Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE BIOMEDICHE

Ciclo XXVII

Settore Concorsuale di afferenza: 06/3D

Settore Scientifico disciplinare: MED/15

NEXT-GENERATION SEQUENCING-BASED MUTATION SCANNING STRATEGY OF THE BCR-ABL KINASE DOMAIN IN PATIENTS WITH PHILADELPHIA-CHROMOSOME POSITIVE LEUKEMIAS TREATED WITH TYROSINE KINASE INHIBITORS

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Esame finale anno 2013/ 2014

SET UP AND VALIDATION OF AN ULTRA-DEEP, NEXT GENERATION SEQUENCING-BASED MUTATION SCANNING STRATEGY OF THE BCR-ABL KINASE DOMAIN IN PATIENTS WITH PHILADELPHIA-CHROMOSOME POSITIVE LEUKEMIAS TREATED WITH TYROSINE KINASE INHIBITORS.

INTRODUCTION

The Philadelphia chromosome

Chronic myeloid leukemia (CML) is a rare, clonal myeloproliferative disorder characterized by enhanced proliferative capacity, prolonged survival of hematopoietic stem cells and reduced apoptosis. CML has relatively low incidence rates, from 0.6 to 2.0 cases per 100 000 persons and is most common in older people, with a median age at diagnosis of around 65 years.[1] Men are affected more frequently than are women, but women seem to have a survival advantage over men.[2] Its incidence does not appear to differ between Western and East Asian countries. Diagnosis of CML is generally straightforward and in the most cases it can be made on the basis of characteristic blood count and differential (excessive granulocytosis wich typical left shift of granulopoiesis). On sedimentation, the white blood cells form a whitish buffy coat from which the name leukemia is derived. The disease usually arise with an initial chronic phase (CP) which, if not adequately treated, advances through an accelerated phase (AP) to a finally blast crisis (BC). The initial "chronic phase", with a median duration of 3-5 years, is characterized by a massive expansion and increase of the myeloid lineage, but these cells mature and function normally. Leukocytosis of more than 100 000/µL with continuous left shift leading to myeloblasts or promyelocytes and splenomegaly are characteristic of the chronic phase. Over time, the acquisition of additional genetic and/or epigenetic abnormalities causes the progression of disease from an intermediated stage, termed "accelerated phase", to a final phase known as "blast crisis" that matches the clinical picture of an acute leukemia. The blast crisis is characterized by a block of cell differentiation that results in the presence of 30% or more myeloid or lymphoid blast cells in peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blast cells. Although it is clear that additional genetic abnormalities are responsible for blast crisis, the specific genetic lesions that cause disease progression are poorly characterized. The study of CML affords an unique opportunity to understand the process of cancer development because this was the first human cancer in which a consistent genetic abnormality was demonstrated to cause the disease. In the 1854 CML was first described as a distinct clinical entity by the pathologists Bennet, Craigie, and Virchow. The hallmark genetic abnormality of CML was provided in the 1960 by Nowell & Hungerford that described for the first time the "Philadelphia chromosome" (Ph), a

minute acrocentric chromosome in the bone marrow cells of patients with CML.[3] In the 1973, the Ph chromosome was recognized by Rowley and colleagues to result from the t(9;22)(q34;q11) reciprocal chromosomal translocation. [4] (Figure 1) In the 80' years it became apparent that the t(9;22) reciprocal translocation resulted in the juxtaposition of the c-abl proto-oncogene (ABL1), 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.[5] The advances in chromosome mapping and molecular biology enabled the discovery of a Philadelphia chromosome also in patients with B-cell acute lymphoblastic leukemia (B-ALL): a smaller 7.0 kb mRNA, as opposed to a CML Ph chromosome 8.5 kb mRNA product, was formed.[6-8] The difference in the Ph chromosome gene product in B-ALL versus CML were due to differences within the BCR breakpoints. [9]

The Bcr-Abl fusion protein

The BCR–ABL fusion results in the production of a constitutively active cytoplasmic tyrosine kinase protein that enhances the proliferative advantage and/or aberrant differentiation capacity over its normal counterparts, giving rise to the expansion of myeloid compartment.[10] The Bcr-Abl oncogene is essential in the development of over 95% of CML and 20% ALL patients. [11,12]. Several groups have reported that BCR-ABL chimeric proteins could induced a CML like disease in mice transplanted with bone marrow infected with a BCR-ABL retrovirus. [13,14] In addition Bcr-Abl is able to transform immature hematopoietic cells, some fibroblast cells lines and hematopoietic cell lines rendering them growth factor independent.[15,16] The ABL gene is the human homologue of the v-abl oncogene carried by the Abelson murine leukemia virus (A-MuLV), and encodes a non-receptor tyrosine kinase. Human Abl is expressed ubiquitously in cells of various tissues and the protein may be found both at the cytoplasmic and nuclear level. This non-receptor tyrosine kinase has been shown to function as a transducer of a variety of cell extrinsic and intrinsic signals including those from growth factors, cell adhesion, inflammatory cytokines, oxidative stress, and DNA damage.[17-19] Studies in transgenic mice have led to the discovery that an homozygous deletion of the ABL gene and the absence of the c-Abl protein is incompatible with the normal development of the animal. Activated Abl tyrosine kinase regulates cytoskeletal function, cell cycle progression, myogenic differentiation and cell death. This diverse array of biological activities is dictated by several modular functional domains, which determine the subcellular localization and the interaction partners of Abl. The N-terminal region of Abl resembles the Src-family of tyrosine

kinases in that it contains the Src-homology SH3, SH2 and tyrosine kinase domains. The SH2 and SH3 domains play key roles in mediating auto-inhibition and allow the interaction with other proteins.[20] The C-terminal region of Abl, not found in Src-family members, contains three nuclear localization signals (NLS), one nuclear export signal (NES), binding sites for G-actin and F-actin as well as binding site for double stranded A/T-rich DNA. The Bcr protein is a 160-kd phosphor-protein, with a novel serine/threonine kinase activity that, like Abl, is ubiquitously expressed. [21,22]. Several structural motifs can be delineated; BCR contains a coiled-coil (CC) oligomerization domain, a serine/threonine (S/T) kinase domain, a Dbl/CDC24 guanine-nucleotide exchange factor homology (DH) domain and a pleckstrin homology (PH) domain, a putative calcium-dependent lipid binding site (CaLB) and a RAC guanosine triphosphatase-activating protein (RAC-GAP) domain. BCR also contains binding sites for growth factor receptor-bound protein 2 (GRB2) at tyrosine 177 (Y177), as well as for the GRB10, 14-3-3 and the ABL proteins, through its SH2 domain.[23,24] Although these data argue for a role of Bcr in signal transduction, his physiological function is not well understood and their true biologic relevance remains to be determined. The breakpoints within the ABL gene at 9q34 can occur anywhere, over a large area, at its 5^{I} end, either upstream of the first alternative exon Ib, downstream of the second alternative exon Ia, or, more frequently, between the two. Regardless of the exact location of the breakpoint, splicing of the primary hybrid transcript yields an mRNA molecule in which BCR sequences are fused to ABL exon a2. In contrast to ABL, the breakpoints within the BCR gene on chromosome 22 are found within three regions defined breakpoint cluster regions (bcr). In 95% of patients with CML and approximately one third of patients with ALL, the BCR gene is truncated within a 5.8-kb region known as the major breakpoint cluster region (M-bcr). This region contains five exons, originally named b1 to b5, but now referred to as e12 to e16, according to their true positions in the gene. Most breakpoints form within introns immediately downstream of exon 13 (b2) or exon 14 (b3). Because processing of BCR-ABL mRNA results in the joining of BCR exons to ABL exon a2, hybrid transcripts are produced that have an e13a2 (b2a2) or an e14a2 (b3a2) junction. In both cases, the mRNA consists of an 8.5-kb sequence that encodes a 210-kd fusion protein, $p210^{Bcr\text{-}Abl}$.[25] In two-thirds of patients with Ph-positive ALL and in rare cases of CML the breakpoint in BCR are further upstream in the 54.4 kb region between the alternative BCR exon $e2^{I}$ and e2, termed the minor breakpoint cluster region (m-bcr). The resultant e1a2 mRNA is translated into a 190-kd protein $p190^{Bcr\text{-}Abl}$. Recently a third breakpoint cluster region within the BCR gene was named micro breakpoint cluster region (μ-bcr). In this case, the breaks occur within a 3^{I} segment of the BCR gene between exons e19 and

e20 giving rise to a 230-kd fusion protein $p230^{Bcr-Abl}$ (19a2 BCR-ABL) associated with the rare Ph-positive chronic neutrophilic leukemia.[26] Although all 3 major Bcr-Abl fusion proteins induce a CML-like syndrome in mice, they differ in their ability to induce lymphoid leukemia, and, in contrast to $p190^{Bcr-Abl}$ and $p210^{Bcr-Abl}$, transformation to growth factor independence by $p230^{Bcr-Abl}$ is still incomplete. [27,28] (Figure 2) The malignant transformation by Bcr-Abl involves several key structures of the chimeric protein: the SH1-SH2 domains and the actin-binding domains in ABL and a coiled-coil motif contained amino-acids 1-63, the tyrosine at position 177 and phosphor-serine-threonine-rich sequences between amino acids 192-242 and 298-413 in BCR. Several mechanisms have been implicated in the malignant transformation by Bcr-Abl, namely a) altered adhesion to stroma cells and extracellular matrix, b) constitutively active mitogenic signaling c) reduced apoptosis and d) degradation of inhibitory proteins. Schematic representation of malignant Bcr-Abl transformation were represented in Figure 3.

a) Altered adhesion properties: CML progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix. Recent data suggest an important role for β-integrins in the interaction between stroma and progenitor cells; CML cells express an adhesion-inhibitory variant of β1-integrin that is not found in normal progenitors. [29] 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 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 focal adhesion proteins such as paxillin and the focal adhesion kinase Fak, p130Cas.[30,31]

b) Activation of mitogenic signaling: multiple signals initiated by Bcr-Abl have proliferative and anti-apoptotic effects. *Stat pathway:* The constitutive phosphorylation of Stat transcription factors (Stat1 and Stat5) has since been reported in several Bcr-Abl-positive cell lines and in primary CML cells. [32,33] Stat5 activation appears to contribute to malignant transformation and its effect in BCR-ABL–transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of Bcl-xL. [34] In contrast to the activation of the Jak-Stat pathway by physiologic stimuli, Bcr-Abl may directly activate Stat1 and Stat5 without prior phosphorylation of Jak proteins. RAS and the MAP kinase pathways: Several

links between Bcr-Abl and Ras have been defined. Auto-phosphorylation of tyrosine 177 provides a docking site for the adapter molecule Grb-2 that, after binding to the Sos protein, stabilizes Ras in its active GTP-bound form. Two other adapter molecules, Shc and Crkl, can also activate Ras. Both are substrates of Bcr-Abl and bind Bcr-Abl through their SH2 or SH3 domains. [35,36] There is still dispute as to which mitogen-activated protein (MAP) kinase pathway is downstream of Ras in Ph-positive cells. Stimulation of cytokine receptors such as IL-3 leads to the activation of Ras and the subsequent recruitment of the serine–threonine kinase Raf to the cell membrane. Raf initiates a signaling cascade through the serine–threonine kinases Mek1/Mek2 and Erk, which ultimately leads to the activation of gene transcription.[37,38] Moreover, activation of the Jnk/Sapk pathway by Bcr-Abl has been demonstrated and is required for malignant transformation; thus, signaling from Ras may be relayed through the GTP–GDP exchange factor Rac to Gckr (germinal center kinase related) and further down to Jnk/Sapk.[39-41] There is also some evidence that p38, the third pillar of the MAP kinase pathway, is also activated in Bcr-Abl–transformed cells, and there are other pathways with mitogenic potential. PI3 kinase pathway: PI3 kinase activity is required for the proliferation of Bcr-Abl -positive cells.[42] Bcr-Abl forms multimeric complexes with PI3 kinase, Cbl, and the adapter molecules Crk and Crkl, in which PI3 kinase is activated. The next relevant substrate in this cascade appears to be the serine–threonine kinase that had previously been implicated in antiapoptotic signaling cascade; 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.[43] When phosphorylated Bad became 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 PI3 kinase-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. [44] 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. Myc pathway: Overexpression of Myc has been demonstrated in many human malignancies. It is thought to act as a transcription factor, though its target genes are largely unknown. Activation of Myc by Bcr-Abl is dependent on the SH2 domain, and the overexpression of Myc partially rescues transformation-defective SH2 deletion mutants whereas the overexpression of a dominant negative mutant suppresses transformation. The pathway linking Myc to the SH2 domain of

Bcr-Abl is still unknown. However, results obtained in v-abl–transformed cells suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinases (cdks), and E2F transcription factors that ultimately activate the MYC promoter.[45] Similar results were reported for BCR-ABL–transformed murine myeloid cells.[46] How these findings relate to human Ph-positive cells is unknown. It seems likely that the effects of Myc in Ph-positive cells are probably not different from those in other tumors and depending on the cellular context, Myc may constitute a proliferative or an apoptotic signal.

c) Inhibition of apoptosis: Several studies showed that BCR-ABL–positive cell lines are resistant to apoptosis induced by DNA damage.[47,48] The underlying biologic mechanisms are still not well understood but seems to be that Bcr-Abl may block the release of cytochrome C from the mitochondria and thus the activation of caspases.[49,50] This effect upstream of caspase activation might be mediated by the Bcl-2 family of proteins. Another link between BCR-ABL and the inhibition of apoptosis might be the phosphorylation of the pro-apoptotic protein Bad. In addition to Akt, Raf-1, immediately downstream of Ras, phosphorylates Bad on 2 serine residues.[51,52] Two recent studies provided evidence that the survival signal provided by Bcr-Abl is at least partially mediated by Bad and requires targeting of Raf-1 to the mitochondria.[53,54] It is also possible that Bcr-Abl inhibits apoptosis by down-regulating interferon consensus sequence binding protein (ICSBP). It becomes clear that the multiple signals initiated by Bcr-Abl have proliferative and anti-apoptotic qualities that are frequently difficult to separate. Thus, Bcr-Abl may shift the balance toward the inhibition of apoptosis while simultaneously providing a proliferative stimulus. This is in line with the concept that a proliferative signal leads to apoptosis unless it is counterbalanced by an anti-apoptotic signal, and Bcr-Abl fulfills both requirements at the same time.[55]

d) Degradation of inhibitory proteins: The recent discovery that Bcr-Abl induces the proteasome-mediated degradation of Abi-1 and Abi-2, two proteins with inhibitory function, may be the first indication of yet another way by which Bcr-Abl induces cellular transformation. [56] Most convincingly, the degradation of Abi-1 and Abi-2 is specific for Ph+ ALL whereas it is not seen in Ph-negative samples of comparable phenotype. The overall significance of this observation remains to be seen, and one must bear in mind that the data refer to acute leukemias and not to chronic phase CML. It is nevertheless tempting to speculate that other proteins, whose level of expression is regulated through the proteasome pathway, may also be degraded.

The pre-BCR-ABL tyrosine kinase inhibitors era

For many years CML was treated with chemioterapy agents such as Busulfan, an oral alkylating agent that was the mainstay of CML therapy. The mechanism of action of busulfan is via hydrolyzation with the release of methanesulfonate groups that produces carbonium ions that alkylate and disrupt DNA structure. Busulfan was able to control the clinical manifestation of the disease but it was rarely capable to eliminate the malignant clone; in addition this agent is characterized by high cytotoxicity. In the 1970s busulfan was largely replaced by hydroxyurea, a drug that produces rapid but transient hematologic control with fewer adverse events. In a randomized trial patients treated with hydroxyurea lived longer than those treated with busulfan.[57] The management of CML was revolutionized by introduction of allogeneic stem cell transplant (HSCT) in the 1970. Although available to only a fraction of patients with CML, this therapy has demonstrated long term disease free survival of 50–80% after 3–10 years of follow-up. Today HSCT is indicated only in a minority of patients who do not achieve an adequate response to first, second or third generation agents. In CML-AP patients HSCT have a lower chance to induce an optimal response, on the other end, for patients in blast phase transplantation remains the only therapy with curative potential. The introduction of interferon alpha (IFN-a), in the 1980, started a new era of CML management. 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. 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.[58,59]

The Bcr-Abl inhibitor imatinib mesylate

The identification of the aberrant activity of the Bcr-Abl protein has led to the development of a large number of molecules targeting Bcr-Abl protein opening the way for the creation of the first example of effective molecular targeted therapy. The Bcr-Abl protein itself is the ideal target since it has a central role in CML pathogenesis and it is not expressed by normal cells. The first breakthrough in the treatment of CML has been the development of imatinib mesylate (Glivec/Gleevec; STI571; Novartis), a 2-phenyl-amino pyrimidine derivative with tyrosine kinase inhibitor activity against ABL, BCR-ABL, PDGFRA, and c-KIT. Functionally imatinib binds the ATP-binding site of Bcr-Abl in its inactive conformation thus preventing tyrosine autophosphorylation

and, in turn, phosphorylation of its substrates. [60,61] This process ultimately results in "switching-off" of the downstream signaling pathways that promote leukemogenesis. To evaluate the in vitro efficacy of imatinib various techniques were used, including the measurement of the enzymatic activity of an immunoprecipitate of the chimeric protein Bcr-Abl or, alternatively, the evaluation of the degree of the autophosphorylation or of phosphorylation of Crlk protein by Western Blot 54. In patients with Ph+ chronic or acute leukemia these techniques show that within 24-72 hours after initiation of therapy the activity of the chimeric protein Bcr-Abl is greatly reduced. Standard-dose imatinib was one of the first targeted therapies to be clinically evaluated in patients with CML. In a phase I clinical trials patients with CML-CP who had failed IFN-α were treated with imatinib in increasing does, from 25 mg/d up to 1000 mg/d. In this setting, CHR were achieved in 95% of patients who were treated with imatinib in doses of 300 mg/day or more and a MCyR were achieved in 60% of patients within a median follow-up of 18 months. At this time point, CML had not progressed to AP or BP in an estimated 89% of patients and 95% of patients were still alive. [62] At 6 years the estimated rate of freedom from progression to AP/BP was 61%, with an overall survival rate of 76%. [63] Adverse side effects from imatinib were minimal, with the most common being nausea, myalgias, edema, and diarrhea. An higher frequency of grade 3 to 4 adverse events were identified at doses of 750 mg/d or higher. Imatinib also induced responses in a significant percentage of patients with AP or BP CML, although these transient. In a phase II clinical trials, where patients with AP/BP received 600 mg/d, the CHR rates were 37% and 15%, MCyR rates were 28% and 16%, and CCyR rates were 19% and 7%, respectively. [64] Based on these studies the recommended daily dose for patients with CML-AP was set at 600 mg/d, and the recommendation of increasing the imatinib dose to 600 and 800 mg/d was rapidly extended also to patients in CP with unsatisfactory response to 400 mg/d or response loss. The utility of standard-dose imatinib as first-line therapy for CML was definitively established in the phase III International Randomized Study of Interferon and STI571 (IRIS). Patients with newly diagnosed CML were randomized to imatinib 400 mg/d or IFN-α in combination with cytarabine. After a median follow-up of 19 months, patients treated initially with imatinib had significantly higher MCyR rates (87% vs. 35%; $P < 0.001$) and CCyR rates (76% vs. 15%; $P < 0.001$). At 18 months, the estimated rate of freedom from progression to accelerated-phase or blast-crisis CML was 96% percent in the imatinib group and 91% in the combination-therapy group ($P<0.001$). Imatinib was better tolerated than combination therapy. [65] The good tolerability and the excellent efficacy of Imatinib obtained in the phase I and II clinical trials resulted in the rapid approval of Imatinib in the 2001 by the Food and

Drug Administration (FDA) and later by the European Medicines Agency (EMA). At 6-year follow-up of IRIS trial, Imatinib induced CHR in 98% of patients in chronic phase and CCyR in 87% patients. [66] Definitions of response were listed in Box1. In the setting of Ph+ ALL patients the tyrosine kinase inhibitor (TKI) imatinib has been widely studied as an addition to initial therapy (allogeneic hematopoietic stem cell transplant following induction chemotherapy). Several studies have now reported higher rates of CR with the potential for improved long-term outcomes. [67-69] The large international United Kingdom (UK) ALL XII/Eastern Cooperative Oncology Group (ECOG) 2993 trial marks the end of more than a decade of imatinib-based studies in adult Ph ALL patients and demonstrated that the addition of imatinib to therapy would improve CR rate and enhance long-term outcomes in a large series of patients. [70]

Therapeutic strategies to address imatinib-resistant disease: 2nd and 3third generation tyrosine kinase inhibitors

Nevertheless treatment with first-line imatinib is associated with high hematologic response rates, a proportion of patients may developed primary or secondary resistance to Imatinib. Over the past few years, second-generation TKIs have been rapidly assessed and introduced into clinical practice in order to circumvent resistance and to improve the outcome of resistant-CML patients. Preclinical data and some observations from single arm studies suggested that higher doses of imatinib, in the initial phase of therapy, could be more effective than standard 400 mg/d dose, and may provide a better disease control. Subsequently was demonstrated that, when compared with standard- dose imatinib, the dose of 800 mg/d was associated with achievement of significantly better rates of CCyR, MMR and CMR. Transformation-free survival in this cohort was significantly better with high-dose imatinib and similar frequency of common adverse events with that seen with standard-dose imatinib were reported.[71] 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, Nilotinib, Bosutinib and Ponatinib (Figure 4). Dasatinib (Sprycel®; BMS-354825; Bristol Myers Squibb) is a potent, second-generation, multi-target kinase inhibitor, that was initially developed as an inhibitor of the Src family of kinases such as Fyn, Yes, and Lyk. It also inhibits BCR-ABL, EphA2, platelet derived growth factor receptor, and c-Kit. [72] Actually dasatinib is approved for first line use in patients with chronic myeloid leukemia in any phase and in Philadelphia chromosome-positive acute lymphoblastic leukemia patients that are refractory or resistant to prior therapy including

imatinib[73,74] In vitro studies have shown that dasatinib is 325-fold more potent than imatinib against WT Bcr-Abl and retains activity against most of the clinically relevant imatinib-resistant Bcr-Abl mutant forms. In murine models dasatinib was shown to inhibit leukemic cell growth and to prolong the survival of mice harboring wild-type Bcr-Abl and M351T, except those carrying the T315I mutation. It has also been suggested that dasatinib significantly suppress CML colony-forming cells and long-term culture-initiating cells. [75,76] The crystal structure showed that the drug binds, unlike imatinib, the active conformation of the ABL kinase domain. [77] In a phase I clinical trial dasatinib was administered in 84 patients with CML or Ph+ ALL who were resistant or intolerant to imatinib, at doses ranging from 15 to 240 mg/d orally once or twice daily over a 4-week treatment cycle. Results showed that the response rate was dependent on the CML phase: in CP-CML patients the rates of CHR and MCyR were 92% and 45% respectively, in patients with CML-AP were 82% and 27%. 80% of CML patients with lymphoid blast crisis or Ph+ ALL had a MHR and a MCyR. Overall, 95% of patients in the chronic phase had a durable response compared with 82% of patients in the accelerated phase. Unfortunately, within 6 months of therapy, almost all patients with Ph+ ALL had relapsed.[78] Several phase II clinical trial for dasatinib in all phases of CML and Ph+ ALL have been reported [79-81] The effects of dasatinib in different stages of CML and Ph+ ALL were explored in the START study (Src/ABL Tyrosine Kinase Inhibition Activity Research Trials of dasatinib) This study included a total of 445 patients with CML in AP, myCB and lyBC, and Ph+ALL, who were resistant or intolerant to prior therapy, including imatinib. The patients were randomized into 5 arms: START-A (accelerated phase), START-B (myeloid blast crisis), START-C (chronic phase), START-L (lymphoid blast crisis), and START-R (dasatinib vs high-dose imatinib). Preliminary results of 4 of the 5 single arm phase II studies (A, B, C and L) constituted the basis for the new drug application for the indication of dasatinib in CML. [82] A phase III clinical trial explored whether dasatinib administered twice daily would be better tolerated compared to daily dosing in patients with intolerance or resistance to imatinib. The trial enrolled 670 CP-CML patients with resistance or intolerance to imatinib who were randomised to one of four treatment arms: 100 mg once daily, 50 mg twice daily, 140 mg once daily and 70 mg twice daily. The rates of MCyR response ranged from 54% to 59% and CCyR responses ranged from 41% to 45% across the four arms. The HR rate ranged from 86% to 92%. There were no significant differences in progression-free survival, time to response and duration of cytogenetic response among the four arms. The daily 100 mg dose was associated with a lower incidence of toxicities, such as pleural effusion and thrombocytopenia. Grade 3–4 myelosuppression is more frequent in patients with accelerated or

