHR	CCgR	4	**T315I**
13	F	26	CML/lyBC	resistance	E255K	CHR	NR	3	E255K, **T315I**
14	F	60	CML/lyBC	intolerance	D276G	CHR	CCgR	7	**T315I**
15	M	37	CML/lyBC	resistance	WT	CHR	PCgR	9	**F317L**
16	M	41	Ph+ ALL	resistance	E255K	CHR	N.E.	4	**T315I**
17	M	18	Ph+ ALL	resistance	Y253H	CHR	CCgR	13	**T315A**, **F317L**
18	M	64	Ph+ ALL	resistance	M351T	CHR	CCgR	13	M351T, **F317L**
19	M	73	Ph+ ALL	intolerance	WT	CHR	CCgR	6	**F317I**
20	F	55	Ph+ ALL	intolerance	WT	CHR	CCgR	4	K356R
21	F	63	Ph+ ALL	resistance	L387M	CHR	CCgR	5	**F317L**, L387M

Table 2 – Patients with acquired resistance to dasatinib: characteristics and response to dasatinib according to mutational status. Definitions of hematologic and cytogenetic response as reported in [72]. Abbreviations: Pt, patient; M, male; F, female; CP, chronic phase; myBC, myeloid blast crisis; lyBC, lymphoid blast crisis; WT, wild-type; CHR, complete hematologic response; NEL, no evidence of leukemia; mCgR, minor cytogenetic response; NR, no response; CCgR, complete cytogenetic response; PCgR, partial cytogenetic response; N.E., not evaluated.

Figures

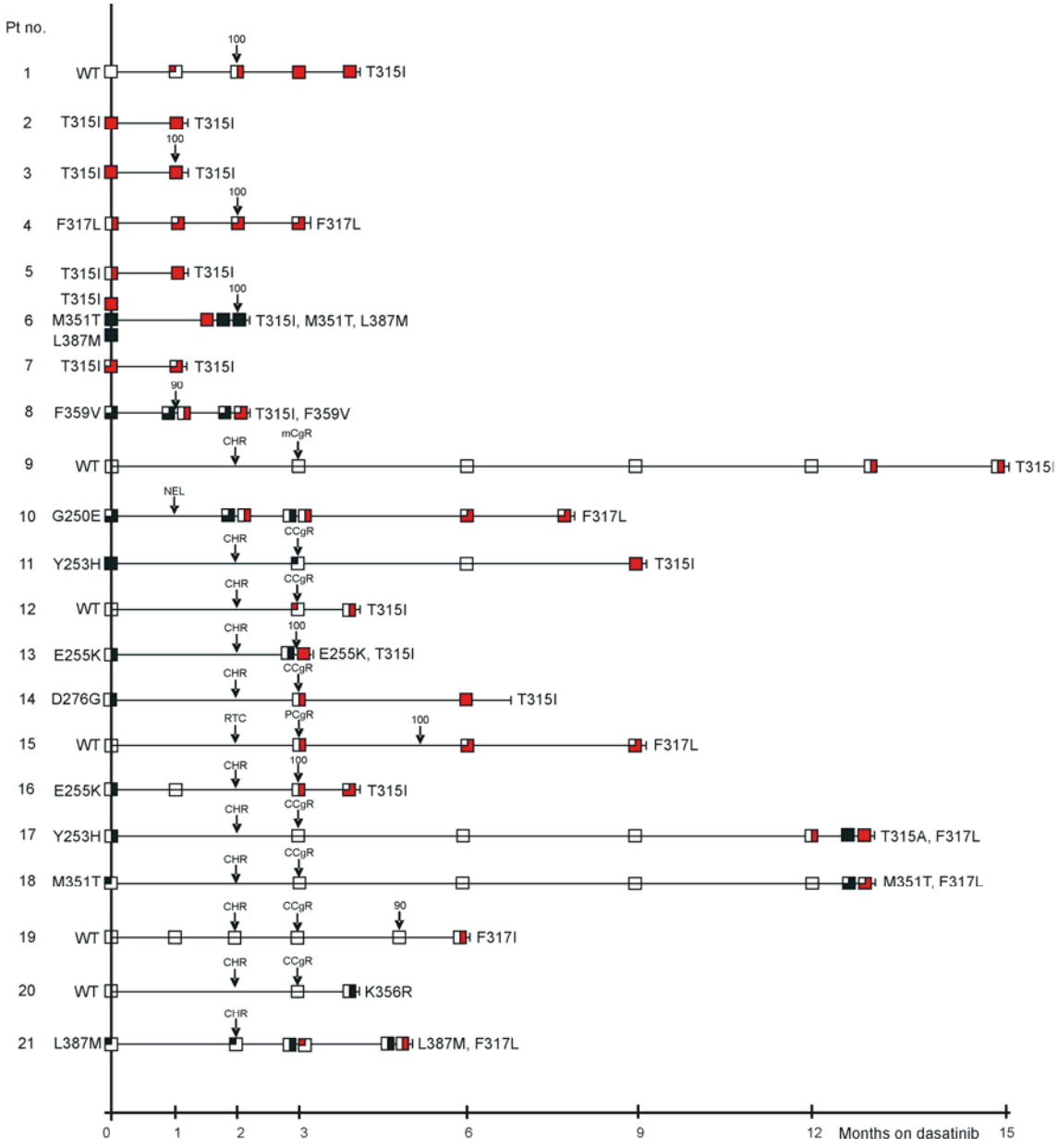


Figure 1 – Mutation monitoring and follow-up of patients. Open squares represent wild-type *ABL*; full squares represent mutated *ABL* as detected by direct sequencing. Mutations at codons 315 and 317 are highlighted in red. The degree of shading indicates the relative proportion of mutant with respect to wild-type, as estimated by relative peak heights in sequence chromatograms. Dasatinib dose increase to 90 or 100 mg twice daily was attempted in nine cases, as indicated. The time the best hematologic and cytogenetic responses were achieved is indicated by an arrow. Abbreviations as in Tables 1 and 2.

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