blast phase CML and constitutes the most common reason for drug interruption. [83] Based on these findings, dasatinib was approved by the FDA at 100 mg once daily in patients with chronic phase CML and at 70 mg twice daily in patients with accelerated or blast phase CML. Nilotinib (Tasigna®; AMN107, Novartis Pharma) is a phenylamino-pyrimidine derivative structurally related to Imatinib. As a Bcr-Abl kinase inhibitor is 20- to 30-fold more potent than imatinib and is highly selective for Bcr-Abl. [84] Is an highly potent inhibitor of KIT, PDGFRA/B, DDR1/2 and the small oxidoreductase NQO2. Nilotinib binds the inactive conformation of the ABL tyrosine kinase and is active against most imatinib-resistant mutations of BCR-ABL, although some P-loop mutations are only partially inhibited. Actually Nilotinib is approved for adults with CML-CP or AP that is refractory or resistant to prior therapy including imatinib. Nilotinib induces a durable cytogenetic responses in approximately 50% of patients in chronic phase when used as second-line therapy but responses in patients in advanced phase tend to be transient.[85] Efficacy of Nilotinib front-line has been compared with Imatinib in the Phase II clinical trial of GIMEMA: 73 patients received nilotinib at a dose of 400 mg twice daily and were followed up for a mean of 30 months. The result confirmed the remarkable efficacy of nilotinib, with CCyR, MMR, and CMR response rates at 24 months of 96%, 85%, and 12%, respectively. Only four patients (5%) discontinued nilotinib because of toxicity. [86] The efficacy of front-line Nilotinib at 400 mg twice daily was also tested in patients with newly diagnosed of CML; the results showed that at 24 months, 93% of patients achieved a CCyR and 79% had a MMR. [87] Treatment was well tolerated with the most frequent grade 3/4 adverse events being neutropenia and thrombocytopenia in 11% of patients. In the Phase III randomized clinical trial ENESTnd (Evaluating Nilotinib Efficacy and Safety in Clinical Trials Newly Diagnosed Philadelphia Chromosome Positive) were compared nilotinib versus imatinib in newly diagnosed CML-early CP. Imatinib at 400 mg twice daily was compared with nilotinib at 300 mg or 400 mg twice daily as first-line therapy in patients with CML-early CP. At 12 months, major molecular response rates for nilotinib (44% for the 300 mg dose and 43% for the 400 mg dose) were significantly superior to that for imatinib (22%, P , 0.001). In addition, on extended follow-up at 24 months, the survival analyses indicated nilotinib 300 mg twice daily as the optimal treatment arm. Compared with imatinib, nilotinib 300 mg twice daily resulted in superior PFS (98% versus 95.2%; P = 0.0437), and improved CCyR and MMR rates (87% versus 77%, P = 0.0018, and 71% versus 44%, P = 0.0001, respectively). In addition a significant lower rate of progression to accelerated/blastic phases was recorded in the nilotinib arms. The more frequent side effects were skin rash, myalgia, and increases in bilirubin, lipase and blood glucose on nilotinib, and fatigue, myalgia, and fluid

retention on imatinib. [88] While nilotinib is associated with an increase in corrected QT interval (QTc), the incidence of cardiac-related adverse events in nilotinib recipients in the ENESTnd study was low. Recent Phase II and III clinical trials have provided strong evidence for the efficacy and tolerability of nilotinib as first-line treatment for patients with CML, especially those in chronic phase. The faster and more profound therapeutic effects of nilotinib, when compared with imatinib, suggest the possibility of longer event-free and overall survival, as well as a higher number of cured patients. Bosutinib (Bosulif; SKI-606; Pfizer), is an anilino-quinolinecarbonitrile that, like dasatinib, belongs to the class of dual Src/Abl inihibitors; was approved by the Food and Drug Administration on September 2012 for the second-line treatment of CML patients in chronic phase, accelerated phase and blast phase. Bosutinib proved to be an active inhibitor of Bcr- Abl in several CML cell lines and transfectants, with IC50 values in the low nanomolar range, 1 to 2 logs lower than those obtained with imatinib. Bosutinib retained activity in imatinib-resistant cell lines displaying BCR-ABL gene amplification as well as in BaF3 cell expressing Y253F, E255K and D276G point mutations. [89] In patients with intolerance or resistance to imatinib or after failure of two prior TKIs Bosutinib allows to obtained response rates comparable to those observed in the same patient population treated with dasatinib or nilotinib. The ongoing Phase III study (BELA) comparing bosutinib versus imatinib in newly diagnosed CML-CP patients has failed to meet the primary end point (CCyR at 12 months), although higher rates of MMR at 12 months and faster times to MMR and CCyR were observed similar to what had been observed for dasatinib and nilotinib before. To date, very limited information on mutations retaining insensitivity to bosutinib has been made available; however the lack of activity against T315I has been evident from first in vitro studies. [90] Currently available clinical trials suggest that bosutinib is generally a safe and effective treatment option for patients with CML who have failed first-line TKIs and who do not express the T315I mutation; however tolerability may be problematic for some patients. Common adverse events associated with bosutinib include diarrhea, nausea, and vomiting. Grade 3 and 4 adverse events were reported in at least 5% of bosutinib-treated patients include elevated serum lipase and liver aminotransferases, anemia, thrombocytopenia and neutropenia. [91] Although 2nd generation TKIs have shown efficacy against many of the clinically relevant mutations for which imatinib is ineffective, neither has shown the ability to inhibit BCR-ABL with the T315I mutation. In order to overcome this issue and to further improve the inhibitory effect a third-generation inhibitor Ponatinib, a tyrosine kinase inhibitor specifically direct for patients with T315I mutations, was developed. Ponatinib (ICLUSIG®; AP24534; Ariad Pharmaceutical) an orally available pan-BCR-

ABL tyrosine kinase inhibitor was structurally designed with a carbon-carbon triple bond to accommodate the T315I mutation in the ABL kinase domain. Actually is the first tyrosine-kinase inhibitor that has shown preclinical and clinical activity against a spectrum of mutants including T315I. Ponatinib inhibits also other kinases beyond BCR/ABL such as PDGFR, FGFR, KIT, RET, and FLT3. [92,93] In 2012 ponatinib was approved by the US Food and Drug Administration for the treatment of adults with T315I-positive CML in any phase and Ph+ ALL who are resistant or intolerant to prior tyrosine kinase inhibitor therapy or for whom no other tyrosine kinase inhibitor therapy is indicated. Initial preclinical testing demonstrating that ponatinib could both decrease auto-phosphorylation of the BCR-ABL kinase as well as inhibit the proliferation of cells dependent on BCR-ABL. From these studies, it was found that ponatinib inhibits both wild-type and mutant BCR-ABL kinases with IC50 (50% inhibitory concentration of the drug) values between 0.37–2.0 Nm. Further, cell proliferation assays demonstrated inhibition with IC50 values ranging from 0.3–0.5 nM for non-mutated and up to 36 nM for mutant BCR-ABL. It was also found that a dose of 40 nM was able to completely suppress the emergence of BCR-ABL resistant mutants in vitro, although efficacy may be limited particularly in examples with the E255V point mutation (IC50 of 36 nM). The maximum tolerated or recommended daily dose of ponatinib, the safety, anti-leukemic activity, and pharmacokinetic/pharmacodynamic profile of the drug were investigated in a Phase I clinical trial involving CML and Ph +ALL patients who were resistant or had relapsed during TKIs therapy. The anti-leukemic activity of the drug was also promising; 42/43 (98%) of patients with chronic phase (CP)-CML had a CHR, 31/43 (72%) had a MCyR, 27/43 (63%) had a CCyR, and 19 patients had a MMR. Further, all 12 patients with CP-CML and the T315I mutation had a CHR, and 8/12 (67%) had a MMR. [94,95] To test the efficacy of ponatinib on any and all BCR-ABL TKI resistant clones researchers proceeded to Phase II clinical trial using the 45 mg/d dose. The Phase II clinical trial (PACE) enrolled 449 patients resistant or with unacceptable side effects to dasatinib or nilotinib, or who harbored the T315I mutation. [96] At 12 months after the initiation of ponatinib therapy, 56% of patients with CP-CML had a MCyR, 46% had CCyR, and 34% had MMR. Interestingly, higher response rates were reached for those with the T315I mutation. In patients with AP-CML and BC-CML or Ph+ ALL, rates of major hematologic response at 6 months were 39% and 31%, respectively. Serious non-hematologic and hematologic adverse drug events (ADEs) were observed. The most common non hematologic adverse events included pancreatitis, abdominal pain, and myocardial infarction (MI) whereas the most common hematologic adverse events were thrombocytopenia (in 37% of patients), neutropenia (in 19%), and anemia (in 13%). Serious-grade

arterial thrombotic events including cardiovascular, cerebrovascular, and peripheral vascular events were seen in only 8.9% of the patients who received ponatinib. At the time of the drug's approval, the label noted that 8% of patients had cardiovascular, cerebrovascular, and peripheral vascular thrombosis including fatal myocardial infarction and strokes. At a follow-up of the Phase II study (median time of 1.3 years), 24% of patients had an MI, stroke, limb ischemia, or stenosis of the vessels of the heart, extremities or brain requiring revascularization. As the increased cardiovascular risks associated with ponatinib were uncovered, it was removed from the market in October 2013. Additionally, the phase III clinical trial for the Evaluation of Ponatinib versus Imatinib in Chronic Myeloid Leukemia (EPIC) investigating the use of ponatinib versus imatinib for first-line use, was halted following this decision. The FDA and ARIAD Pharmaceuticals Inc. "mutually agreed the trial should be terminated" due to the now understood increased cardiovascular risks associated with ponatinib. (Ariad Ends Phase III Leukemia Drug Trial. Genetic Engineering and Biotechnology News. October 18, 2013). In December 2013 Ariad Pharmaceuticals is allowed to resume sales of ponatinib with expanded black box warning and to limited patient population.

The open issues: the drug-resistance in CML and Ph+ ALL patients

Despite the remarkable effectiveness of 1st and 2nd generation TKIs in different clinical trials one-third of CML patients on first-line imatinib therapy require other therapy due to imatinib intolerance or resistance. [97] The existence of patients resistant to imatinib was evident soon after the introduction of the drug in clinical practice; thus it was soon recognized that further improvement was necessary. Different studies both in vitro, using resistant cell lines generated through exposure to gradual increases of the concentration of imatinib, and in vivo, using cells from resistant-patients were performed. In the IRIS study 13% of patients receiving imatinib did not achieve a MCyR and 24% of patients did not achieve a CCyR during 19 months of follow-up. [98] Furthermore, after a median follow-up of 60 months, 6% of those originally assigned to imatinib had progressed to AP or BP, 3% had hematologic relapse and 5% had lost their MCyR. [99] Resistance has been defined using the European LeukemiaNet (ELN) criteria for failure of first-line TKI therapy and according to the National Comprehensive Cancer Network (NCCN) guidelines. Therapeutic resistance to TKIs can be classified as primary or secondary resistance according to the time of onset. Primary resistance refers to the lack of initial response most commonly due to the presence of baseline mutations in the kinase domain, on the other hand, secondary resistance refers to the

loss of a previously achieved hematological or cytogenetic responses and progression from CP to advanced-stage disease often due to the prolonged exposure to the TKI. [100] Whether it is primary or secondary, the most common and well understood mechanism of resistance against all TKIs is the emergence of point mutations within the kinase domain of Abl that is considered the main mechanism responsible for the reactivation of the kinase activity in resistant leukemia cells. [101] These mutations prevent the binding of imatinib to the Bcr-Abl in some cases by distrupting critical elements of their interaction or by making changes to the conformation of the protein. The sequencing of the ATP binding site and of the activation loop of the BCR-ABL kinase domain performed in 9 patients with CML-BC relapsed after Imatinib treatment, had shown an identical cytosine to thymidine substitution, in six of nine patients. [102] Additional studies performed in Imatinib-resistant CML patients in advanced phase, indicated that the mutation at residue 315 in the ABL KD was an important determinants of the Bcr-Abl reactivation within the leukemic clone. This mutation resulted in a single amino-acid change at position 315; the hydroxyl group of threonine 315 of Bcr-Abl, also called "gatekeeper" residue, forms a crucial hydrogen bond with imatinib; in addition the side chain present at position 315 sterically controls the binding of the inhibitor to hydrophobic regions near to the ATP-binding. The absence of hydroxyl group normally provided by the side chain of the isoleucine prevent the formation of the hydrogen bond required for high affinity inhibitor binding; furthermore the substitution of threonine with a more hydrophobic isoleucine induces a steric clash interfering with Imatinib positioning whitin the ATP-binding site. The majority of the CML patients in advanced phase (66%) and some in the chronic phase (5%) relapse after imatinib treatment, developing c-Abl dependent and independent mechanisms of resistance.[103] Approximately 30-50% of the relapsed patients acquire point mutations. In subsequent years there was an exponential increase in the number of point mutations that were found in CML patients who either lost or did not achieve response to imatinib therapy. Actually more than 90 different amino acid substitutions at more than 60 positions scattered all over the kinase domain have now been reported. (A summary of the *BCR-ABL* KD amino acid substitutions identified in clinical samples from patients reported to be resistant to the currently approved TKIs may be found in Table 1 and figure 5). These kinase domain mutations occurs at strategic key regions of the protein, such as the P-loop (or phosphate-binding loop), which faces the imatinib-binding pocket, and the activation loop, a molecular switch controlling the shift between the inactive and the active conformation of the kinase, to which imatinib is unable to bind. In some cases, mutations will occur directly in the kinase active site, sterically

hindering TKI binding, in other cases, mutations will occur at residues that affect the overall three-dimensional structure of the kinase. Because imatinib and nilotinib bind BCR-ABL in its inactive conformation, mutations may occur in resistant patients and destabilize or prevent adoption of the inactive conformation of the kinase. Failure to adopt this conformation prevents the binding and activity of imatinib and nilotinib. [104] In vitro studies, that attempted to measure the degree of sensitivity to imatinib of the most recurrent Bcr–Abl mutant forms in terms of IC50, have suggested that not all mutations could be equally challenging and to different mutations correspond different IC50 values (Figure 6) [105] 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 IC50 and might therefore be overcome by a dose increase. Controversy remains over the point in time when such mutations are acquired; 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. Bcr-ABL KD mutated clones most probably achieve dominance as a consequence of the selective pressure of TKIs, in a process known as clonal selection. In addition, withdrawal of TKI therapy has been reported to alter the dynamics of Bcr–Abl-positive clones deselecting, at least temporarily, the mutated ones· Repeated deselection and reselection of mutated clones in patients who resumed therapy with TKI after periods of cessation, which suggests that mutated clones are reduced below the lower detection limit of the method used for their detection, rather than completely eliminated. A recent study on 19 patients who failed therapy with imatinib and switched to non-TKI-based therapy upon showed consistent regression of most mutations over a period of months, although with different kinetics and to different extents. For example under non-specific chemotherapy the Y253F/H and E255K showed an almost complete regression of mutant transcripts, the M351T-mutated transcripts remained at high levels; T315I had a heterogeneous behavior, since it became undetectable in two patients, whereas persisted at 100% in three other patients. [106] Probably in the absence of TKI selective pressure it is unlikely that a small mutated clone can outgrow at the expense of the unmutated Bcr–Abl-positive population, given that the majority of them seem to have decreased phosphorylating activity and/or leukemogenicity as compared with unmutated Bcr–Abl. [107-109] Beside KD mutations several Bcr-Abl independent mechanism of resistance to TKIs were described. Early studies on the pharmacokinetics of imatinib showed that the blood-levels of the drugs is

influenced by inter-individual variations in genes coding for metabolic enzymes and specialized membrane transport systems involved in the influx-efflux of drugs in the target cell, suggesting that the bioavailability of TKIs were tightly modulated by binding to several proteins. The efflux mechanisms, that play a critical role in limiting the absorption and accumulation of the drugs, were extensively studied in the 70s. The over-expression on the cell surface of the transporter ABCB1, better known as "P-glycoprotein", encoded by the gene MDR-1, appears to be one of the possible mechanisms of resistance to imatinib described in the early 1970's. [110] In one study, investigators reported an increases of this multidrug resistance in a subclone of a resistant human leukemia cell line (Lama-84R) derived by growing the cells in increasing concentrations of imatinib; in addition was demonstrated that imatinib resistant cell lines overexpressed the P-glycoprotein (Pgp) efflux pump. [111]. Multiple reports have provided evidence that deregulation of the organic cation transporter hOCT1 can impede the influx of imatinib. Using hOCT inhibitors on different imatinib-sensitive CML cells caused a reduced uptake of imatinib. [112] This finding was further supported by clinical data showing that patients who display a minimal response to imatinib also express a significantly lower amount of hOCT. [113] [114] An additional study investigated a panel of polymorphisms in the transporter hOCT1 that can be hypothesized to influence imatinib transport and metabolism in a large series of newly diagnosed, previously untreated CML patients receiving imatinib in the framework of the TOPS phase III trial. In the overall population, the OCTN1 *C* allele (rs1050152), in the hOCT1 gene, had a significantly favorable impact on achievement of MMR and a weak effect on CMR (P=0.03 and P=0.07, respectively). [115] Another method by which tumors bypass the inhibitory effects of TKI is by the sequestration of such drugs by plasma proteins, such as the plasma protein-1 acid glycoprotein (AGP). The 89-96% of imatinib bind mainly to AGP (alpha-1-acid-glycoprotein) a plasma protein which has the ability to inhibit and to sequester Imatinib in the plasma thus making it biologically inactive and this binding decreases imatinib's ability to inhibit c-ABL in a dose-dependent manner. [116]A recent study had shown that low plasma concentrations of the drug significantly differ from patient to patient and this can be associated with a different rate of response to the therapy [117]; most plasma concentration of imatinib has occurred mainly in patients who achieved a CCYR and a MMR. Resistance or disease progression beyond chronic phase has long been associated with clonal evolution, that represent the acquisition of additional chromosomal abnormalities (ACAs) in the Ph-chromosome-positive cell population. [118,119] Approximately 10%-12% of patients with CML-CP have additional chromosomal findings at diagnosis whereas the percentage of proportion of patients with ACAs rises during the

course of the disease to approximately 80% in CML-BC. The most frequent aberrations detected in advanced CML were trisomy 8, a second Ph-chromosome and a partial trisomy of the long arm with partial monosomy of the short arm of chromosome 17 [isochromosome (17)(q10)], which were designated "major-route of karyotypic evolution" [120] ACAs that were rarely observed in AP or BC, such as t(3;12), t(4;6), t(2;16), and t(1;21), were designated minor-route ACAs. [121] A recent study performed on from 1151 CML patients randomized to the German CML Study IV showed that the impact of additional cytogenetic findings at diagnosis correlates with a decreased response to imatinib in terms of complete cytogenetic response (CCyR), major molecular remission (MMR), progression-free (PFS) and overall survival (OS). [122]

Emergence of BCR-ABL KD mutations with current TKIs

a) **Prognostic implications of BCR-ABL KD mutations under imatinib first line.** Approximately 21%-48% of CML patients who experienced primary resistance to imatinib and 10-68% of cases with secondary resistance developed BCR-ABL1 mutations with the most frequently T315I, G250E, M244V, M351T, and E255K/V**.** Several studies have demonstrated that during first-line imatinib treatment emergence of BCR-ABL1 KD mutations predicts loss of CCyR, shorter progression-free survival (PFS), shorter time to AP/BP progression, and shorter overall survival (OS). [123-126.] Before the advent of dasatinib and nilotinib, the CML/002/STI571 GIMEMA trial, a retrospective molecular analysis conducted on 40 late CP-CML patients intolerant or resistant to interferon-a, and with cytogenetic resistance to imatinib, have demonstrated that all 40 patients failed to obtain MCyR at 12 months and 19 (48%) patients had turned positive for mutations. In addition the presence of BCR-ABL1 KD mutations was associated with significantly shorter time to AP/BP progression (P = 0.0002) and shorter survival (P = 0.001).

b) **Prognostic implications of BCR-ABL KD mutations under dasatinib/nilotinb second line**. In the CA180-034 trial and in the CAMN107A2101 14%-33% of CML treated with second-line dasatinib or nilotinib develop new BCR-ABL mutations. In this cases the most common mutations that confer resistance to dasatinib were T315I, F317L, andV299L whereas the most common mutations with nilotinib were E255K/V, T315I, F359C/V, G250E, and Y253H. Several studies found that CP-CML patients who harbored mutants less sensitive to dasatinib or nilotinib in vitro achieving lower rates of CCyR and PFS than patients harboring mutants with high or unknown in vitro sensitivity to dasatinib or nilotinib. Patients with CP-

CML treated with second-line dasatinib, who participated in the clinical trials START-C, START-R, and CA180-034, were analyzed at 2-year follow-up. Among 121 patients who had a BCR-ABL1 mutant with an intermediate in vitro sensitivity to dasatinib (IC50>3 nM), 32% had CCyR with a PFS of 67%. Among patients who had a mutant with a high in vitro sensitivity to dasatinib (IC50≤ 3 nM) or unknown IC50, 53% and 51% achieved CCyR, and the PFS rates were 75% and 80%, respectively. Patients who participated in the phase II clinical, nilotinib second-line registration trial, were analyzed at the 1-year follow-up. Among 26 patients who had a BCR-ABL1 mutant with an intermediate in vitro sensitivity to nilotinib (IC50> 150 nM), none achieved CCyR and the PFS was 31%. Among patients who had a mutant with a high in vitro sensitivity to nilotinib (IC50≤ 150 Nm) or unknown IC50, 40% and 48% achieved CCyR, and the PFS was 64% and 59%, respectively. [127-131] (Table 2)

c) **Prognostic implications of BCR-ABL KD mutations under dasatinib/nilotinb first line.** Based on the DASISION S0325 (Intergroup study of dasatinib versus imatinib in the first-line setting), therapy with dasatinib front-line is associate with fewer failure than imatinib. In the 2-year follow-up of the DASISION trial, 9% (22/258) of CP-CML patients discontinued first-line dasatinib because of disease progression or treatment failure, compared with 11% (28/258) treated with imatinib and 2% (2/123) of CP-CML patients discontinued first-line dasatinib because of disease progression or treatment failure, compared with 7% (8/123) treated with imatinib.[132,133,134] The frequency of emerging mutations among patients who discontinued treatment was similar between dasatinib-treated and imatinib-treated patients. At the 2-year follow-up, 26% (10/38) of patients treated with dasatinib who had an evaluable mutational analysis performed at discontinuation had a mutation compared with 21% (10/48) of patients treated with imatinib.[132. 88,133,134] Specifically, the 10 dasatinib-treated patients carrying mutations had the following mutations: T315I (n= 7), F317L (n =2), and F317I/V299L (n =1). [132] In 22 patients with evidence of resistant disease or with hematologic or cytogenetic relapse or progression 1 of 9 (11%) dasatinib treated patients and 2/13 (15%) of imatinib-treated patients had a mutation. Based on the ENESTnd trials, where CML-CP patient was treated with nilotinib first-line at 300 mg and 400 mg bid arms, the 2-year follow-up showed that 9% (26/282) and 3% (9/281) of CP-CML patients discontinued first-line nilotinib respectively because of disease progression, treatment failure or suboptimal response, compared with 17% (48/283) treated with imatinib. The 3-year follow-up showed that 10% (28/282) and 5% (15/281) of CP-CML patients

discontinued first-line nilotinib (300 mg and 400 mg bid arms, respectively) because of disease progression, treatment failure or suboptimal response, compared with 20% (57/283) treated with imatinib. [133] In patients with baseline mutations mutation analysis was performed every 3 months. [135,136] The frequency of emerging mutations during treatment was lower in the nilotinib arms than in the imatinib arm of the ENESTnd trial. At the 3-year minimum follow-up, 5% (11/228)and 5% (11/215) of patients with at least one post baseline mutation analysis treated with nilotinib (300 mg and 400 mg bid arms, respectively) had a mutation compared with 9% (21/237) treated with imatinib. [135]. The 11 patients in the 300 mg bid nilotinib group carrying new mutations (3 patients had multiple mutations) had the following mutations: Y253H (n = 4), F359V (n = 4),T315I (n = 3), G250E (n = 1), E255K (n = 1), E459K (n = 1). The 11 patients in the 400 mg bid nilotinib group carrying new mutations (2 patients had multiple mutations) had the following mutations:Y253H (n = 4), E255K/V (n = 3/1), T315I (n = 2), F359V (n = 2), Q252H(n = 1). Development of both nilotinib and dasatinib proved successful in reducing the Achilles heels of imatinib in terms of insensitive mutations. In vitro studies reporting the IC50 of dasatinib and nilotinib for the main Bcr–Abl mutant forms initially predicted them to be active against all mutations but are inactive against the T315I. Clinical experience with dasatinib or nilotinib first line in imatinib-resistant patients demonstrated that, besides the T315I, each compound has a small and unique spectrum of additional insensitive mutations For nilotinib this spectrum includes some mutations already known to confer resistance to imatinib: the P-loop mutations Y253H and E255K/V, and the F359V/I/C mutations. For dasatinib, this spectrum includes both mutations already known to confer resistance to imatinib (F317L/I) and some novel, TKI-specific mutations: V299L, T315A and F317V/C [137]. When already present at the time of imatinib resistance, before the switch to dasatinib or nilotinib, these mutations together with T315I were invariably found to correlate with poor response rates and were currently found in patients who relapsed after an initial response to dasatinib or nilotinib.

d) **Prognostic implications of BCR-ABL KD mutations under second-line bosutinib.** 288 CP-CML imatinib resistant/intolerant patients were treated with bosutinib during the first registration clinical trial. Among imatinib-resistance and imatinib-intolerant patients 23% (45/200) and 7% (6/88) respectively discontinued therapy due to disease progression or unsatisfactory response. Mutational analysis at baseline performed in 115 patients,

showed that 65 (57%) of patients had at least one mutation. The most common mutations were M351T(n = 7), F359V (n = 7), F317L (n = 4), L248V (n = 4), G250E (n = 3),M244V (n = 3), and T315I (n = 3). [138]

e) **Prognostic implications of BCR-ABL KD mutations under ponatinib.** While ponatinib should still inhibit a number of compound mutants, recent preclinical studies have shown that a select few clinically reported compound mutants may confer varying resistance. [92] Proliferation assays with Ponatinib were performed with BaF3 cells expressing a clinically reported BCR-ABL1 compound mutations. The most resistant mutant, E255V/T315I (IC50: 659.5 nM), exhibited 11.9- and 22.7-fold higher ponatinib resistance than E255V (IC50: 55.6 nM) or T315I (IC50: 29.1 nM), respectively. The IC50 for E255V/T315I is >6.5-fold the average steady-state plasma concentration (101 nM) for patients receiving ponatinib at the PACE starting dose of 45 mg/day. The Q252H/T315I, T315I/M351T, T315I/F359V and T315I/H396R mutants exhibited marginal ponatinib sensitivity (IC50: 84.8-114.3 nM). M244V/T315I was the only T315I-inclusive compound mutant in the panel predicted to be sensitive to ponatinib at clinically achievable levels. [94]

f) **Compound mutations.** A risk of sequential TKI treatment is the selection of Bcr-Abl compound mutants, defined as harboring ≥2 mutations in the same Bcr-Abl allele. (Figure 7) Recently several studies have demonstrated that this compound mutations have the potential to confer resistance to multiple TKIs, highlight their presence how an emerging clinical problem for patients receiving sequential TKIs. In patients with Ph+ leukemia the management of TKIs resistance due to Bcr-Abl single mutants is now achievable, but the ability of clinically available TKIs to target Bcr-Abl compound mutants has yet to be thoroughly investigated. Patients with multiple mutations, if compared with those with no or one mutation are characterized by poor prognosis [139]. A study conducted at the MD Anderson Cancer Center on 207 imatinib-resistant CML patients treated with a second-generation TKI (nilotinib, dasatinib, bosutinib) has demonstrated that 7 (3%) of patients had more than one mutation detected by direct sequencing and 4 (4%) patients had multiple mutations. Surprisingly this study showed that patients with more than one mutation have a significantly worse outcome than those with no or one mutation. The 4-year PFS rates were 56% (no mutation), 49% (1 mutation), and 0% (more than 1 mutation; P = 0.02). [140] Again, mutational analysis performed by sequencing and mass spectrometry on 220 patients treated with nilotinib or dasatinib, after imatinib resistance, revealed that

multiple mutations were present in 31 patients (14%) after imatinib failure, with one case with mutations clinically resistant to both nilotinib and dasatinib by sequencing; by mass spectrometry, 60 patients (27%) had multiple mutations with 5 cases with mutations known to confer clinical resistance to both nilotinib and dasatinib. In patients without mutations clinically resistant to nilotinib or dasatinib multiple sensitive mutations detectable only by mass spectrometry and detected after failure of imatinib treatment were associated with lower CCyR rates achieved after second-line TKI therapy, compared with no mutation or one mutation (21% vs 50%, P = 0.003). [139, 141] Importantly, ultra-deep sequencing of serial samples from Ph+ leukemia patients who had received sequential TKIs treatment showed that the majority (76%) of Bcr-Abl compound mutations were two-component mutations, as compared to 21% triple and 3% quadruple mutations. [142] Not surprisingly, the composition of the compound mutations noted in this study reflected the TKIs treatment history, where one or more mutational components were associated with a typical clinical or in vitro resistance profile to the specific TKIs.

The role of Bcr–Abl KD mutation analysis in the optimal management of CML patients

When to perform mutation analysis. Now that multiple therapeutic options are available, the knowledge of the presence/absence and the type of mutation are two precious pieces of information to be integrated in the decision algorithm aimed at tailoring the best therapeutic choice. Whatever their contribution to the resistant phenotype the presence of a mutation should never overlooked since their presence is a warning sign of genetic instability that is the engine of disease evolution towards a more aggressive and less manageable phenotype. More importantly, the presence of specific type of mutations guides the therapeutic decisions. (Box 2). For this reason the BCR-ABL KD mutation analysis has entered in the panel of molecular tests for CML patients, as quantitative PCR for the monitoring of residual Bcr–Abl transcript levels did years ago. In order to educate physicians as to when mutation analysis should be prescribed, which techniques provide the most informative results and how these results should be interpreted and translated into clinical decisions, a panel of European experts was appointed by the ELN with the aim to compile a series of recommendations to optimize the use of this molecular test in the context of routine management of CML patients. [143,144] The specific recommendations about when

BCR-ABL KD mutation analysis should be performed in CML patients treated with TKI were summarized in box 3

Different strategies to perform mutational analysis. Today the gold standard as well as the currently recommended methodology for BCR-ABL KD mutation analysis is direct Sanger sequencing. Sequencing of PCR products has been widely used to search for known and unknow ABL kinase domain variants in CML and Ph+ ALL patients although it is known to have several potential drawbacks: the relatively low sensitivity (in ≥20% of Ph+ cells), the difficulty to precisely quantitate the mutated subclone and the lack of informativity regarding the clonal composition when multiple mutations are present. Subcloning of PCR products followed by sequencing being able to achieved a very low detection limits and to identify compound mutations but this method is very labor intensive and therefore not suitable for routine diagnostics. Additional alternative methods with sensitivities ranging between 1% and 10% have been set up and proposed by several authors. Denaturing high-performance liquid chromatography (D-HPLC) combined with direct sequencing allows prescreening for sequence variations, reducing the number of samples to be sequenced and improves the limit of detection to 1%, but alone does not allow characterization of the precise sequence.[145] A screening method combining PCR with single-strand conformation polymorphism analysis (PCR-SSCP) was also described in a cohort of Ph+ ALL previously evaluated by direct sequencing. Although PCR with single-strand conformation polymorphism analysis can generally find conformational changes of DNA with a minimum detection limit of 2–5%, the authors reported that it was inferior to sequencing. [146] Alternative methods that allow the detection of specific ABL mutations have been developed. They include PCR-RFLP analysis, Allele-specific oligonucleotide (ASO)-(PCR) and a PNA-based PCR clamping technique. With the restriction fragment length polymorphism (RFLP)-based assays analysis a detection limit of 5% can be reached, but the assay is limited to a very small number of ABL mutations (Y253F/H, E255K/V, T315I, and 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; it relies on the artificial introduction of a TaqI restriction site in the mutant, but not in the wild-type sequences, by use of 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 was very sensitive, being able to reliably detect artificially generated mutated amplicons in the presence of 1000-fold excess of wild-type molecules. [147] Other extremely sensitive technique for detection of specific point mutations

(F311L, T315I, and M351T) is the ASO-PCR. A recent study have demonstrated the presence of rare cells bearing ABL mutations prior to STI571 therapy with a detection limit of 0.01%. [148] More recently a PNA-based PCR clamping technique with a detection limit of 1:500 (0.2%) for Y253H, E255K, and T315I mutations was described. With this method, the E255K mutation was again retrospectively detected at diagnosis in a single AP-CML patient who never achieved either cytogenetic or hematologic remission. [149] Despite the fact that the methods cited above were highly sensitive when used for longitudinal screening of resistant patients known to have a specific point mutation, some drawbacks exist. First: they are more sensitive but are limited by their specificity for a definite and limited spectrum of specific mutations and seem not to be suitable for large-scale screening of the entire spectrum of mutations occurring in the kinase domain of ABL; more importantly ASO-PCR and PNA-based PCR clamping techniques are characterized by generation of several disadvantage how the generation of false-positive or very expensive costs. Newer technologies to detect mutations at higher sensitivity are still experimental but not yet incorporated into clinical practice. Some of these technologies include: mass spectrometry, digital PCR and NGS or ultra-deep sequencing. The Mass spectrometry is a very sensitive technique, with a detection limit ranging from 0.05% to 0.5% depending on the mutation (0.2% on average). It has been used to detect 31 specified most frequent and clinically relevant mutations, including all of the nilotinib- and/or dasatinib-resistant mutations and the most common imatinib-resistant mutations (detected in ≥1% of patients with mutations) demonstrating that low level mutations may actually be detected in a proportion of patients negative by SS allowing a more appropriate selection of the second-line treatment strategy. [139,141] Additionally this study showed that low-level nilotinib-/dasatinib-resistant mutations are associated with poor response and high risk of failure; 16% of patients with low-level mutations resistant to nilotinib or dasatinib, detected upon imatinib failure, achieved low CCyR rates if compared with patients with other mutations (41%) or patients with no mutations (49%). Patients with low-level resistant mutations had extremely poor failure-free survival at 18 months after therapy with nilotinib or dasatinib (0%), compared with 51% for patients with other low-level mutations and 45% for those with no mutations. The Fluidigim quantitative real-time digital PCR is a nano-fluidic based method characterized by sectioning the sample into thousands of independent reaction chambers, increasing the detection of rare mutations This technology is particular suitable to assess the kinetics of mutation development, allowing earlier detection of mutations and potentially allowing correlating kinetics of mutation development with outcomes. It was initially used to identify and quantify the T315I

mutation in 28 CML patients analyzed both before and at the time of relapse. With this method the T315I mutation was detected before relapse in 8 patients in whom it was detected at relapse.[150] (A comprehensive list of several mutation scanning strategies is represented in Figure 8). During the last 25 years the field of DNA sequencing has profoundly changed due to the development of several revolutionary technologies of sequencing, the so-called next-generation sequencing technologies. These new methods have expanded previously focused readouts from a variety of DNA preparation protocols to a genome-wide scale and have fine-tuned their resolution to single base precision. The next-generation technologies, first introduced to the market in 2005, have been used for standard sequencing applications, such as genome sequencing and re-sequencing, and for novel applications previously unexplored by Sanger sequencing. Several platforms for massively parallel DNA sequencing read are commercially available: the Roche/454 GS Junior system, the Illumina/Solexa Genome Analyzer, the Applied Biosystems SOLiDTM System and the Ion Torrent. By different approaches each technology seeks to amplify single strands of a fragment library and perform sequencing reactions on the amplified strands. The fragment libraries are obtained by annealing platform-specific linkers to blunt-ended fragments generated directly from a genome or DNA source of interest. Although the initial machines could read only ∼110 bp, current machines with improved design and chemistry can routinely generate 400–500 bp reads and up to 600Mbp of raw sequence per 10-h run. The recently improvements should allow for ∼1000 bp read. The yield of sequence reads and total bases per instrument run is significantly higher than the 96 reads of up to 750 bp each produced by a capillary sequencer run, and can vary from several hundred thousand reads (Roche/454) to tens of millions of reads (Illumina and Applied Biosystems SOLiD).

The Roche/454 GS Junior instruments The typical application of the “amplicon deep sequencing” of the Roche 454 GS Junior Sequencing System is the target re-sequencing which provide an efficient method to quickly interrogate particular genomic regions of interest, from single PCR products up to 50 Mb regions via hybridization capture technologies. The clonal nature of 454 Sequencing Systems allows unambiguous allele resolution of variation in complex regions of the genome, along with quantitative detection of variants present in less than 1% of a mixture. The preparations of “Basic Amplicon Library” for the GS Junior System consists of a simple PCR reaction using the DNA template of interest and a pair of special fusion primers defined “Fusion primers”. (Figure 9) The fusion primers used to generate are each composed of two or three parts,

fused together. The 5'-portion is a 25-mer whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA Capture Beads, and for annealing the emPCR Amplification Primers and the Sequencing Primer; in addition, this 5'-part must always end with the sequencing key "TCAG" used for Amplicon sequencing. The exact, required sequences are as shown in blue (Adaptor) and red (key). The 3'-portion of each primer is designed to anneal with a specific sequence on either side of the target of interest on the DNA sample, delineating the margins of the amplicon that will be produced. Fusion primers are conjugated with multiplex identifiers – allowing us to pool and sequence different and multiple samples in a single run . After the production of amplicons the next step are the "emulsion PCR" followed by "pyrosequencing" (Figure 10). Ideally, during the emulsion-PCR a single template will be ligated to adapters, separated into single strands and attached to small capture beads leading to uniform clusters on each bead. The fragments will then be amplified by a technique defined "PCR-emulsion", whereby each bead is isolated within a droplet of a PCR reaction mixture – in oil emulsion. The droplets act as individual amplification reactors, producing $\sim 10^7$ clonal copies of a unique DNA template per bead thus, at the end of amplification, each bead carries several million copies of a unique DNA fragment. Then, the emulsion is broken, the DNA will be denatured and the beads are then deposited onto an array of picoliterscale wells. The use of the picotiter plate in parallel, massively increasing the sequencing throughput. The plate contains millions of wells that are individual reactors for the sequencing reactions that are catalysed by the Bacillus stearothermophilus (Bst) DNA-polymerase. The diameter of the wells is manufactured so that only one bead can be accepted in each well support the concept "one beads-one fragment". After these preparatory steps, the sequencing begins using a pyrosequencing approach. The pyrosequencing is a sequencing-by-synthesis technique that measures the release of inorganic pyrophosphate (PPi) by chemiluminescence. The template DNA is immobilized, and solutions of dNTPs are added one at a time; the release of PPi, whenever the complementary nucleotide is incorporated, is detectable by light produced by a chemiluminescent enzyme present in the reaction mix. The light is detected using a CCD sensor and software detects wells containing template DNA producing a "pyrogram," which corresponds to the order of correct nucleotides that had been incorporated. The data analysis is performed with the GS Amplicon Variant Analyzer (AVA) software that allows to provides an easy and robust analysis for the identification and quantitation of known or novel sequence variants. The current state-of-the-art 454 platform marketed by Roche Applied Science is capable of generating 80–120

Mb of sequence in 200- to 300-bp but the recent software upgrade of GS Junior will enable to achieve read lengths of 800 bases.

FIGURES

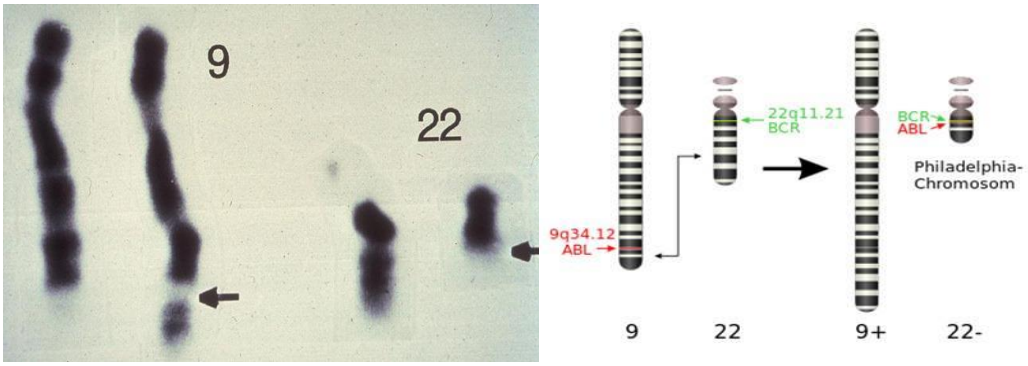


Figure 1 – Philadelphia translocation t(9;22)(q34;q11.2) seen in chronic myeloid leukemia. Discovered in 1960, the diagnostic karyotypic abnormality for chronic myelogenous leukemia is shown in this picture of the banded chromosomes 9 and 22. The chromosomal defect in the Philadelphia chromosome is a translocation, in which parts of two chromosomes, 9 and 22, swap places. The result is that a fusion gene is created by juxtapositioning the Abl gene on chromosome 9 (region q34) to a part of the BCR ("breakpoint cluster region") gene on chromosome 22 (region q11). This is a reciprocal translocation, creating an elongated chromosome 9 (termed a derivative chromosome, or der 9), and a truncated chromosome 22 (the Philadelphia chromosome).

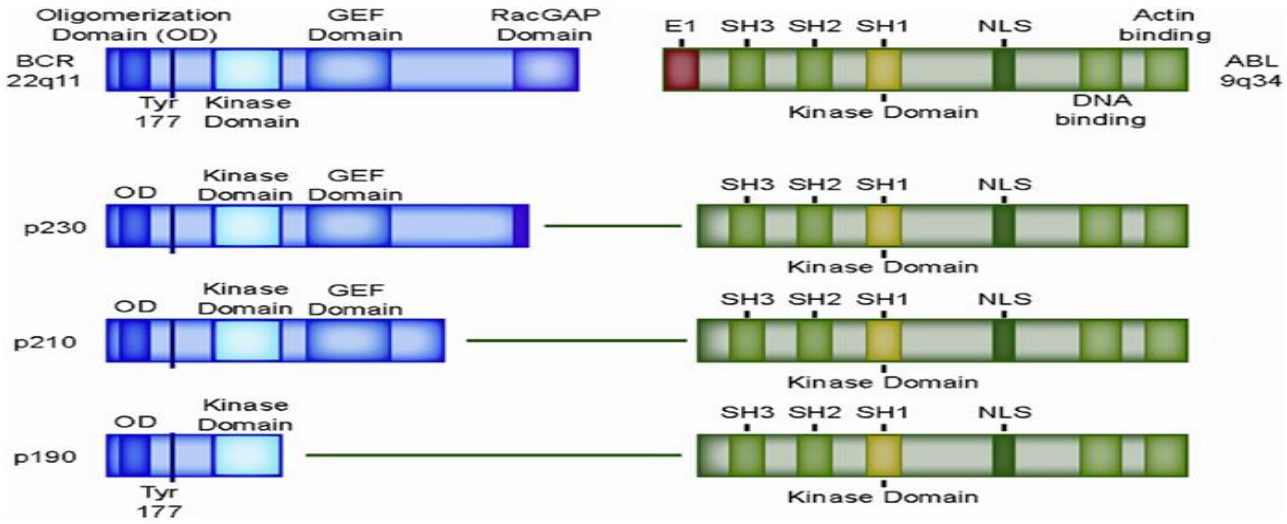


Figure 2 - Structure of the most common BCR–ABL1 fusion genes. Domain structure of wild type BCR and wild type ABL1 protein, as well as retained domains in the three most common BCR–ABL1 variants, p230, p210, and p190. OD, oligomerization domain (coiled-coil domain) mediating oligomerization; Tyr177, tyrosine 177 when phosphorylated serves as a docking site for the adaptor protein GRB-2; SH2-domain, SRC homology 2 (binding to phosphorylated tyrosine residues, including BCR exon 1); SH3-domain, SRC homology 2 (binding to proline rich peptides); SH1-domain, SRC homology 1 (ABL1 catalytic domain); GEF-domain, guanine nucleotide exchange factors (G-protein signaling); E1, exon 1 of ABL1, contains the inhibitory N-terminal "cap" that binds the catalytic domain (SH1) of ABL1 and prevents autophosphorylation; NLS, nuclear localization signal.

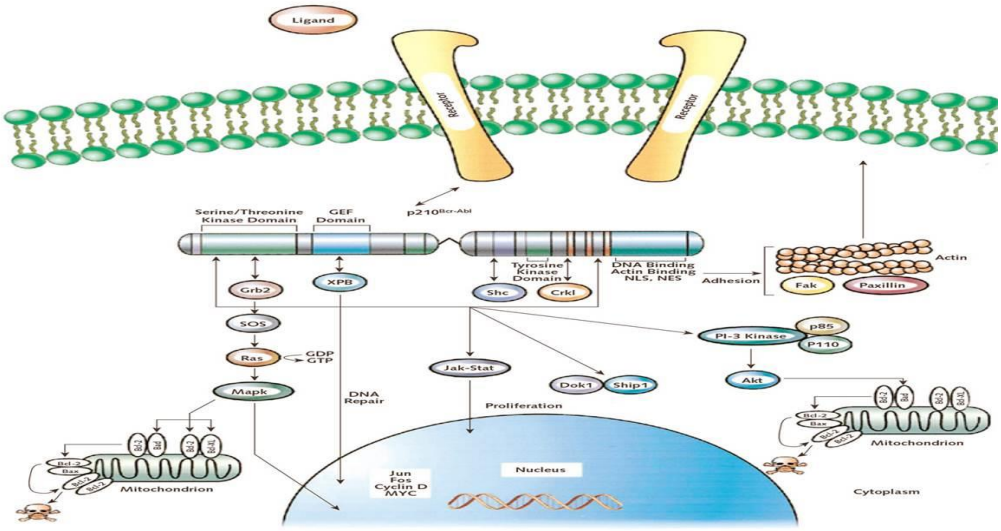


Figure 3 - Some of the cellular events triggered by Bcr-Abl kinase activity. Ras activation in Bcr–Abl-expressing cells is mediated by Bcr–Abl interaction with the adaptor signalling molecules Grb2, Shc, Sos and Dok. The sum of these interactions results in the favouring of Ras in its active GTP-bound 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-XL, to an anti-apoptotic signal mediated by Bcl-XL. 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 re-organisation. 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.

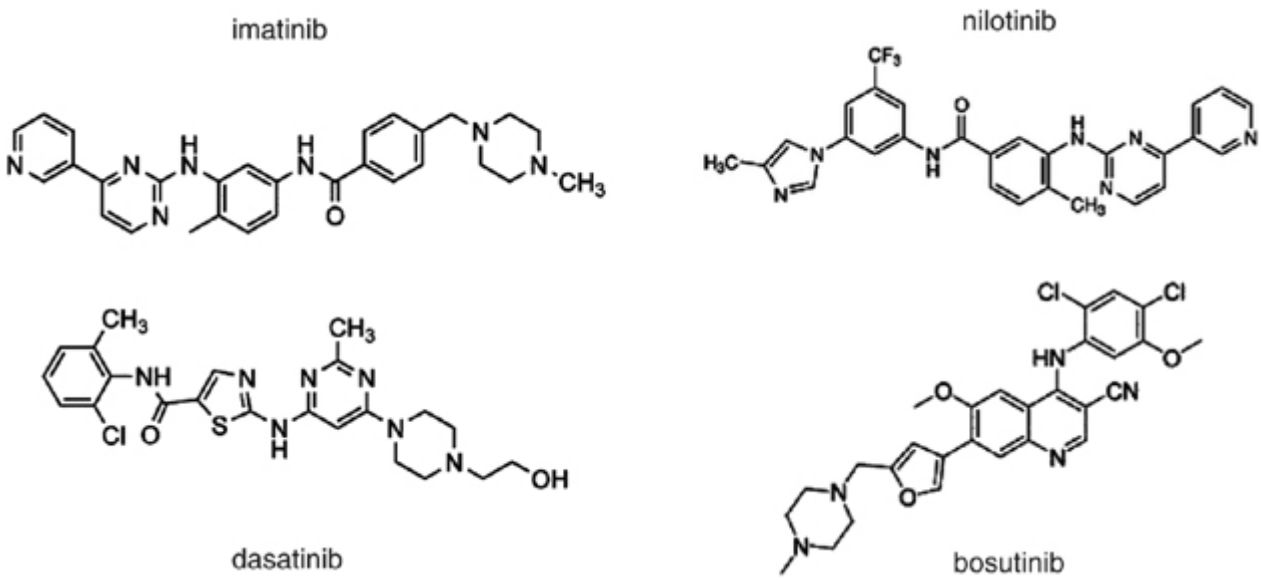


Figure 4 - Chemical structure of imatinib and the second-generation tyrosine kinase inhibitors (TKIs) nilotinib, dasatinib and bosutinib.

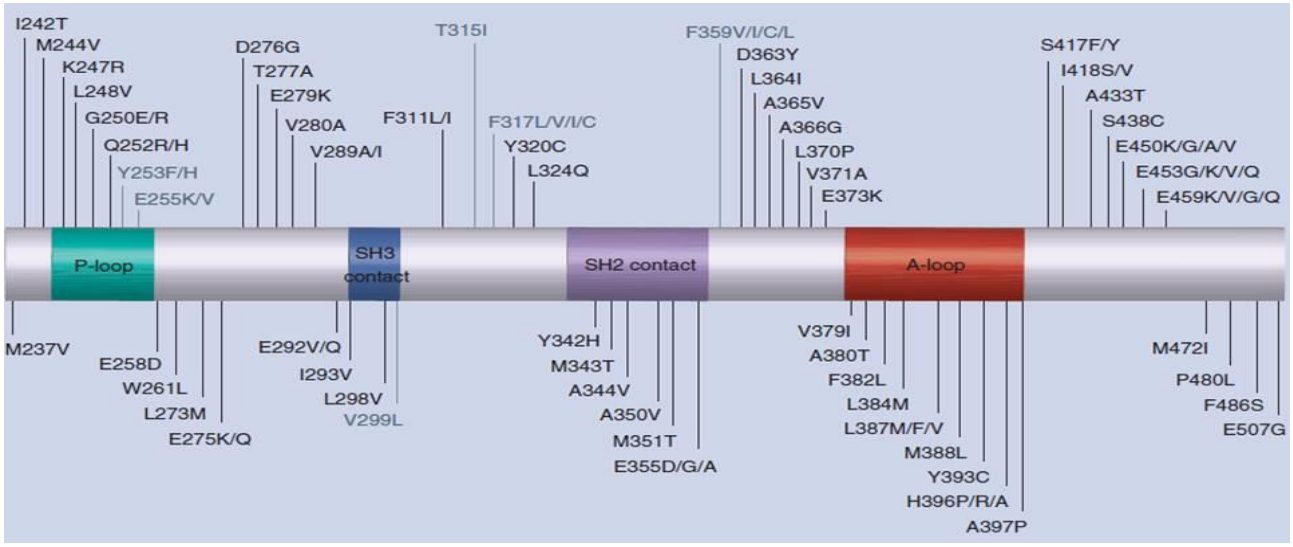
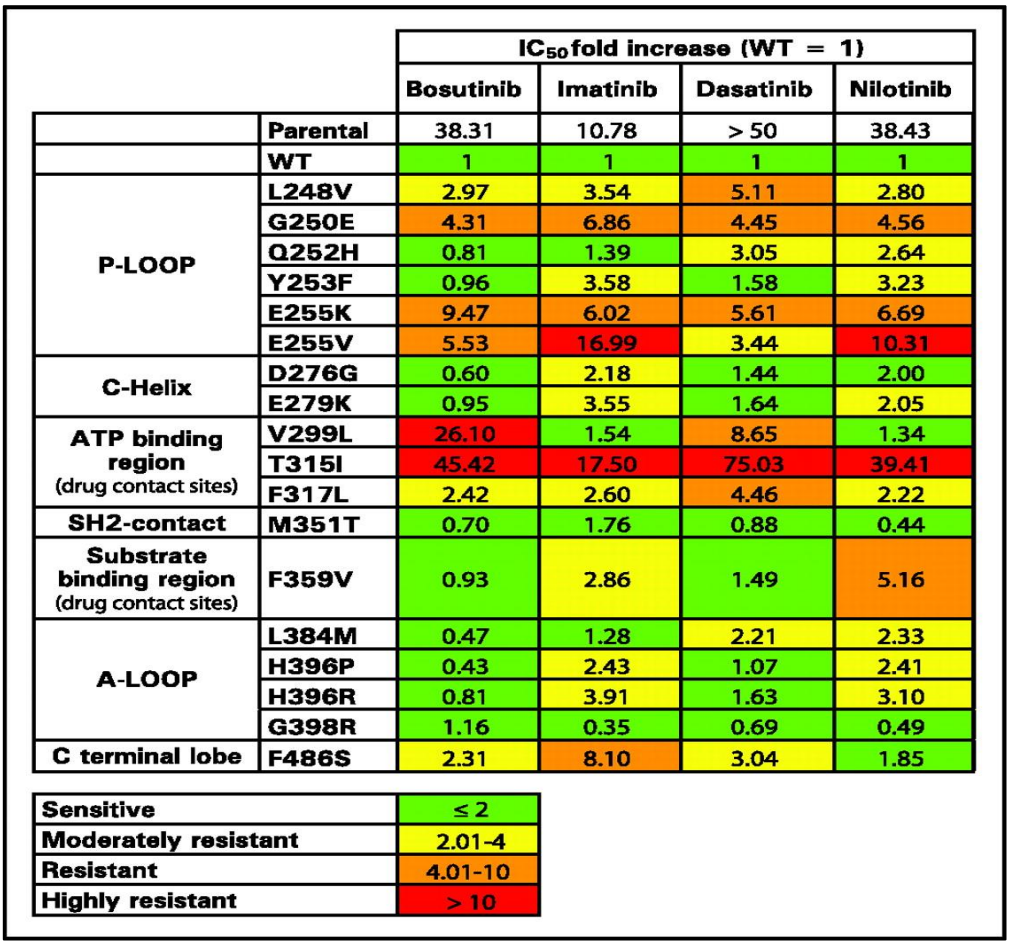


Figure 5 - Map of mutations in the BCR-ABL1 KD identified in clinical samples from patients resistant to imatinib. Key structural motifs within the KD are indicated: P-loop indicates phosphate binding loop, SH2 contact and SH3 contact represent the contact regions with SH2 and SH3 domain-containing proteins, and A-loop indicates the activation loop. K247R and Y320C are in italics because they have been reported to be single-nucleotide polymorphisms. Numbering of residues is according to ABL1a isoform.



		IC_{50} fold increase (WT = 1)			
		Bosutinib	Imatinib	Dasatinib	Nilotinib
	Parental	38.31	10.78	> 50	38.43
	WT	1	1	1	1
P-LOOP	L248V	2.97	3.54	5.11	2.80
	G250E	4.31	6.86	4.45	4.56
	Q252H	0.81	1.39	3.05	2.64
	Y253F	0.96	3.58	1.58	3.23
	E255K	9.47	6.02	5.61	6.69
	E255V	5.53	16.99	3.44	10.31
C-Helix	D276G	0.60	2.18	1.44	2.00
	E279K	0.95	3.55	1.64	2.05
ATP binding region (drug contact sites)	V299L	26.10	1.54	8.65	1.34
	T315I	45.42	17.50	75.03	39.41
	F317L	2.42	2.60	4.46	2.22
SH2-contact	M351T	0.70	1.76	0.88	0.44
Substrate binding region (drug contact sites)	F359V	0.93	2.86	1.49	5.16
A-LOOP	L384M	0.47	1.28	2.21	2.33
	H396P	0.43	2.43	1.07	2.41
	H396R	0.81	3.91	1.63	3.10
	G398R	1.16	0.35	0.69	0.49
C terminal lobe	F486S	2.31	8.10	3.04	1.85

Sensitive	≤ 2
Moderately resistant	2.01-4
Resistant	4.01-10
Highly resistant	> 10

Figure 6 - IC50 values from Bosutinib, Imatinib, Dasatinib and Nilotinib against 18 mutated forms of Bcr-Abl expressed in transfects Ba/F3 cells. IC50, relative concentration that inhibits 50%; WT, wild type; P loop, phosphate-binding loop; ATP, adenosine triphosphate; SH2, Src homology 2; A loop, activation loop. For details see reference [105].

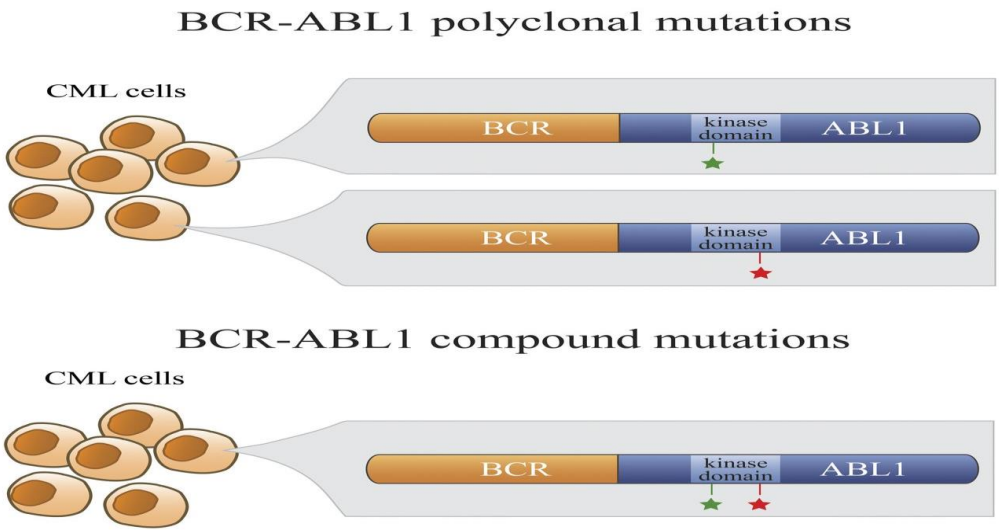


Figure 7 - Polyclonal versus compound mutations. In a subset of patients who develop clinical resistance to ABL TKIs, more than 1 point mutation in the kinase domain of BCR-ABL is detectable by direct sequencing. In the case of polyclonal mutations, these BCR-ABL mutations (green and red stars; top panel) exist separately in different clones. In contrast, BCR-ABL compound mutants exhibit 2 mutations within the same BCR-ABL molecule (green and red stars; bottom panel).

Strategy	Lower detection limit
Allowing to screen the entire KD for any sequence variation	
Direct sequencing	15-25%
Cloning+sequencing	Depends on the number of clones
D-HPLC (Transgenomic Wave™)	1-10%
High-Resolution Melting (HRM)	5%
Allowing to detect a limited number of specific mutations	
Restriction Fragment Length Polymorphism (RFLP)	1-10%
Denaturing Gradient Gel Electrophoresis (DGGE)	2-5%
Pyrosequencing	5%
Qualitative allele-specific oligonucleotide (ASO)-PCR	0.01%
Real-time allele-specific oligonucleotide (ASO)-PCR	0.1-0.001%
Amplification Refractory Mutational System (ARMS)-PCR	0.1–1%
Ligation (L)- or Ligation Dependent (LD)-PCR	0.05-0.1%
Invader-PCR	1-5%
Scorpion-PCR	1%
Real-time PCR with Fluorescence Resonance Energy Transfer (FRET)-probes	5%
Peptide-nucleic acid (PNA)-clamping PCR	0.2%
Mass Spectrometry (Sequenom™)	0.05-0.5%
Digital PCR on nanofluidic arrays (Fluidigm Biomark™)	?

Figure 8 - Comprehensive list of several mutation scanning strategies.

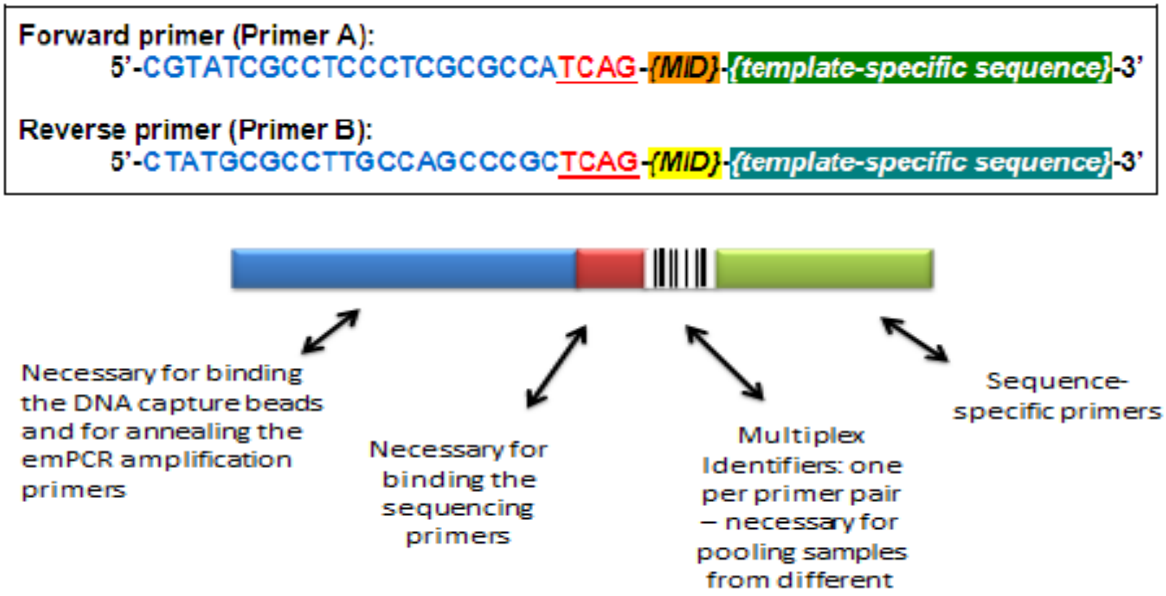


Figure 9 - Components of fusion primers for 'Basic' Amplicon sequencing. The primers used to generate 'Basic' Amplicon libraries are each composed of several parts fused together. The Adaptor (blue), at 5'-portion, is a 25-nt sequence dictate by the requirements of the 454 Sequencing System for hybridizing to the DNA Capture Beads and for annealing the emPCR Amplification, Primers and the Sequencing Primer. There exist two kinds of such primers, termed "Primer A" and "Primer B", allowing for the directional sequencing of the target sequence from either end. The sequencing key "TCAG" (red) is used for Amplicon sequencing. A template specific primer (green and blue-green) at the 3'-portion is designed to anneal with a specific sequence on either side of the target of interest on the DNA sample. This specific template are typically 20-25 nt in length. Multiplex Identifiers (MIDs), place immediately after the sequencing key, can be used to "barcode" the amplicons or samples. They are shown on orange or yellow.

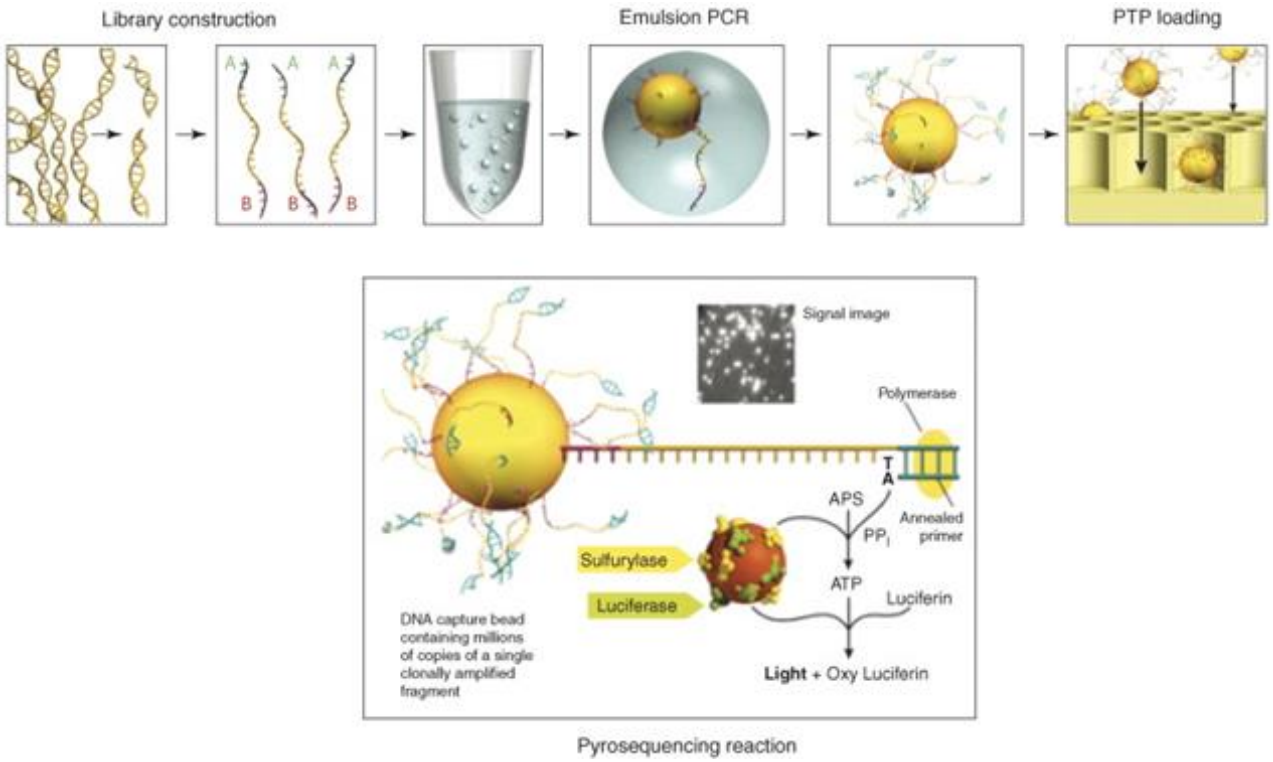


Figure 10 – Roche 454 Workflow. Library Adaptors are ligated to the DNA and then attached to Capture Beads by adaptors. Each bead carries a unique single-stranded library fragment. Beads are then emulsified with amplification reagents in a water-in-oil mixture to trap individual beads in amplification microreactors. During emPCR millions of copies of each clone is generated using adaptor specific primer and attached to each bead. Amplified beads are then loaded to PicoTiterPlate, a specialized plate designed to load one bead per well. Individual nucleotides are flowed in sequence across the wells. Each incorporation of a nucleotide complementary to the template strand results in a chemiluminescent light signal recorded by the camera. The output format of this platform is SFF (Standard File Format) can be used to analysis and assemble data of 454 platform.

BOX and TABLES

Complete hematologic response

- Platelet count <450 cells per µl
- WBC count <10 cells per µl
- No immature granulocytes
- Basophils <5% of blood count
- No palpable spleen

Cytogenetic response†

- CCyR = 0% Ph+ metaphases
- PCyR = 1–35% Ph+ metaphases
- mCyR = 36–65% Ph+ metaphases
- Minimal = 66–95% Ph+ metaphases
- None = >95% Ph+ metaphases
- MCyR = complete + partial

Molecular response‡

- Major (MMR or MR3) = 3-log reduction from a standardized baseline = Bcr–Abl transcript level <0.1% on the international scale
- Complete (CMR or MR4) = 4-log reduction from a standardized baseline = Bcr–Abl transcript level <0.01% on the international scale

Box 1 - Definitions of hematologic, cytogenetic and molecular response in chronic myeloid leukemia. †As assessed by conventional chromosome G-banding analysis examining at least 20 bone marrow metaphases. ‡As assessed by real-time reverse transcription PCR for Bcr–Abl transcript levels. CCyR: Complete cytogenetic response; CMR: Complete molecular response; mCyR: Minor cytogenetic response; MCyR: Major cytogenetic response; MMR: Major molecular response; MR: Molecular response; PCyR: Partial cytogenetic response; Ph+: Philadelphia chromosome-positive; WBC: White blood cell.

T315I

- Hematopoietic stem cell transplant or investigational drugs

V299L, T315A & F317L/V/I/C

- Consider nilotinib rather than dasatinib

Y253H, E255K/V & F359V/C/I

- Consider dasatinib rather than nilotinib

Any other mutation

- Consider high-dose imatinib†, or dasatinib or nilotinib

Box 2 – Summary of the most appropriate alternative therapeutic option for imatinib resistance-patients based on the BCR-ABL kinase domain mutation status.

At diagnosis

- In patients presenting in accelerated phase/blast crisis (optional)

During first-line therapy with imatinib

- In case of failure
- In case of an increase in Bcr–Abl transcript levels leading to a MMR loss
- In any other case of suboptimal response

During second-line dasatinib or nilotinib therapy

- In case of hematologic or cytogenetic failure

Box 3 – Recommendations for mutation analysis. Based on a critical review of the literature and expert opinion, the ELN panel agreed on a series of recommendations as to when to perform a mutational analysis in CML patients.

IMATINIB						NILOTINIB	DASATINIB	BOSUTINIB	PONATINIB
M237V	L273M	F311L	E355D/G	V379I	A397P	Y253F/H	V299L	V299L	?
M244V	E275K/Q	T315I	F359V/I/C	A380T	S417F/Y	E255K/V	T315I	T315I	
L248R	D276G	F317L/V/I/C	D363Y	F382L	I418S/V	T315I	F317L/V/I/C	?	
G250E/R	T277A	F359V/I/C	L364I	L384M	S438C	F359V/I/C			
Q252R/H	E279K	Y342H	A365V	L387M/F	E453G/K				
Y253F/H	V280A/I	M343T	L370P	M388L	E459K/V				
E255K/V	V289A	A344V	V371A	Y393C	P480L				
E258D	V299L	M351T	E373K	H396R/P	F486S				

Table 1 – Summary of the *BCR-ABL* KD amino acid substitutions identified in clinical samples from patients reported to be resistant to the currently approved TKIs. Imatinib, dasatinib and nilotinib are approved both by FDA (Food and Drug Administration) and EMA (European Medicines Agency) for first or subsequent-line use. Busutinib and ponatinib have been approved by FDA for patients with resistance (or intolerance) to prior TKI therapy. V299L and F317L/V/I/C (dark grey) retain insensitivity also to dasatinib; Y253F/H, E255K/V, F359V/I/C (light grey) retain insensitivity also to nilotinib. T315I (black) is a pan-resistant mutation retaining insensitivity to dasatinib, nilotinib and bosutinib. The question marks indicate that bosutinib-resistant mutations other than T315I and ponatinib-resistant mutations, if any, still need to be assessed.

Trial	Frequency of *BCR-ABL1* mutations (n/N)[a]	Frequency of mutations among patients with mutations[b]
Dasatinib second-line		
START-C, START-R, and CA180-034	27% (47/174)[c]	T315I (53%) F317L (21%) V299L (15%)
Nilotinib second-line		
Phase II nilotinib registration trial	24% (47/192)[d,e,f]	E255K/V (28%) T315I (26%) F359C/V (15%) G250E (15%) Y253H (13%)
Dasatinib/nilotinib second-line		
MD Anderson Cancer Center	21% (23/110):[g] 14% (8/56) for dasatinib 28% (15/54) for nilotinib	Y253H (13%) F317L (13%) T315I (9%) V299L (9%) F311I (9%)
Italian study	33% (31/95)[h]	T315I (36%)[i] F317L (20%)[i] E255V/K (11%)[i] V299L (9%)[i] Y253H (9%)[i]

Table 2 – Summary of mutation analysis conducted in a series of clinical trials of dasatinib/nilotinib in the second-line setting.

[n] is the number of patients with mutations and N is the number of patients in whom mutation analysis was performed.

[b] The percentage indicates the number of patients with a specific mutation divided by the total number of patients with mutations; the most frequent mutationsare reported.

[c] Mutation analysis was performed at the time of progression or discontinuation.

[d] Only patients resistant to imatinib have been included.

[e] Mutation analysis was performed during nilotinib therapy.

[f] Frequency of new BCR-ABL1 mutations in patients who experienced pro-gression: 25/64 (39%); most frequent mutations: E255K/V (28%), T315I (28%),E459G/E459K (12%).

[g] Mutation analysis was performed after treatment with a second TKI.

[h] Mutation analysis was performed during treatment with a second TKI.

[l] Includes patients who relapsed on dasatinib or nilotinib as second or third TKI.

AIMS

The spectra of imatinib, dasatinib and nilotinib sensitive and resistant mutations are nowadays quite well characterized. While some mutations are associated with a moderate degree of resistance and may therefore be overcome by a dose escalation of Tirosine Kinase Inhibitors (TKIs), other confer an highly resistant phenotype. In patients who fail or have a suboptimal response to TKIs or show a trend of increasing Bcr-Abl, 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

i. set up, validate and assess the routine applicability of an high-throughput Ultra-Deep Sequencing-based mutation screening strategy of the BCR-ABL KD based on the Roche GS Junior Instrument;
ii. resolve qualitatively and quantitatively the complexity of mutated populations surviving TKIs in a large series of Ph+ leukemia patients;
iii. investigate their clonal structure and evolution over time in relation to therapeutic intervention;
iv. study the dynamics of expansion of BCR-ABL1 KD mutations in Ph+ leukemia patients developing resistance to TKI-based therapies;
v. test the ability of UDS to highlight emerging clones harboring TKI-resistant mutations earlier than conventional sequencing and assess if pre-existent Abl KD mutations may be challenging for the clinical efficacy of the second-generation tyrosine kinase inhibitor

PART I

SET UP AND VALIDATION OF AN ULTRA DEEP, NGS SEQUENCING-BASED, MUTATION SCANNING OF THE BCR-ABL KINASE DOMAIN IN PATIENTS WITH PH+ LEUKEMIAS TREATED WITH TIROSINE KINASE INHIBITORS.

Background

Although BCR-ABL Kinase domain mutations can frequently be identified in patients who develop resistance to tyrosine kinase inhibitors in Philadelphia-positive (Ph+) leukemia small insertions and deletions within the Bcr-Abl kinase domain (KD) have occasionally been reported in patients who failed TKI therapy and have been hypothesized to have a causative role in drug resistance. [151,152] Some were in-frame insertions and deletions, others were predicted to result in truncated Bcr-Abl proteins. However, the detection of these sequence variations is hampered by the fact that they are almost always confined to subclones co-exisiting with full length BCR-ABL. This has most likely resulted in an underestimation of their frequency and complexity, since i) they can be confused with background noise/reduced quality readings in direct sequencing chromatograms and ii) cloning would be needed to better resolve overlapping sequences in these samples. UDS enables greater sensitivity, quantitation of sequence variant abundance and clonal analysis of a given DNA region. We thus decided to set up and validate an Ultra-Deep Sequencing (UDS) BCR-ABL KD mutation screening assay in Ph+ leukemia patients with response or resistance to TKIs therapy in order to fully characterized the spectrum of minor variants (<10%-15%) as point mutations, insertion and deletions. A series of additional experiments aimed to investigate the technical precision, robustness and reproducibility of our approach were performed on the Roche GS Junior instrument.

Patients and methods

Patients and samples. 175 samples of bone marrow or pheripheral blood from 77 CML and 24 Ph+ ALL who received one or multiple lines of TKI therapy, sent to the " Institute of Hematology and clinical oncology L. e Ariosto Seragnoli" for diagnostic assessment, were selected for this study. These samples, collected at different timepoint of disease, had also been selected according to their known mutational status previously assessed by conventional Sanger sequencing. All patients

provided informed consent for participation in this study. Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation and then stored at –20°C in guanidinium thiocyanate until use.

Positive and negative controls. BaF3 cell lines carrying the T315I Abl mutations and a wild-type cell lines for Abl mutations (kindly provided by Michael Deininger and Brian Druker, Oregon Health and Science University, Portland, OR) were used as positive and negative controls. From two up four RNA samples, randomly selected from our patients, and two RNA samples, from Ph+ ALL patients already analyzed by UDS on a Roche GS Junior instrument in Prague, were also used as positive control.

RNA extraction, c-DNA sinthesys and 1st step amplification. Total cellular RNA was extracted from mononuclear cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and spectrophotometrically quantified by 260 nm absorbance. Its integrity was also assessed by electrophoresis on ethidium bromidestained 2% agarose gel. One microgram of total cellular RNA was reverse transcribed to cDNA in 20 µL final volume using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). 1st amplification step was performed using 2.5 µl of c-DNA and the following primers: either Fwd-BCR-p190 (located on BCR mRNA exon 1a on) or Fwd-BCR-p210 (located on BCR mRNA exons 12/13) and a Rev-ABL (located on Abl mRNA exon 10) (Figure 1). This procedure ensure that the normal, non-rearrange ABL transcript, was not analysed. The first-step amplification was performed with the Fast Start High Fidelity PCR System kit (Roche). Reactions were carried out in a 25-µL final volume containing 2.5U Fast Start High Fidelity polymerase, 2.5 µL 10X Buffer with 18 mM $MgCl_2$, 0.5µL dNTP mix (10 µM each), and 12.5 pmoles of each primer. An initial denaturation step of 5 min at 95 °C was followed by amplification for 30 cycles (denaturation for 30'' at 95 °C, annealing for 30'' at 60 °C, extension for 2'30'' at 72 °C) and final extension for 7 min at 72 °C.

Amplicon library preparation. The optimal length of fragments for Roche GS Junior analysis is 400 bp; give that a certain degree of overlap between fragments needs to be ensured we decided that the optimal number of amplicons to be created to ensure a reliable and comprehensive mutation scanning strategy is four. Thus, a 2nd amplification step was then performed to generate 4 partially overlapping amplicons that covering the whole kinase domain of ABL: Amplicon1 (358 bp), Amplicon2 (357 bp), Amplicon3 (309 bp) and Amplicon 4 (337 bp). Samples harboring multiple mutations were analyzed to gain further insights into the clonal architecture of mutated

populations. To this purpose, two further ad hoc" amplicons (Amplicon 5 and Amplicon 6), able to mapping multiple mutations fell in KD regions <450 bp, were designed allowing to generate clonal reads for cases harbouring a P-loop mutation plus a threonine 315 or phenylalanine 317 mutation, and for cases harbouring a threonine 315 or phenylalanine 317 mutation plus a methionine 315, phenylalanine 359 or A-loop region mutation: the most frequent mutation combination (Figure 2). Re-amplification of 1 µl of the first PCR product was performed using the FastStart High Fidelity PCR System kit (Roche Applied Science, Mannheim, Germany) and specific fusion-primers. For our analysis MID sequences were selected among those provided in the Technical Bullettin no.005-2009. 2nd step amplification reactions were carried out in a 50-µL final volume containing 2.5 µL cDNA, 2.5U Fast Start High Fidelity polymerase, 2.5 µL 10X Buffer with 18 mM $MgCl_2$, 0.5 µL dNTP mix (10 µM each) and 10 pmoles of each primer. An initial denaturation step of 5 min at 95 °C was followed by amplification for 30 cycles (denaturation for 20'' at 95 °C, annealing for 20'' at 69 °C, extension for 20'' at 72 °C) and final extension for 7 min at 72 °C. The presence of aspecific products or primer dimers was ruled out by gel-electrophoresis on a 2% agarose gel. 1st step PCR primers sequence, fusion primer sequence and MIDs are detailed in Table 1-2-3.

Purification and quantification of library. The amplicons were first purified with the Agencourt AMPure XP magnetic beads (Beckman Coulter) that bind to dsDNA molecules greater than 100 bp in size, and subsequently quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) for generating the standard curve to ensure that the products fall within the quantifiable range. The procedure of amplification, purification and quantification was done according to the Amplicon Library Preparation Method manual, GS Junior Titanium Series, May 2010 (454 Life Sciences, Branford, CT).

UDS of the BCR-ABL KD. Equimolar pooling of amplicons was followed by clonal amplification of single DNA molecules on beads according to the emPCR Amplification Method Manual, Lib-A (454 Life Sciences). GS Amplicon Variant Analyzer (AVA) software (version 2.7; 454 Life Sciences) and Sequence Pilot software (version 4.0.1; JSI Medical Systems, Kippenheim, Germany) were used to align sequence reads to the ABL reference sequence (Genbank accession no. X16416) and calculate variant frequencies. Reads from both orientations and from overlapping amplicons were combined into a single alignment and primer regions were automatically trimmed to avoid artifacts deriving from nucleotide synthesis errors. The presence of all relevant mutations was also manually verified by inspection of individual flowgrams at the corresponding positions with particular attention to homopolymeric regions. Emulsion breaking and bead recovery, enriched

DNA-containing beads were performed according to the Sequencing Method Manual for the Titanium sequencing kit (454 Life Sciences).

Conventional sequencing of the BCR-ABL KD. Direct sequencing was performed on all the samples using an ABI PRISM 3730 (Applied Biosystems). The nested PCR product were purified (Qiaquick PCR Purification Kit, Qiagen) and sequenced, both strands for each fragment, using Big Dye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems) according to manufacturer's instructions. Removal of unincorporated dideoxynucleotides 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).

Results

Sensitivity and limit of detection of the UDS approach. Initial experiments were performed to test the sensitivity and to assess the lower detection limit of our method. Six distinct amplicon libraries generated by serial dilution of a T315I-positive BaF3 cell line (at the following ratios: 50%:50%; 25%:75%; 10%:90%; 5%:95%; 2%:98%; 1%:99%) into an unmutated cell line were pooled and sequenced in the same run. Each amplicon carrying the T315I mutation had been prepared using a separate molecular barcode (MID) and the wild-type reference sample had been prepared with the corresponding matching MID. Our initial results showed high linearity of mutation calling and concordance in detection and quantitation of the T315I mutation over the entire range of dilutions with a lower detection limit at least of 0.1%. As demonstrated in Figure 3 for the T315I mutation the massively parallel sequencing assay revealed an optimal correlation between the measured relative frequency and the distinct dilutions steps. In detail, the mutation load decreased from 54.47% (50% dilution), 22.11 % (25% dilution), 8.28% (10% dilution) 5.04% (5% dilution), 1.75% (2% dilution), 0.95% (1% dilution). By fitting a regression line to this data, we obtained a slope β of 1.079 and a R^2 of 0.99, which indicates a near perfect decrease of the mutation load of the T315I mutation.

Technical precision and reproducibility of the UDS approach. Different experimental were performed to assess the technical reproducibility of our UDS approach. In the first experiment (experiment #1) two RNA samples from Ph+ ALL patients already analyzed by UDS on a Roche GS Junior instrument in Prague and known to be positive for mutations (T315I in sample 1 and 2; S481T in sample 1) were selected. After reverse-transcription the 1st amplification was split to generate, in parallel, six distinct amplicon libraries with six different MIDs. The resulting twelve

libraries (48 amplicons) were then pooled and sequenced in the same run (Figure 4). Results showed that the expected “major” mutation (T315I in Samples 1 and 2) and “minor” mutation (S481T in Sample 1) could be detected and quantified with high concordance across the six distinct library replicates. (Results are given in Figure 5 and Table 4) Importantly, the achieved coverage of the different MIDs used, did not affect the linear relationship. It is important to note that the entire sequencing workflow contains multiple critical steps, e.g. RNA extraction or two different step of amplification may introduce variability and errors during amplification reactions. We then studied whether reproducibility could be maintained when replicating the analysis from the beginning or whether the reverse transcription and first amplification steps, preceding library generation, could introduce variability in the type and/or abundance of variants detected. Therefore, in a second experiment (experiment #2) four RNA samples randomly selected among those to be included in the study (ALL-27-04: T315I, E255K, E255V; LBC-17-01:L387M; MBC-12-01: F359V, L387M, M351T,V379I; CP-05-02: F317L,G250E, C305C, K274E) were thus processed in triplicate starting from the reverse-transcription step. The 12 resulting amplicon libraries had been prepared using 12 different molecular barcode (MID) and then sequenced in the same run (Figure 6). Moreover the entire workflow was independently replicate, restarting from the reverse transcription of the RNA samples to cDNA, also to check for inter-run reproducibility. The first analysis was based on the ALL-27-04 patients harboring three distinct mutations T315I, E255K, E255V with different abundance. The mean of the T315 variant frequency was 61.66% with a standard deviation of 1.17%. For the E255K point mutation the mean of the observed variant frequency was 24.20% with a SD of 3.01% while for the last mutation, an E255V, the mean of variant frequency was 1.84% with a standard deviation of 0.22%. Results showing the mean and variance of the obtained mutation loads are given in Figures 7-8 and Table 5. In a third experiment 15 samples randomly selected among those in which minor (e.g. undetectable by SS; abundance between 1 and 15%) mutations had been detected by UDS and for whom residual RNA for the repetition of reverse-transcription and 1st PCR steps was available, were re-sequenced. Low level mutations previously detected could be confirmed.

Detection of insertion and deletions. After the encouraging results about sensitivity and reproducibility of our analysis we decided to take advantage of UDS approach in order to explore the Bcr-Abl kinase domain aberrations focusing on the detection of small insertions or deletions. Our results revealed a 35-base pair (bp) insertion in 56/77 (73%) CML and 21/24 (87%) Ph+ ALL patients with a variable abundance ranging from 0.1%-96% of all Bcr-Abl transcripts. This sequence

variation, already reported in the literature as '35INS', consists in the retention of 35 nucleotides from intron 8 at the exon 8 to exon 9 border that leads to a truncated BCR-ABL variant having 10 a.a. encoded by intron 8 sequences but lacking 653 C-terminal a.a., including 22 a.a. of the Kinase domain, along with the entire C-terminal region. (Figures 9-10) Re-sequencing a set of samples in the same and independent runs confirmed the presence of the 35INS and demonstrated that this variant was not a PCR or sequencing artifact. Longitudinal analysis showed that the expression of 35INS fluctuated over time with no apparent correlation with response levels. In addition, UDS detected one in-frame deletion consisted of a 72bp deletion (nt.1233-1304) at the junction of exon 6 to exon 7, that causes the loss of 24 residues (a.a. 359-383) of the KD. (Figure 11) This previously unreported variant was found in 26/77 (33%) CML patients and 14/24 (58%) Ph+ ALL patients, with an abundance ranging from 0.2% to 19%. Additional studies about this deletion were ongoing.

IRON II-International study. The robustness, sensitivity and reproducibility of the ultra-deep amplicon sequencing approach based on the 454 technology has been previously explored in several hematologic malignancies. [142,153,154] The pivotal IRON study (Interlaboratory Robustness of Next-generation sequencing) had recently provided first evidence of technical feasibility and high concordance of results across 10 laboratories from 8 countries. [155] More recently robustness and clinical utility of 454 technology were also addressed in an even greater detail in several studies that concordantly demonstrated high reliability and reproducibility for variants with an abundance >1%. [156,157] Currently, the ongoing IRON-II study is setting-up and testing UDS strategies for the detection of a wide spectrum of mutations, including the BCR-ABL kinase domain screening strategy herein presented. In this framework a total of 554 clinical samples (2,216 amplicons) were analyzed by the 10 laboratories - including 517 clinical samples analyzed in parallel by NGS and SS and 30 clinical samples analyzed in parallel by NGS, SS and conventional pyrosequencing. Three hundred and ninety-four of 398 (99%) variants detected by SS were also detected by NGS. In addition, comparison between NGS, SS and conventional pyrosequencing results showed very good concordance with respect to the estimation of variant abundance.

Discussion

We investigated the robustness of an UDS BCR-ABL KD kinase domain approach in chronic myeloid leukemia and Ph+ Acute Lymphoblastic Leukemia patients with response or resistance to Tyrosine Kinase inhibitors. First of all we sequenced serial dilution of a T315I-positive BaF3 cell line into an

unmutated cell line to assess the lower detection limit of our assay and subsequently a series of experiment were performed to explore the reproducibility and precision of our method. Repeated measurements were performed using different molecular barcodes for two representative cases harboring different clinical point mutations. High precision was demonstrated when replicating the libraries on two different GS Junior Sequencing instruments starting off with different emulsion PCR reactions; in both centers, results showed a high linearity of mutation calling and accuracy of mutation detection. The intra and inter run reproducibility were confirmed by a series of experiments in which a set of samples were re-subject to reverse-transcription and 1st amplification step, multiplexed and loaded into the same or different sequencing run. Of note, the 454 amplicon deep-sequencing assay also is capable of detecting larger insertions (exemplary 35ins insertion and 72bp deletion) further underline that this technologies allow a more accurate sequence characterization in comparison to conventional methods. Minor clones harboring insertions or deletions (always involving intron/exon junctions - which implicates alternative or aberrant splicing mechanisms) were found to be very frequent both in CML and in Ph+ ALL patients but, apparently, they did not correlate with response or resistance to TKI therapy. In line with our findings, a very recent functional study has demonstrated that the truncated BCR-ABL protein resulting from the 35INS is kinase-inactive and should not play any role in TKI-resistance. [158] Next-generation sequencing is a robust, powerful and versatile technology, allowed reliable identification of emerging mutants, accessible to a wider and wider number of diagnostic laboratories.

FIGURES

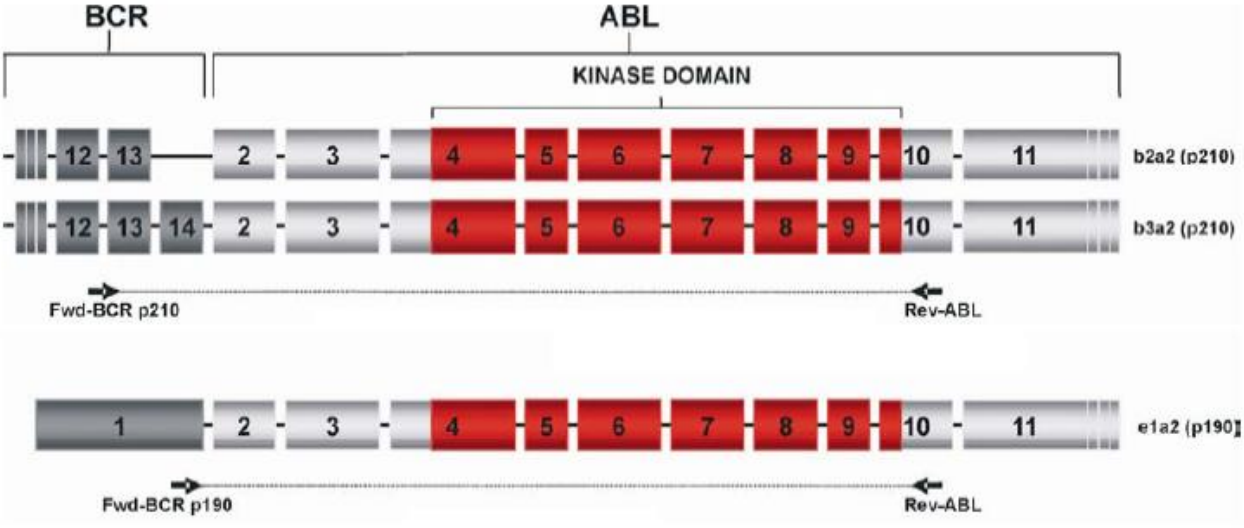


Figure 1 – Map of the first step amplification primers on the ABL KD. Fusion proteins encoded by B*CR-ABL* vary in size due to differences in breakpoint positions and alternative splicing patterns in the *BCR* gene. In most CML and a minority of Ph+ ALL patients, the hybrid transcript displays either 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-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-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).

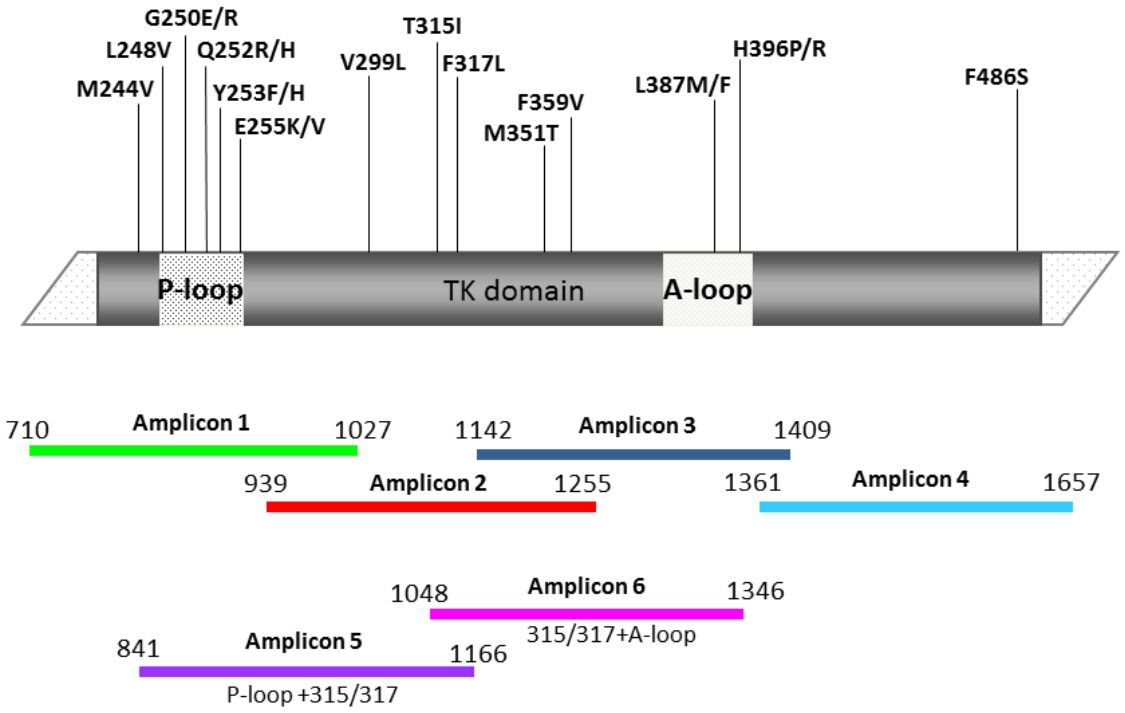


Figure 2 – Map of the sequencing amplicons on the ABL KD. Nucleotide positions are according to the Genbank accession number X16416.1. The position of the most frequent TKI-resistant mutations is indicated, with amino-acid substitutions numbered according to Abl 1a isoform.

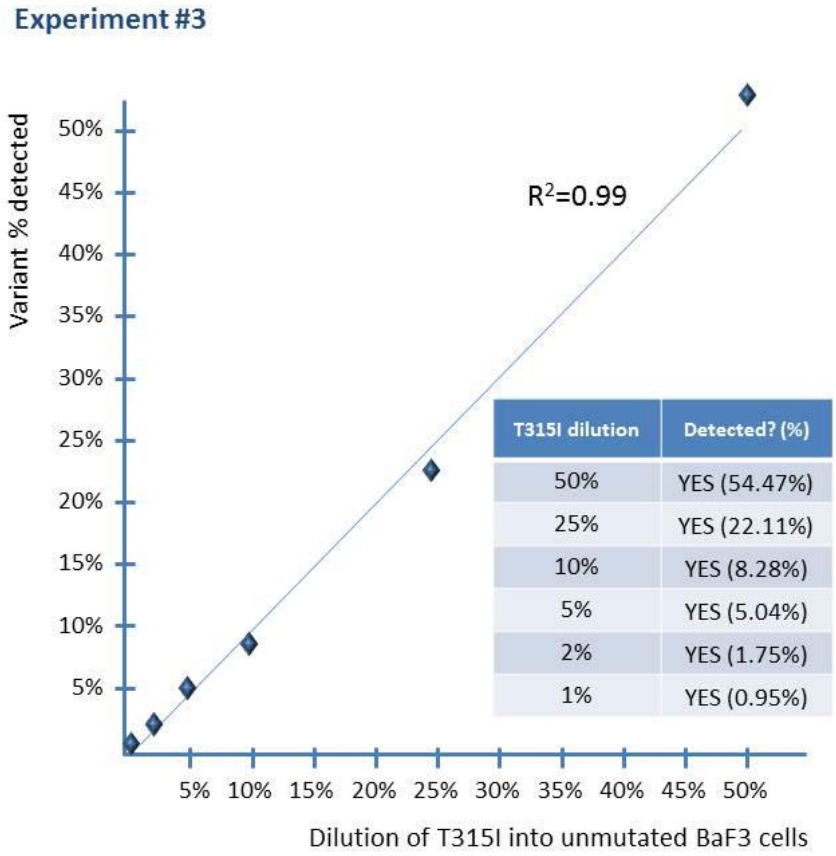


Figure 3 – Linearity and sensitivity of detection. Sequencing of serial dilutions of a BaF3 cell line sample positive for the T315I variant into an unmutated cell line confirmed the applicability of the UDS approach to identify mutations as low as 1% – the lower detection limit that was chosen in this study – and showed very good concordance between dilutions and instrument's assessment of variant abundance (linear regression R^2=0.99, slope 1.079).

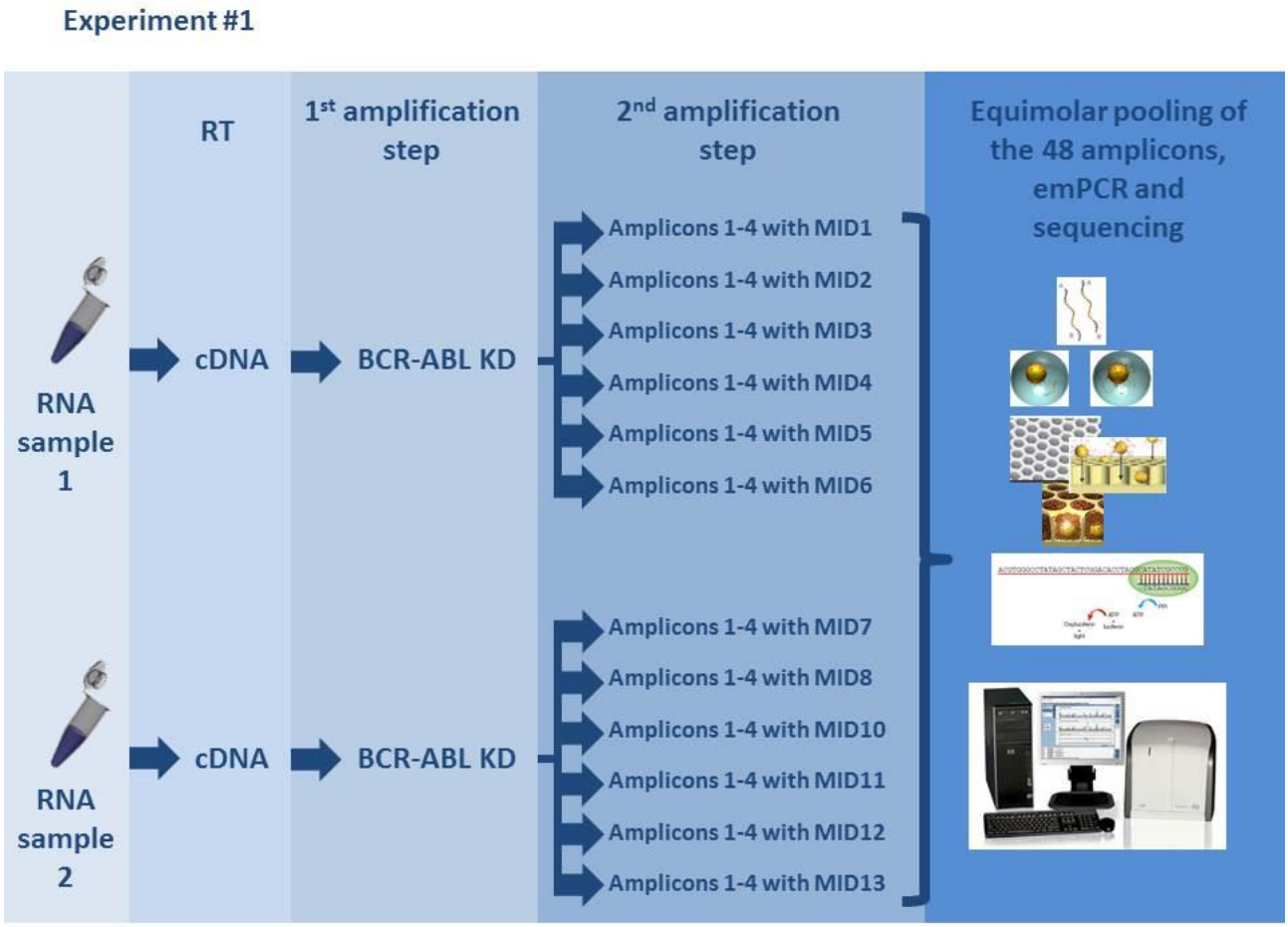


Figure 4 – Outlines of the first experiment on assay reproducibility. Two RNA samples known to be positive for BCR-ABL KD mutations as assessed by SS were converted to cDNA. After the first amplification step, six amplicon libraries were generated in parallel and barcoded with six different MIDs. The twelve libraries (48 amplicons) were then pooled and sequenced in the same run. Abbreviations: RT, reverse transcription; MID; multiplex identifier; emPCR, emulsion polymerase chain reaction.

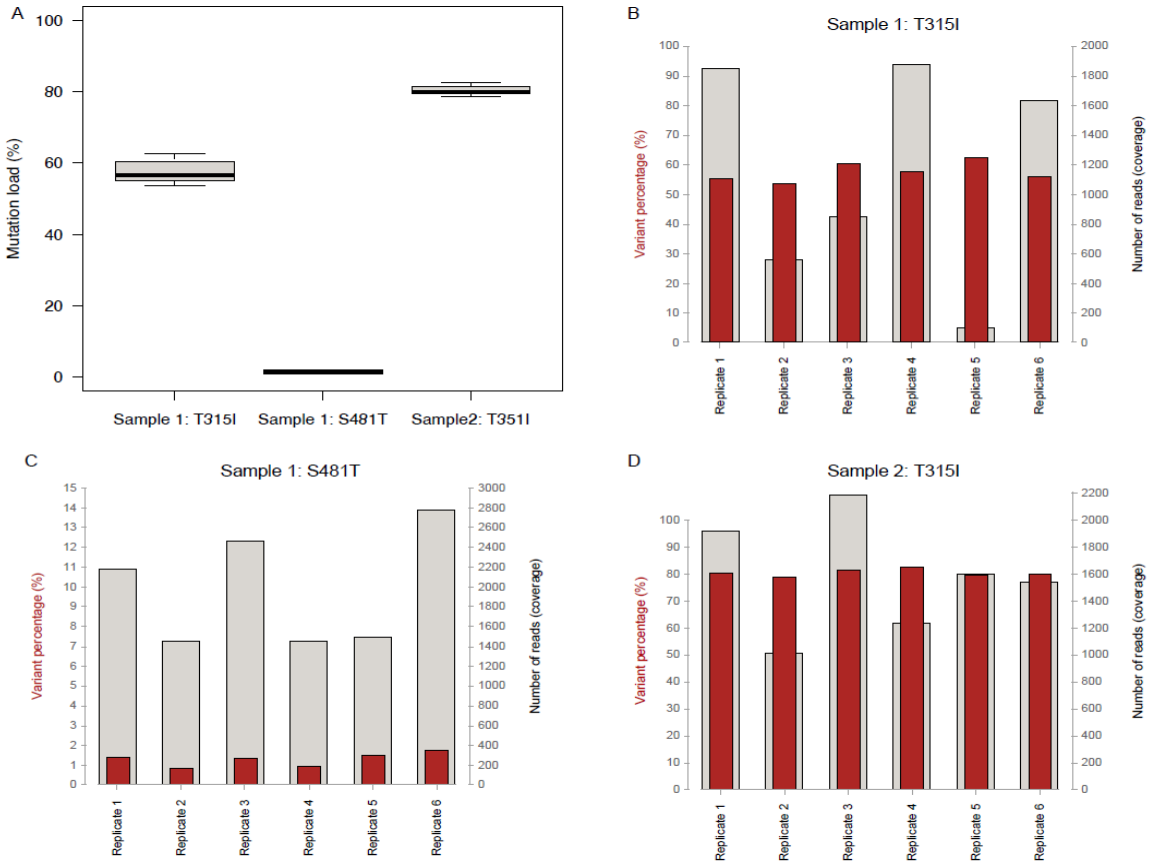


Figure 5 – Data on the first experiment on assay reproducibility. A: Box and whisker plot of the mutation load as measured for Sample 1 and Sample 2. B-D: The left y-axis depicts the percentage of mutated reads as relative variant frequency. The right y-axis indicates the respective coverage that was obtained for the respective replicate samples.

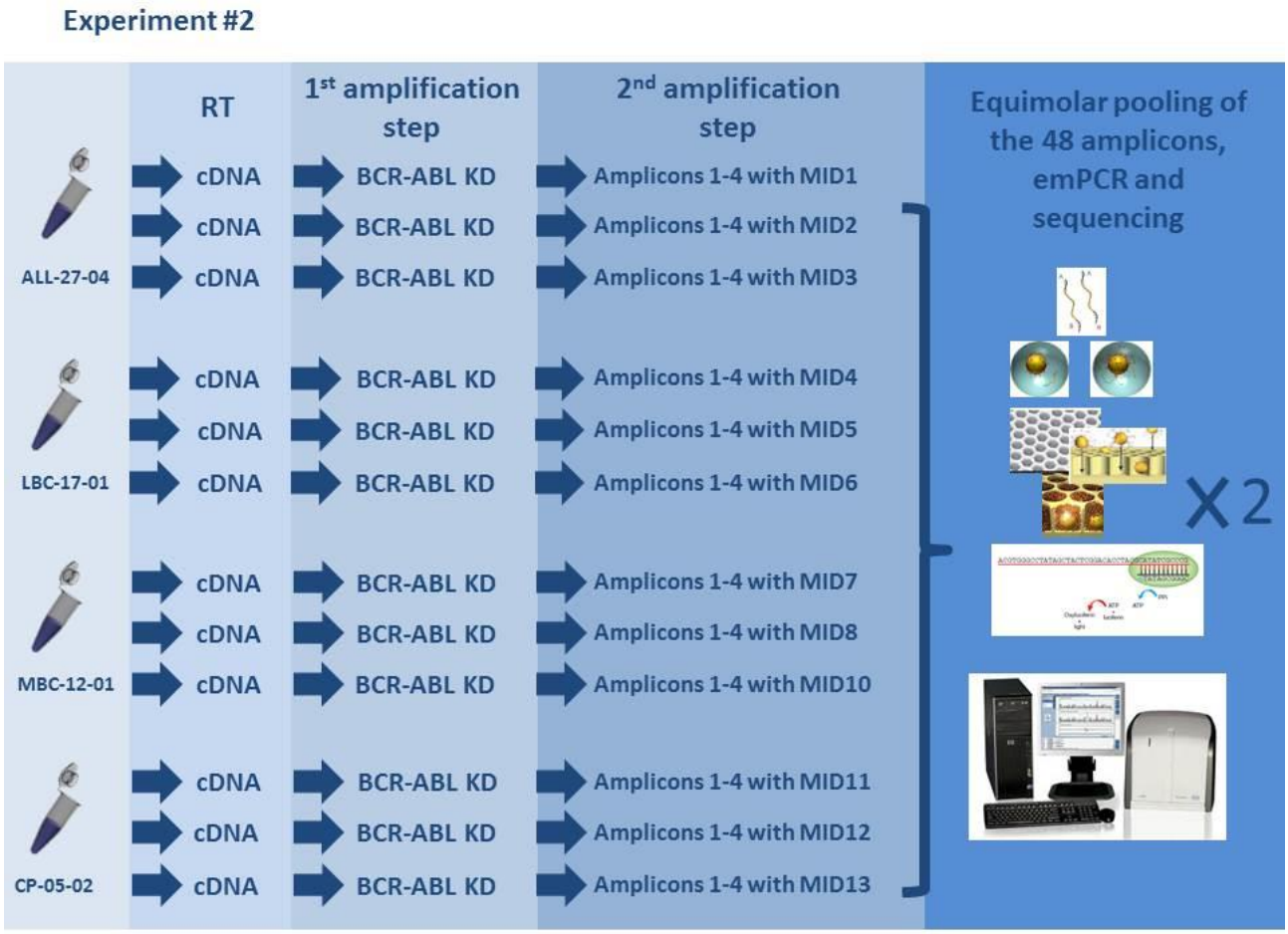


Figure 6 – Outlines of the second experiment on assay reproducibility and robustness of mutation calling. Four RNA samples randomly selected among those to be included in the study were processed in triplicate starting from the RT step. The 12 resulting amplicon libraries were then sequenced in the same run. The entire workflow was repeated twice, restarting from the reverse transcription of the RNA to cDNA, to check also for inter-run reproducibility to check also for inter-run reproducibility. Abbreviations: RT, reverse transcription; MID; multiplex identifier; emPCR, emulsion polymerase chain reaction.

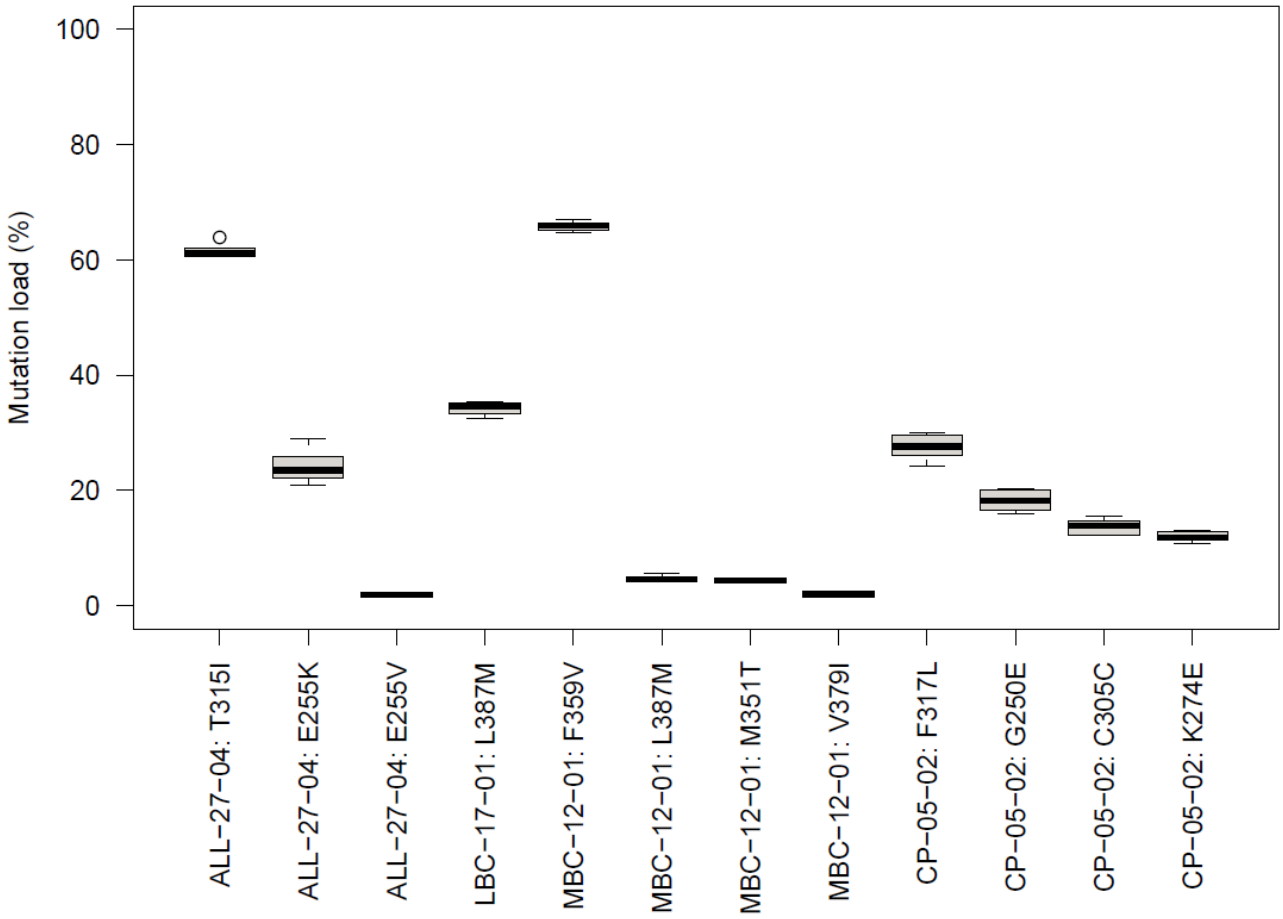


Figure 7 – Box and whiskers plots of the mutation load as measured for samples ALL-27-04, LBC-17-01, MBC-12-01, CP-05-02 in the second experiment on assay reproducibility.

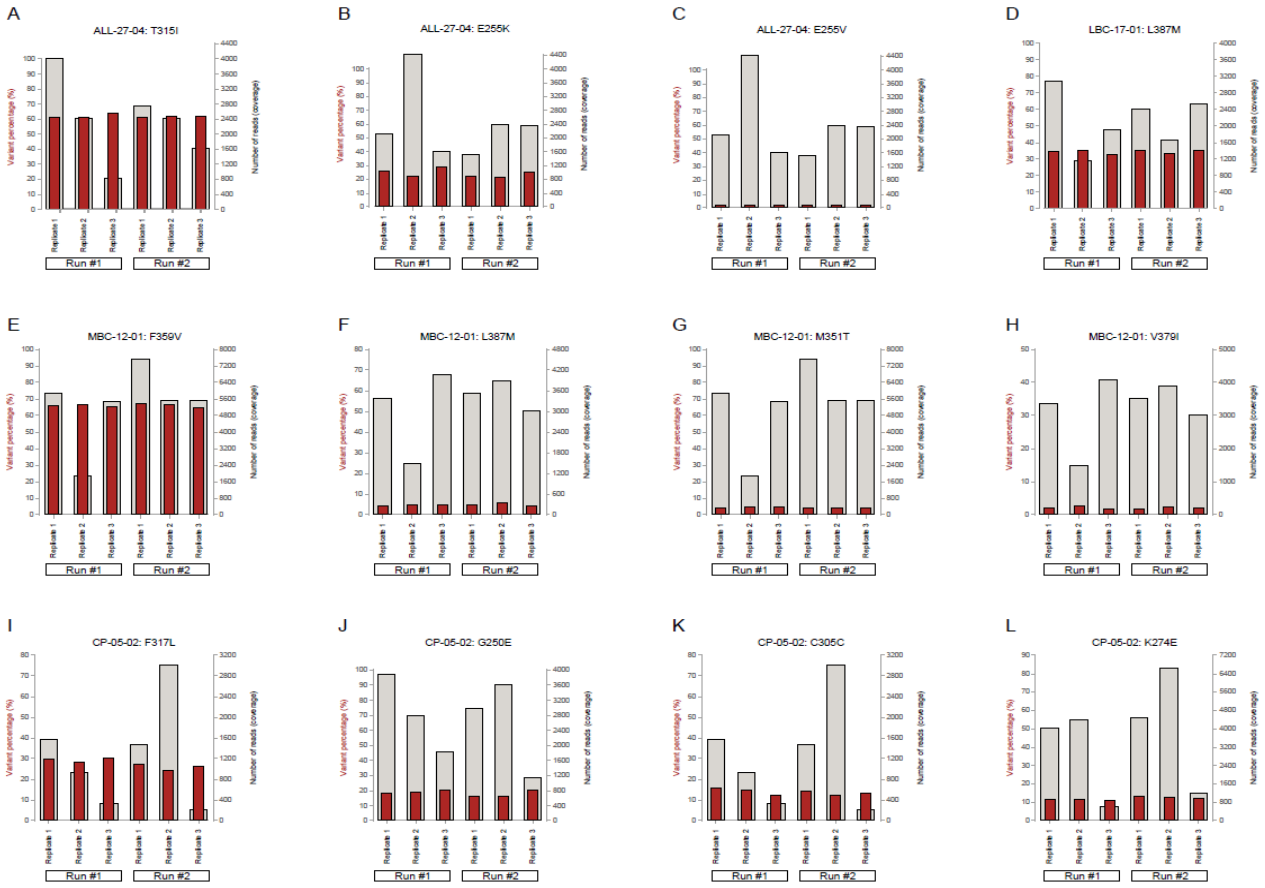


Figure 8 – Panels A-L: relative frequency and coverage for each of the mutations detected in samples ALL-27-04, LBC-17-01, MBC-12-01 and CP-05-02 in the second experiment on assay reproducibility. The left y-axis depicts the percentage of mutated reads as relative variant frequency. The right y-axis indicates the respective coverage that was obtained for the respective replicate samples across the two sequencing runs.

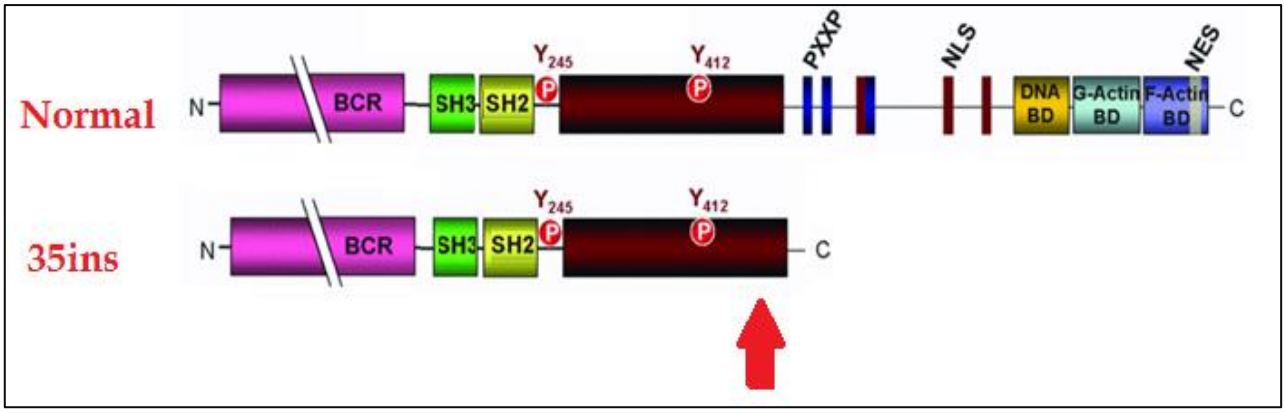


Figure 9 - Normal and 35ins truncated Bcr-Abl protein. Truncated BCR-ABL variant lacking of 22 a.a. of the KD, including the entire C-terminal region which contains a proline-rich domain, 3 Nuclear Localization Signal (NLS), a DNA-binding domain (DNA-BD), two actin-binding domain (G-Actin BD and F-actin BD) and a Nuclear Export Signal (NLS). SH3, Src homology 3 domain; SH2, Src homology 2 domain; Y245 is in the linker region connecting SH2 domain with the kinase domain. Y412 is in the activation loop of the kinase domain.

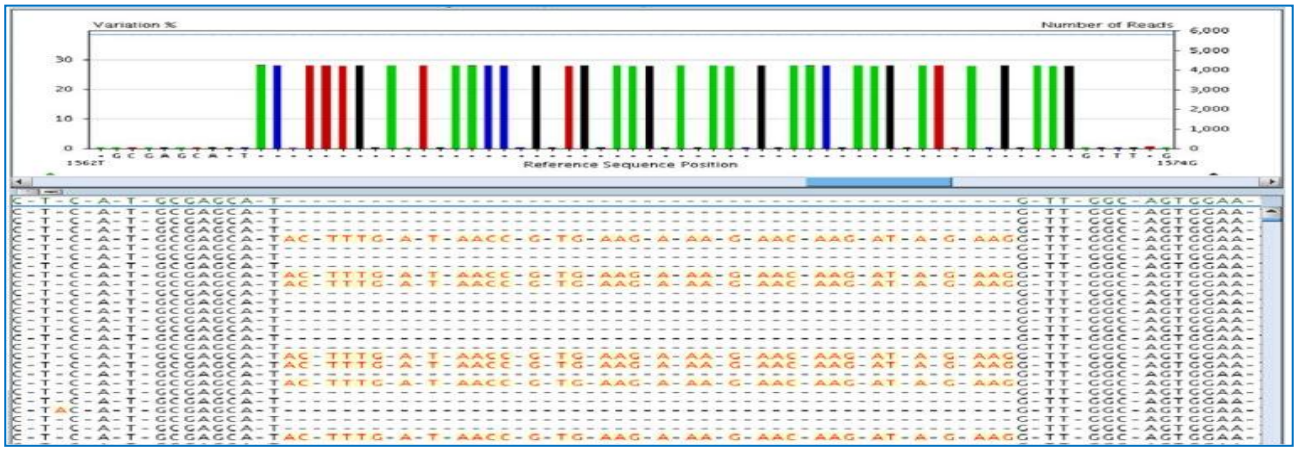


Figure 10 - Screenshot of 35INS sequence reads obtained with AVA software on the Roche GS Junior. At the top a graphical representation of the insertion; at the bottom the 35INS sequence is shown in red.

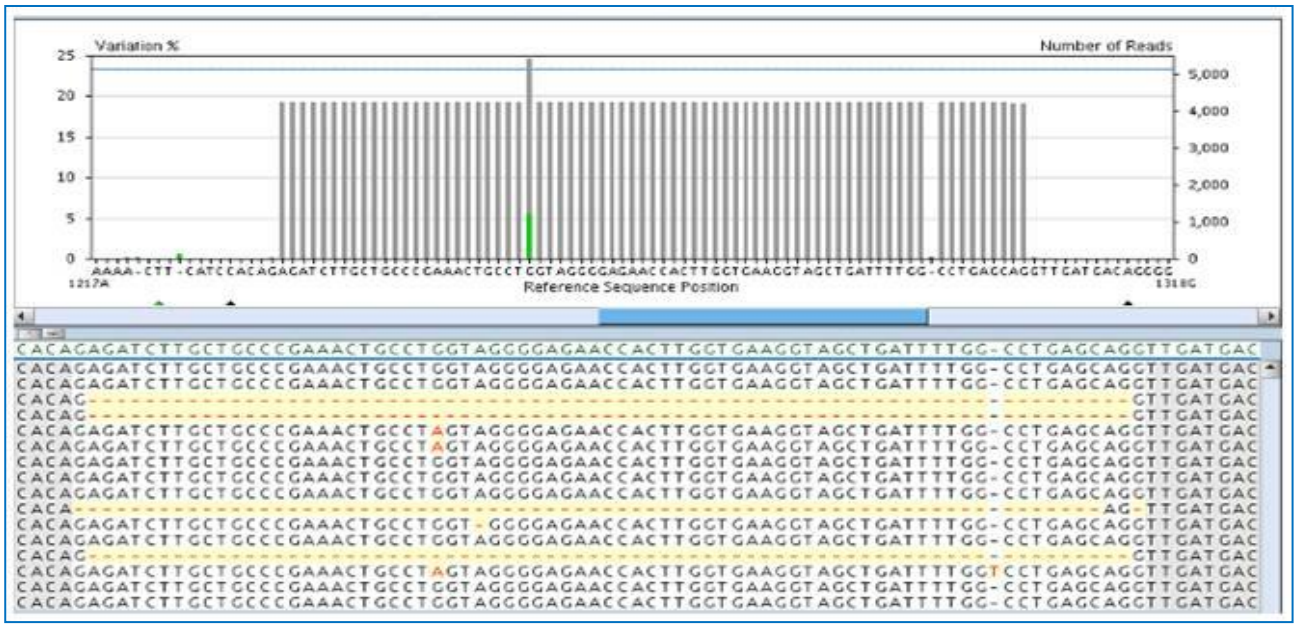


Figure 11 - Screenshot of 72-del sequence reads obtained with AVA software on the Roche GS Junior. At the top a graphical representation of the 72nt deletion; at the bottom the 72-del is shown in yellow.

TABLES

Forward BCR p210 (b2a2- and b3a2)	GAGCAGCAGAAGAAGTGTTTCAGA	**BCR, exons 12/13**
Forward BCR p190 (e1a2)	CAACAGTCCTTCGACAGCAG	**BCR, exon 1**
Rev- ABL	CTTGGAGTGAGGCATCTCAG	**ABL, exon 10**

Table 1 - 1st step amplification primers. 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).

Amplicon 1 (nt.710-1027)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-TGGCAAGCTCTACGTCTCCT-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CTGCACCAGGTTAGGGTGTT-3'
Amplicon 2 (nt.939-1255)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-GAGGGCGTGTGGAAGAAATA-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CAAGTGGTTCTCCCCTACCA-3'
Amplicon 3 (nt.1142-1409)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-ACTACCTGAGGGAGTGCAACC-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-ATACTCCAAATGCCCAGACG-3'
Amplicon 4 (nt.1361-1657)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-AGCCAAGTTCCCCATCAAAT-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CAGCTCCTTTTCCACTTCGT-3'
Amplicon 5 (nt.841-1166)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-TCTATGGTGTGTCCCCCAAC-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-TCTGAGTGGCCATGTACAGC-3'
Amplicon 6 (nt.1048-1346)	**Forward**	5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-AACACCCTAACCTGGTGCAG-3'
	Reverse	5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CAGTCCATTTGATGGGGAAC-3'

Table 2 - Fusion primers for the second step of amplification. 'MID' (multiplex identifier) denotes that a 10 nucleotide sequence was added in this position to 'barcode' amplicons from different samples. Abbreviations: nt., nucleotide.

Amplicons 1-4	MID 1	ACGAGTGCGT
	MID 2	ACGCTCGACA
	MID 3	AGACGCACTC
	MID 4	AGCACTGTAG

Table 3 - MID sequences used in combination with the primers detailed. For our analysis MID sequences were selected among those provided in the Technical Bullettin no.005-2009.

		Repl 1	Repl 2	Repl 3	Repl 4	Repl 5	Repl 6	Mean(%)	Min (%)	Max (%)	SD (%)	CV	95% CI
Sample 1	T315I	55.06	53.67	60.35	57.46	62.50	55.71	57.46	53.67	62.50	3.38	0.06	2.70
	S481T	1.38	0.83	1.34	0.90	1.49	1.69	1.27	0.83	1.69	0.34	0.27	0.27
Sample 2	T315I	80.05	78.68	81.49	82.63	79.47	79.75	80.35	78.68	82.63	1.45	0.02	1.16

Table 4 – Results of the first experiment on assay reproducibility. For the two samples sequenced, as a series of six replicates, a total of three variants are listed. The numbers denote the mutation load, with read depth given in brackets. Abbreviations: Repl, replicate; SD, standard deviation; CV, coefficient of variation; CI, confidence interval.

		Run #1			Run #2								
		Repl 1	**Repl 2**	**Repl 3**	**Repl 1**	**Repl 2**	**Repl 3**	**Mean (%)**	**Min (%)**	**Max (%)**	**SD (%)**	**CV**	**95% CI**
ALL-27-04	T315I	60.86	61.07	63.90	60.94	61.19	61.98	61.66	60.86	63.90	1.17	0.19	0.94
	E255K	25.97	22.19	29.04	22.23	20.97	24.79	24.20	20.97	29.04	3.01	0.12	2.41
	E255V	1.99	1.56	1.70	1.92	1.73	2.15	1.84	1.56	2.15	0.22	0.12	0.17
LBC-17-01	L387M	34.38	35.12	32.57	35.30	33.39	34.74	34.25	32.57	35.30	1.07	0.03	0.85
MBC-12-01	F359V	65.57	66.34	65.16	66.94	66.33	64.81	65.86	64.81	66.94	0.81	0.01	0.65
	L387M	4.35	4.69	4.56	4.82	5.52	4.19	4.69	4.19	5.52	0.47	0.10	0.37
	M351T	4.21	4.67	4.79	4.08	4.40	4.21	4.39	4.08	4.79	0.28	0.06	0.23
	V379I	1.94	2.45	1.64	1.77	2.40	2.03	2.04	1.64	2.45	0.33	0.16	0.26
CP-05-02	F317L	29.56	27.97	30.01	27.22	24.12	26.11	27.50	24.12	30.01	2.20	0.08	1.76
	G250E	17.83	18.59	20.11	16.49	16.03	20.22	18.21	16.03	20.22	1.77	0.10	1.42
	C305C	15.52	14.65	12.14	14.38	12.25	13.48	13.74	12.14	15.52	1.36	0.10	1.09
	K274E	11.35	11.57	10.68	13.11	12.78	12.01	11.92	10.68	13.11	0.91	0.08	0.73

Table 5 – Results of the second experiment on assay reproducibility. Run #1 and Run #2 denotes that the entire workflow was repeated twice in two distinct sequencing runs to check also for inter-run reproducibility. Abbreviations: Repl, replicate; SD, standard deviation; CV, coefficient of variation; CI, confidence interval.

PART II

UNRAVELING THE COMPLEXITY OF TIROSINE KINASE INHIBITORS-RESISTANT POPULATIONS BY AN ULTRA-DEEP SEQUENCING OF THE BCR-ABL KINASE DOMAIN

Background

Although the introduction of the 2nd generation tyrosine kinase inhibitors has revolutionized the outcome of therapy in chronic myeloid leukemias and Philadelphia chromosome–positive (Ph+) acute lymphoblastic leukemias patients, it was observed that the leukemic clone may evolve to evade Bcr-Abl inhibition usually due to selection of point mutation within the kinase domain of BCR-ABL. Now that multiple lines of TKIs were available, in order to assure a more rational therapeutic management of Ph+ Leukemia patients, not only the presence of a mutation but also the actual amino-acid change should be investigated in patients displaying resistance to TKIs. After the encouraging results about the sensitivity, reproducibility and robustness of our UDS-based BCR-ABL KD mutations screening approach we decided to take advantage of this method in order to i) resolve qualitatively and quantitatively the complexity of mutated Ph+ populations surviving TKIs ii) investigate the clonal architecture of mutated populations in the case of multiple mutations occurring within the same amplicon iii) follow the dynamic of this resistant mutations over time in relation to treatment of patients with Ph+ leukemia.

Patients and methods

Patients and sample. We retrospectively selected 33 CML or Ph+ ALL patients who had received sequential treatment with multiple TKIs (among imatinib, dasatinib, nilotinib and ponatinib) and had experienced sequential relapses following the selection of 1 or more TKIs-resistant mutations. Up to 10 samples were analyzed for each patient, for a total of 106 samples. The main characteristics of the patient population analyzed are presented in Table 1. We also randomly selected and analyzed 15 CML patients who had achieved an optimal response to imatinib according to the European leukemia Net (ELN) definitions for comparison. Written informed consent was obtained from all patients, in accordance with the Declaration of Helsinki. Furthermore this study received approval from the Review Board of S Orsola-Malpighi Hospital (Bologna) and of the Institute of Hematology and Blood Transfusion (Prague).

RNA extraction, c-DNA sinthesys and 1st step amplification. RNA extraction, c-DNA synthesis and 1st step amplification were performed as previously described.

Amplicon Library preparation. Amplicon Library preparation was performed as previously described.

UDS of the BCR-ABL KD. UDS was performed using a Roche GS Junior (454-Life Science) as previously described and was designed to enable high sensitivity mutation calling, with a target sequence coverage ranging from 3438 to 9976 independent reads for each nucleotide position with a lower detection limit of 0.2% (with at least 5 independent reads confirming the sequence variation). The target sequence coverage was thus at least 2500 clonal reads for each nucleotide position of interest and ranging from 2850 to 8876. Several published studies have concordantly demonstrated high reliability and reproducibility of 454 pyrosequencing technology for variants present at frequencies ≥1% [159]; for this purpose we decided to reduce the likelihood of false positive results by accepting only variants with an overall abundance > 1%, and to exploit the high coverage only for haplotype and clonal evolution analyses.

Conventional sequencing of the BCR-ABL KD. Direct sequencing of the BCR-ABL KD was performed as previously described.

Results

The BCR-ABL KD mutations status may be more complex than SS show. All the nucleotide substitutions that had been detected by conventional sequencing (defined “major” mutations) were also detected by UDS, with fairly good concordance between the percentage of variant reads assessed by UDS and the mutation abundance estimated from the relative peak height in the SS chromatogram (Table 2). 55% of samples has shown to harboring “minor mutations” (abundance between 1% and 15%) by UDS. The type of minor mutations detected by UDS could frequently be accounted for by TKIs exposure history because half (57 of 111, 51%) could be recognized to be poorly sensitive either to the TKI being administered or to the previous TKI received, or both. A complex clonal texture was observed: in 4 patients (MBC-11, ALL-23, ALL-32, ALL-33). In this patients several Ph+ populations were found to have acquired multiple nucleotide substitutions leading to the same TKI-resistant amino acid change. In two patients UDS was able to distinct two amino-acid substitution: a t>c at the position 1096 and a c>a substitution at position 1098, both resulting in an F317L mutation. This mutations was found in one CML Myeloid-Blast Crisis patient (MBC-11) which not achieved hematologic response to dasatinib and in a Ph+ ALL patient (ALL-32)

that loss cytogenetic response during dasatinib therapy. A g>c and g>t at position 903, both resulting in a Q252H mutation, was found in a Ph+ ALL (ALL-23) with disease progression during dasatinib therapy. Their abundance were 14.19% and 7.49% respectively**.** In the remaining cases, minor mutations were either silent (18% I432I; Y449Y; L340L; E282E) or never reported in association with TKI resistance (28% P465L; T277I; N336S; A399T; L341P; E236G; K262R). Overall, by UDS, up to six (major+minor) mutations could be detected in the samples analyzed. The sequencing of 15 CML patients who had achieved stable optimal response to imatinib were also analyzed by UDS, for comparison, but none were found to harbor point mutations with a cutoff level of >1%.

A complex clonal structure and evolution of mutated population is uncovered by UDS. When multiple mutations fell in KD regions <450 bp, it was possible to design "ad hoc" amplicons mapping to those regions and take advantage of the clonal nature of the sequence reads generated by UDS to see whether multiple mutations belonged to the same (compound mutations) or to different (polyclonal mutations) BCR-ABL transcripts (hence, defined 1 or multiple Ph+ populations) (Figure 1). The great majority of the samples turned out to be a complex mosaic of populations harboring the mutations alone as well as in combination; overall by UDS up to thirteen different mutant subpopulations were found to coexist in the samples analyzed. In general, complexity tended to be higher in samples from patients with advanced-phase (accelerated phase, myeloid and lymphoid blast crisis) CML and from patients with Ph+ ALL. Single mutants were 136 of 274 (49.6%), compound mutants harboring two paired mutations were the most frequent, since they accounted for 78% compound mutants overall identified (Figure 2). Compound mutants harboring three mutations were by far less frequent (10%); only 3 compound mutants harbouring 4 mutations simultaneously were observed. In a patients with Ph+ ALL (ALL-29), with progression disease during treatment with ponatinib, two overlapping peaks at adjacent positions (c>t at 1091 and t>g at 1092) of codon 315 were identified in the SS chromatogram but the resulting amino acid substitution(s) could not be resolved (Figure 3). This patient harboring in addition a E255V mutation. The UDS allow to discriminate between a T315M mutations with an abundance of 28.61% and a T315I mutation with 22.93% of abundance. The dissection of mutated Ph+ clones in this patient identify three distinct and independent clone harboring the T315M, T315I, E255V with an abundance of 25.20%, 20.01% and 5.03% respectively. Surprisingly we observed two Ph+ clones that harboring together a T315M1 and a E255V (3.41% and), and a T315I plus a E255V (2.92%).

The landscape of mutated populations is highly dynamic. Longitudinal quantitative follow-up of mutated populations painted an elaborate picture of how the relative frequency of competing populations can ebb and flow over time and with therapeutic intervention. Some representative examples are illustrated in Figure 4. The switch from a TKI to another determined the fall of previously dominant population(s) and the rise of new dominant one(s), not necessarily preexisting at the time of switchover or, at least, not always detectable at the time of switchover with the level of sensitivity allowed by our experimental approach. A new dominant population could be unrelated to the former, rather arising from an unmutated population (Figure 4 panel A), or could result from the acquisition of new mutations by the former, which generated compound mutants (Figure 4 panel B-C). In several cases, the evolution of the pattern of mutated populations suggested that the same mutation could have been acquired in parallel by independent populations (ie, 1 unmutated and 1 already harboring a mutation) (Figure 4 panel D). In a CML LBC-14, that progressed to lymphoid blast crisis after 32 months of imatinib and never achieving a hematologic response to dasatinb, the UDS approach revealed that mutation populations rise and fall in dominance over time in relation to therapeutic intervention (Figure 4 panel A). The UDS analysis at the time of first relapse detected 7 distinct imatinib-resistant mutated populations: G250E, E255V, E255K, Y253F, G250E+E255V, G250E+E255K and G250E+Y253 with different abundance. Dasatinib treatment cleared these mutants as quickly as in 2 months, but just as quickly a pan-resistant T315I mutant was found to have emerged. The patient achieved a transient hematologic response after 1 month but lost it shortly after detection of T315I. We focused our attention on a Ph+ ALL patients (ALL-32) who first relapse under imatinb with the selection of a Y253H and subsequently relapse on dasatinib therapy following selection of three distinct mutations (Figure 4 panel B). At the time of first relapse, a single imatinib-resistant Y253H mutant that accounted for almost 90% of BCR-ABL–positive cells was detected by SS and UDS. After 3 months of dasatinib therapy, the patient had achieved a complete cytogenetic response although residual disease remained detectable at the molecular level (as assessed by real-time quantitative [RT-Q]-PCR for BCR-ABL transcript). Neither the Y253H (known to be substantially sensitive to dasatinib) nor other mutants were detectable any longer by UDS. After 9 months on dasatinib, the patient was found to have lost the cytogenetic response. UDS showed the coexistence of 3 distinct compound mutants where Y253H was coupled with 3 well-known dasatinib resistant mutations (a T315I and an F317L resulting from 2 different nucleotide substitutions). It might be hypothesized that the original Y253H-positive cells were never completely eliminated by dasatinib and persisted

at very low levels (undetectable by UDS) until they happened to gain a selective advantage again, although de novo acquisition of mutations by previously unmutated cells cannot be ruled out. Interestingly, Y253H and T315I were already detectable by UDS 1 month before. In a patients with CML in lyBC (LBC-15) at the time of second relapse, after 6 months of dasatinib therapy, a dasatinib-resistant F317L mutation was detected (Fig 4 panel C). UDS portrayed a complex scenario with 3 distinct populations where the former imatinib-resistant Y253H and the newly acquired dasatinib-resistant F317L were present alone and in combination, although the Y253H1F317L compound mutant quantitatively dominated over the F317L and Y253H positive ones. It might be hypothesized that the same mutation was acquired in parallel by independent populations (e.g. one unmutated and one already positive for the Y253H). After we analyzed one CML-CP patient (CP-01) that was first treated with dasatinb and subsequently with nilotinib (Figure 4 panel D). After 6 months of second-line dasatinib treatment, during which the patient achieved a complete cytogenetic response but no molecular response, the SS identified a H396R and F317L with an abundance of 50% and 30%. The UDS analysis in addition detected the imatinib-resistant H396R mutant plus 2 additional populations, 1 harboring a dasatinib-resistant F317L and 1 harboring H396R plus F317L together. Three months later, the F317L had become the dominant one, while H396R+F317L and H396R had declined. During dasatinib therapy, a pan-resistant T315I was also acquired because at the time of switchover to nilotinib, a T315I together with H396R+T315I and F317L+T315I compound mutants were already detectable by UDS (4.44%, 0.76%, and 0.22%, respectively). As quickly as in 2 months, nilotinib treatment selected the T315I mutant that expanded, achieving almost full dominance. As represented in figure 5, the clonal analysis of patient with Ph+ ALL in progressive disease has revealed two different point mutations where codons 250 and 253 map. The screenshot shows that part of the sequence reads positive for the G250E mutation (ggg>gag) also carry the Y253H mutation (tac>cac). Selection/deselection of mutated populations could be strikingly rapid. Some compound mutants (M351T+F317L, Y253H+T315I, Y253H+F317L, F359V+T315I) were observed to have higher selective advantage over the respective single mutants. Other compound mutants (H396R+F317L, H396R+T315I, T315I+F317L, E355G+T315I, G250E+F317L, E255V+Y253H, E255V+T315I) were identified that did not overcome the respective single mutants. The E255K+T315I compound mutant was detected in 6 cases: in 3, it became dominant over the E255K and T315I mutants, whereas in another 3 it did not. The triple and quadruple compound mutants detected fluctuated at low levels and were

never able to gain dominance, except in patient LBC-17, suggesting that accumulation of 2 mutations, when tolerated, has almost always limited selective advantage.

Discussion

UDS has revolutionized the way we can approach the study of drug resistant cellular populations highlighting the undetectable minor mutated variants and performing haplotype analysis. Our results suggest that Ph+ leukemia populations there seem to accumulate mutations to escape TKI therapy. In this scenario, testing for resistance-associated mutations is important to guide selection of the most appropriate treatment regimen. We have now found that there may be a high degree of heterogeneity in BCR-ABL KD sequences from patients failing multiple sequential TKIs. In 55% of the samples “major” mutations, with and abundance >10%-15% and detectable by SS), were found to be only “the tip of the iceberg”: UDS revealed that additional “minor” mutations, with an abundance <10%-15% and undetectable by SS might be present, even by using a lower abundance cutoff of 1%. In 51% of the cases, minor mutations could be recognized as poorly sensitive either to the TKI being administered or to the previous TKI received. They most likely corresponded either to outgrowing mutations anticipating an imminent relapse (as for the pan-resistant T315I in samples CP-01-02, AP-10-01, ALL-26-01, ALL-28-01, ALL-29-04) or, more rarely, to “withdrawing” mutants not (yet) entirely deselected by the change in TKI (as in CP-03-06, CP-04-03, CP-06-02, and CP-06-03). In other cases, they could rather be seen as the result of “secondary route(s)” toward resistance followed by some Ph+ cells as an alternative to the one(s) leading to the dominant population(s) (as in AP-10-02, LBC-14-01, ALL-27-03, ALL-32-04, ALL-33-03). One would expect such mutations to be always colocalized (“passengers”) on BCR-ABL molecules already harboring a TKI-resistant mutation (“driver”; as in sample CP-03-05); An even higher degree of complexity emerged when we tried to reconstruct the different haplotypes in the samples harboring multiple mutations (Table 3). Our UDS approach revealed that compound and polyclonal mutations are not 2 mutually exclusive scenarios. Thus, sequential changes in TKI-selective pressure result in heterogeneous mosaics of Ph+ populations harboring different mutations or mutation combinations. Longitudinal observation of the dynamics of these populations in vivo in relation to TKI treatment might suggest that some compound mutants (M351T+F317L, Y253H+T315I, Y253H+F317L) are selectively at an advantage over single mutants, whereas others (H396R+F317L, H396R+T315I, T315I+F317L) are not. On the other hand, the same E255K+T315I (the most frequent compound mutant identified in our samples) was found to

achieve dominance in some cases but not in others, suggesting that the patient-specific context, may shape the fitness of a compound mutant. Our results on the complexity of mutated populations and on their dynamics under sequential TKI treatment concur to depict a model in which evolution of BCR-ABL–positive cells is mainly shaped by TKI-selective pressure (whether microenvironment and/ or the immune surveillance may also be playing a role is currently unknown) and the fitness of each mutated population is the net result of an “absolute” fitness (the ability to survive treatment depending on the intrinsic sensitivity to the specific TKI administered) and of a “relative” fitness (the ability to survive the competition with all other coexisting populations). Here we show that information provided by SS may not always be sufficient to predict responsiveness to a TKI while the information added by UDS would alter TKI selection: in case one of these minor mutated populations were a T315I-positive one, for example, ponatinib might become preferable over dasatinib. However we acknowledge that the clinical significance of low-level mutation detection remains, at present, unclear since it’s difficult to predict their role in relapse and progression. Although it is premature to predict whether UDS will replace SS as the gold standard for BCR-ABL KD mutation analysis further evaluation of this technology is highly warranted.

FIGURES

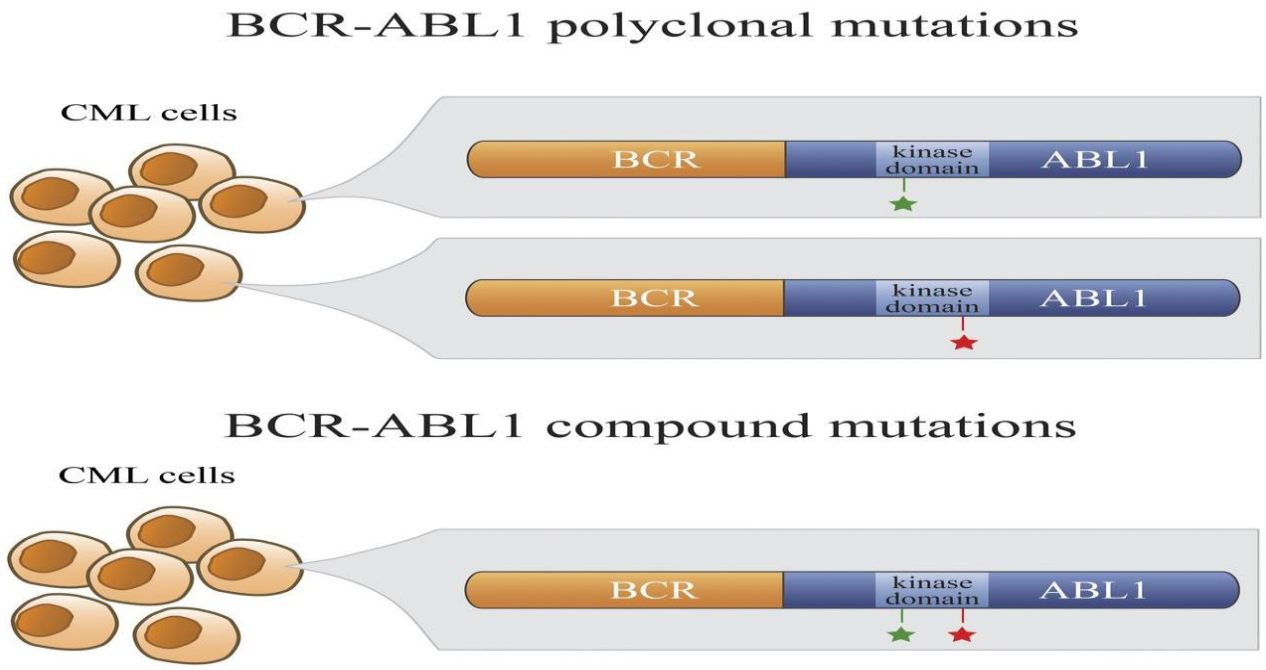


Figure 1 - Polyclonal versus compound mutations. In a subset of patients who develop clinical resistance to ABL1 TKIs, more than 1 point mutation in the kinase domain of BCR-ABL1 is detectable by direct sequencing. In the case of polyclonal mutations, these BCR-ABL1 mutations (green and red stars; top panel) exist separately in different clones. In contrast, BCR-ABL1 compound mutants exhibit 2 mutations within the same BCR-ABL1 molecule (green and red stars; bottom panel)

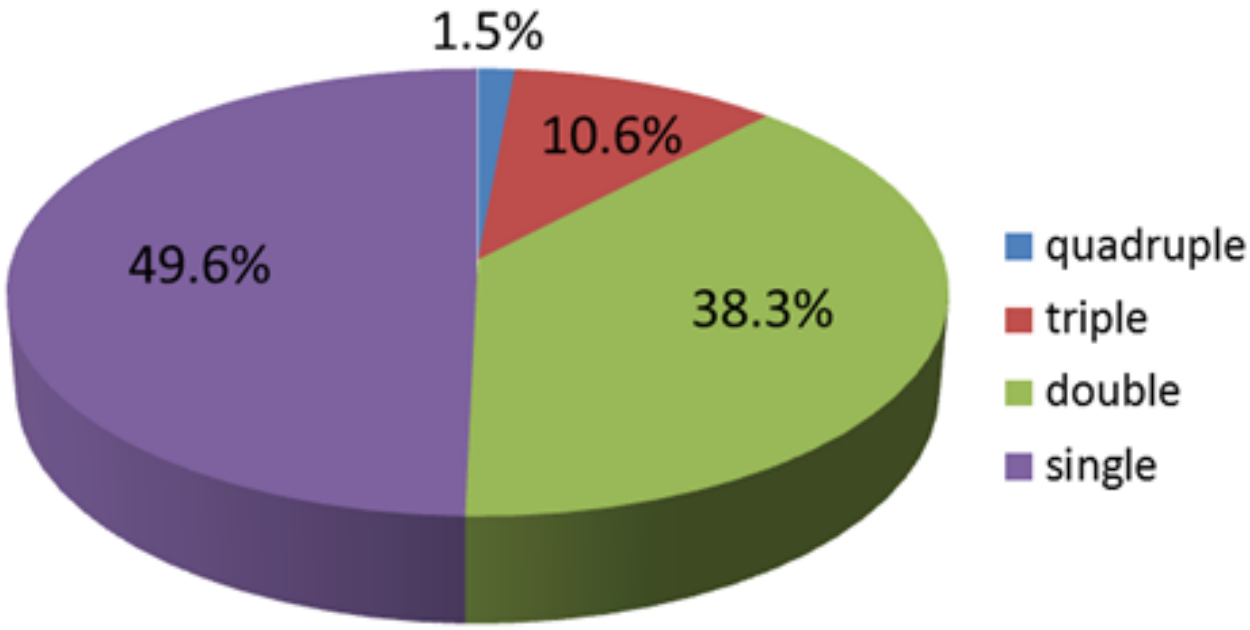


Figure 2 – Relative frequency of single as against compound mutants. Compound mutants harbouring two paired mutations were almost as frequent (38.3%) as single mutants (49.6%) and by far more frequent than triple and quadruple (they accounted for 105 of the 138 (76%) compound mutants overall identified). Mutated Ph+ populations harbouring 3 or 4 mutations in the same *BCR-ABL* molecule were also occasionally detected, but in one case only they were found to have achieved clonal dominance over those with one or two mutations.

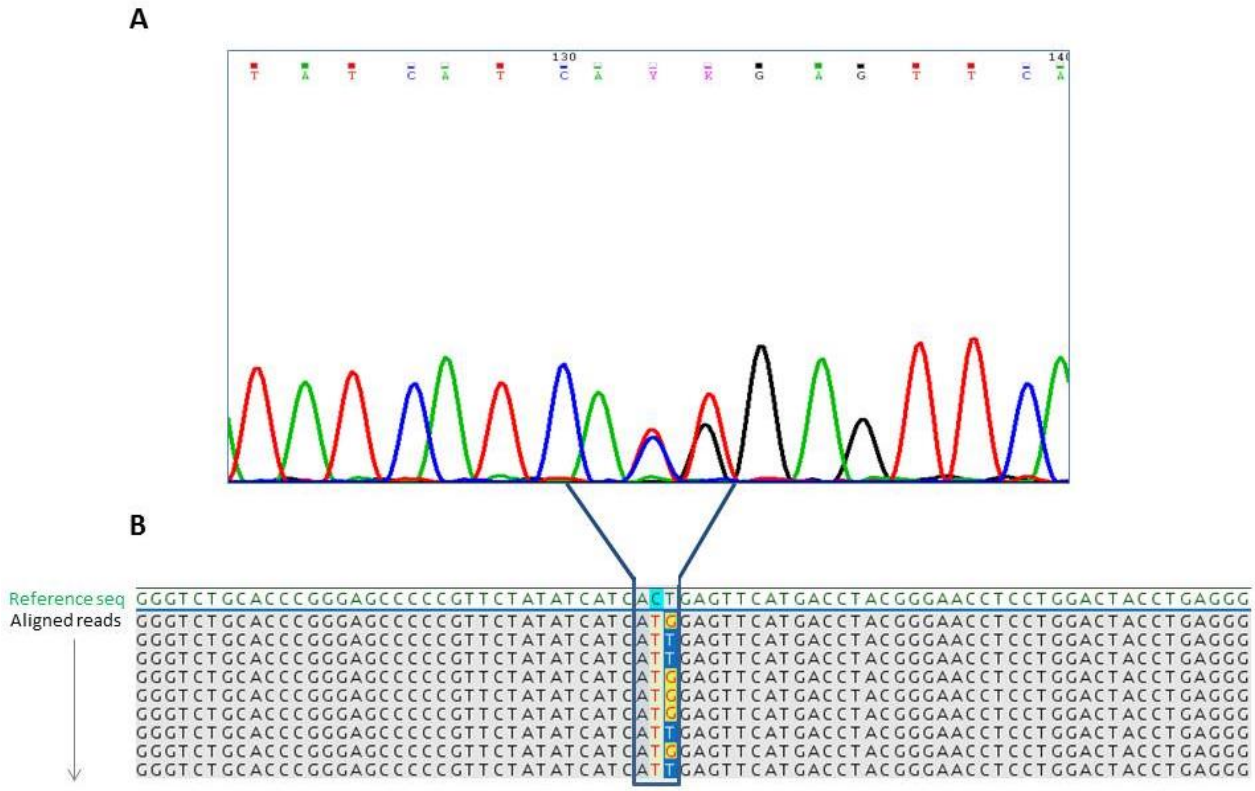


Figure 3 – Example of clonal analysis for sample ALL-29-05. A) conventional sequencing results showing the double nucleotide substitution at codon 315; B) screenshot showing a portion of the global alignment of sequence reads obtained with AVA software, where codon 315 maps. UDS allowed to resolve two distinct populations of mutants at this codon, one harboring the T315I (att) and one harboring the T315M (atg). The reference ABL sequence is shown in green at the top.

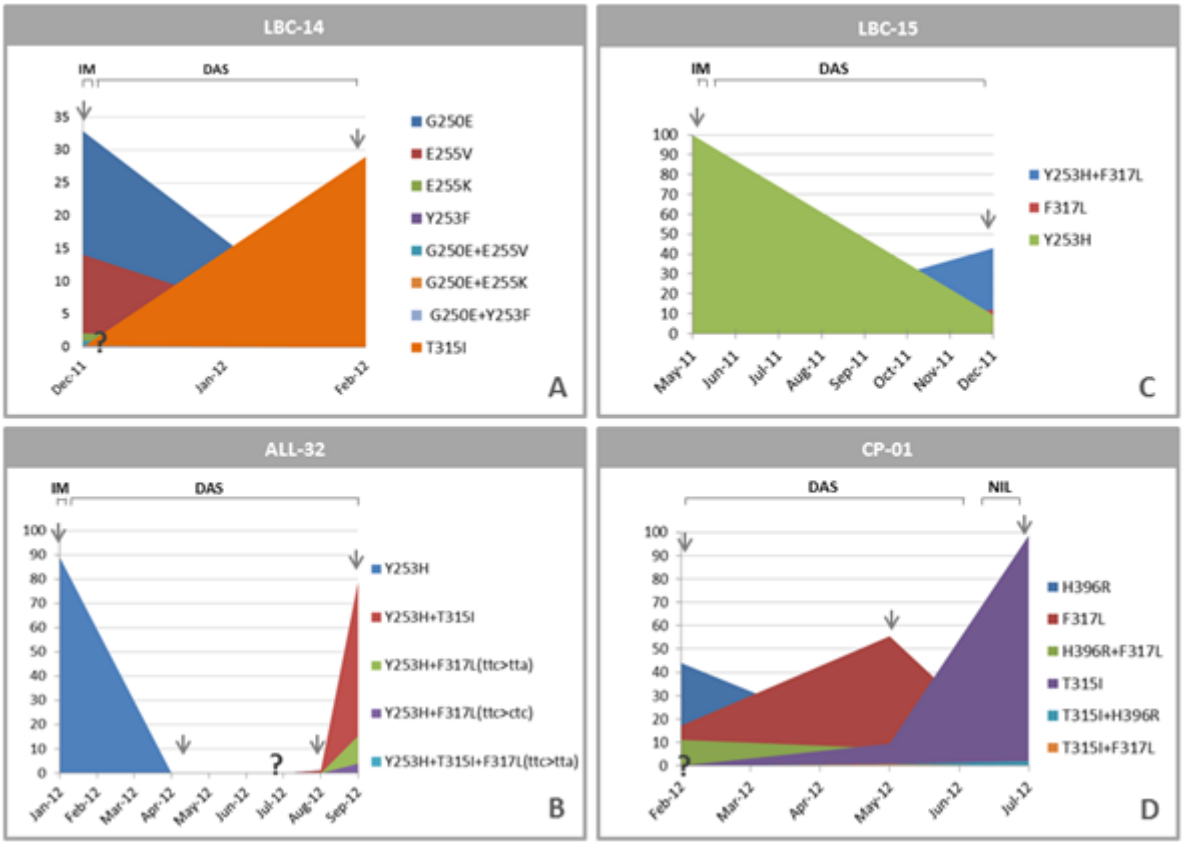


Figure 4 – Mutated populations rise and fall in dominance over time in relation to therapeutic intervention. Graphical illustration of the kinetics of mutated population abundances in four representative cases. Arrows indicate the time-points at which UDS was performed. Patient IDs are as in Table 3.

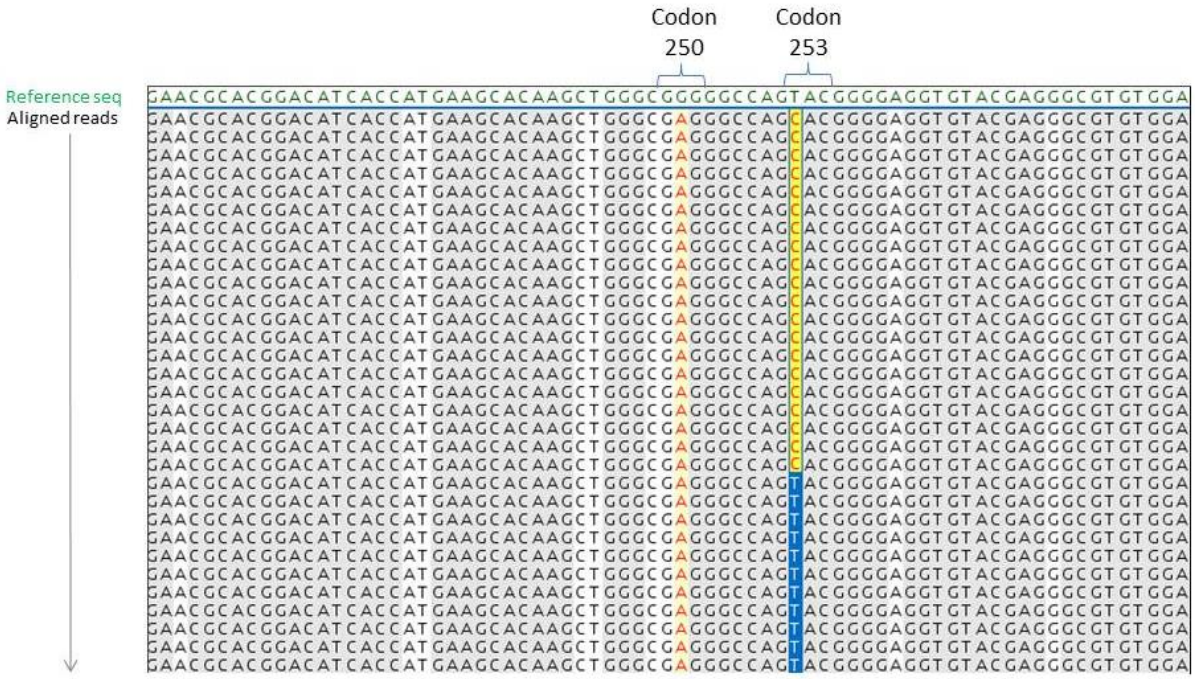


Figure 5 – Example of clonal analysis for sample ALL-33-03. Screenshot showing a portion of the global alignment of sequence reads obtained with AVA software, where codons 250 and 253 map. The reference ABL sequence is shown in green at the top. The screenshot shows that part of the sequence reads positive for the G250E mutation (ggg>gag) also carry the Y253H mutation (tac>cac).

TABLES

Patients, total	33
Median age (range)	52 (18-79)
Male to female ratio	19:14
Disease phase/type:	
- CML	18
- chronic phase	9
- accelerated phase	1
- myeloid blast crisis	3
- lymphoid blast crisis	5
- Ph+ ALL	15
No. of lines of TKI therapy received:	
- 2	22
- 3	8
- 4 or more	3

Table 1 – Features of the patients included in the present study. Abbreviations: TKI, tyrosine kinase inhibitor

Code	Date	TKI	Line	Mutations by SS	Mutations by UDS*	Estimated mutated populations by UDS**	Disease status and response
CP-01-01	29/02/2012	DAS	2	H396R (~50), F317L (~30)	H396R (55.05), F317L (28.23)	H396R (43.99), F317L (17.17), H396R+F317L (11.06)	Complete hematologic response but no cytogenetic response after 3 months on DAS
CP-01-02	02/05/2012	DAS	2	F317L (~70), H396R (~20)	F317L (63.07), H396R (15.74), T315I (5.42)	F317L (55.47),H396R (7.60), H396R+F317L (7.38), T315I (4.44), H396R+T315I (0.76), F317L+T315I (0.22)	Complete hematologic response, cytogenetic response not assessed
CP-01-03	07/07/2012	NIL	3	T315I (~100)	T315I (99.28)	T315I (99.28)	Loss of complete hematologic response
CP-02-01	04/03/2008	IM	1	F359V (~20)	F359V (17.33)	F359V (17.33)	Loss of complete cytogenetic response after 18 months on IM
CP-02-02	02/04/2008	DAS	2	T315I (~100)	T315I (94.80)	T315I (94.80)	Loss of complete hematologic response
CP-03-01	07/03/2005	IM	1	G250E (~100)	G250E (93.72), F317L (1.78)	G250E (92.20), G250E+F317L (1.52), F317L (0.26)	Minor cytogenetic response after 12 months on IM
CP-03-02	14/09/2005	DAS	2	G250E (~70), F317L (~20)	G250E (74.71), F317L (22.51)	G250E (62.00), G250E+F317L (12.71), F317L (9.80)	N.A.
CP-03-03	17/11/2005	DAS	2	G250E (~70), F317L (~30)	G250E (60.73), F317L (27.06)	G250E (46.44), G250E+F317L (14.29), F317L (12.77)	No cytogenetic response
CP-03-04	13/02/2006	DAS	2	G250E (~50), F317L (~40)	G250E (45.47), F317L (37.49), H295H (4.91), C330C (1.48)	G250E (30.46), F317L (20.40), G250E+F317L (12.47), F317L+H295H (2.14), G250E+F317L+H295H (1.19), H295H (0.89), F317L+C330C (0.82), G250E+H295H (0.69), G250E+F317L+C330C (0.47), G250E+C330C (0.19)	No cytogenetic response
CP-03-05	15/05/2006	NIL	3	G250E (~100), E255E (~100)	G250E (87.17), E255E (85.78), F317L (10.44)	G250E+E255E (77.90), G250E+F317L+E255E (7.66), F317L (2.56), G250E (1.61), F317L+E255E (0.22)	No cytogenetic response
CP-04-01	20/10/2005	IM	1	L384M (~100)	L384M (87.04), E255V (15.14)	N.A.	Loss of complete cytogenetic response after 24 months on IM
CP-04-02	22/12/2005	NIL	2	L384M (~70), E255V (~30)	L384M (68.33), E255V (32.02), M351I (2.64)	N.A.	Loss of complete hematologic response
CP-04-03	24/01/2006	NIL	2	E255V (~100)	E255V (80.71), L384M (14.40)	N.A.	No hematologic response
CP-05-01	20/01/2005	IM	1	G250E (~100)	G250E (99.51)	G250E (99.51)	Loss of complete hematologic response after 36 months on IM
CP-05-02	23/03/2005	DAS	2	F317L (~30), G250E (~30)	F317L (24.93), G250E (22.89), C305C (11.70), K274E (10.38)	G250E (16.52), F317L (15.06), C305C (9.30), G250E+F317L (4.30), K274E (4.09) F317L+K274E (3.70), G250E+K274E (1.04), C305C+F317L (0.97), C305+CK274E (0.91), G250E+F317L+K274E (0.51), G250E+C305C+F317L (0.39), G250E+C305C+K274E (0.13)	Complete hematologic response
CP-05-03	20/04/2005	DAS	2	F317L (~100)	F317L (99.50)	F317L (99.50)	No hematologic response
CP-06-01	19/04/2005	IM	1	H396R (~100)	H396R (99.63), A413A (1.71), K247N (1.11)	N.A.	Partial cytogenetic response after 18 months on IM
CP-06-02	17/05/2005	DAS	2	None	H396R (16.07), F317L (7.43)	H396R (16.07), F317L (7.43)	Complete cytogenetic response (=0/200 Ph+ by FISH)
CP-06-03	14/06/2005	DAS	2	F317L (~20)	F317L (20.86), H396R (3.54)	F317L (20.20), H396R (2.88), F317L+H396R (0.66)	Loss of complete cytogenetic response (=30/200 Ph+ by FISH)
CP-	22/03/2007	IM	1	None	A433A (1.48), P408P (1.42),	A433A (1.48), P408P (1.42), K378R (1.32)	No cytogenetic response after 12 months on

07-01					K378R (1.32)		IM
CP-07-02	08/09/2009	NIL	2	T315I (~30)	T315I (25.99), M351T (5.93), T345T (6.37), R332R (5.91)	T315I (20.47), T345T (6.15), R332R (5.75), T315I+M351T (5.30), M351T (0.47), T315I+T345T (0.22), M351T+R332R (0.16)	No cytogenetic response
CP-07-03	04/03/2010	NIL	2	T315I (~70)	T315I (65.85), Y253H (16.65), W235R (3.77), F497L (3.14), T406I (2.43), Q477Q (2.37), F486S (2.28), D363N (1.47)	N.A.	No cytogenetic response; transplanted 6 months later
CP-08-01	22/06/2010	IM	1	M351T (~100), E499E (~100)	M351T (99.84), E499E (99.84)	N.A.	No cytogenetic response after 12 months on IM
CP-08-02	26/04/2012	NIL	2	Y253H (~100), E499E (~100)	Y253H (95.64), E499E (99.67), M351T (3.20)	N.A.	No cytogenetic response
CP-09-01	22/06/2004	IM	1	M244V (~20)	M244V (19.4), H396R (2.88), L298V (1.91), L364I (1.64)	N.A.	No cytogenetic response after 15 months on IM
CP-09-02	14/07/2005	IM	1	M244V (~80)	M244V (79.31), H396R (6.75), L298V (3.75), L364I (3.28)	N.A.	Loss of complete hematologic response
CP-09-03	03/09/2007	DAS	2	M244V (~70), T315A (~60)	M244V (73.36), T315A (57.53), F425S (2.18), T406I (1.75)	N.A.	Complete hematologic response but no cytogenetic response
AP-10-01	07/03/2005	IM	1	E355G (~50)	E355G (43.75), L341P (21.75), F496L (19.63), L428L (11.01), T315I (10.24), Y456Y (4.81)	N.A.	Progression from CP to AP after 9 months on IM
AP-10-02	11/04/2005	DAS	2	T315I (~50)	T315I (42.60), F317L (1.25)	T315I (42.60), F317L (1.25)	Progression to myeloid blast crisis
MBC-11-01	12/04/2010	IM	1	M351T (~100), L248V (~30)	N.D.	N.D.	Loss of complete hematologic response after 6 months on IM
MBC-11-02	23/08/2010	DAS	2	M351T (~100), F317L (~70), L248V (~20)	M351T (100.00), F317L(ttc>tta)(65.52), L248V (19.45), del(248-274) (9.52), F317L(ttc>ctc)(8.52), V299L (1.99)	M351T+F317L(ttc>tta) (52.60), M351T (10.30), M351T+L248V (9.29), M351T+F317L(ttc>tta)+L248V (8.78), M351T+F317L(ttc>ctc) (6.33), M351T+del(248-274)(4.77), M351T+F317L(ttc>tta)+del(248-274)(4.14), M351T+V299L (1.41), M351T+L248V+F317L(ttc>ctc) (1.19), M351T+del(248-274)+F317L(ttc>ctc) (0.61), M351T+F317L(ttc>ctc)+V299L (0.39), M351T+L248V+V299L (0.19)	No hematologic response
MBC-12-01	05/03/2012	IM	1	F359V (~70)	F359V (63.24), L387M (4.18), M351T (3.42), V379I (1.62)	F359V (60.26), L387M (2.47), M351T (2.45), F359V+L387M (1.61), V379I (1.12), F359V+M351T (0.87), F359V+V379I (0.50), M351T+L387M (0.10)	Loss of complete cytogenetic response after 12 months on IM
MBC-12-02	24/09/2012	DAS	2	L387M (~60), T315A (~50), F359V (~20)	L387M (57.43), T315A (57.19), F359V (16.24), T315I (8.22), F317V (3.98), F317L (1.15)	L387M+T315A (55.99), F359V+T315I (8.06), F359V (4.37), F359V+F317V (3.63), T315A (1.20), L387M+F317L (0.97), F317V (0.19), F359V+F317L (0.18), L387M+T315I (0.16), L387M+F317V (0.16), L387M (0.15)	Loss of complete hematologic response
MBC-13-01	28/05/2010	DAS	2	T315A (~100)	T315A (73.75), V299L (8.19)	T315A (72.40), V299L (6.84), T315A +V299L (1.35)	Loss of complete hematologic response after 6 months on DAS
MBC-13-02	09/12/2010	NIL	3	T315A (~100), E255V (~70), G250E (~15)	T315A (92.87), E255V (69.74), G250E (12.22), E255K (1.03)	T315A+E255V (65.07), T315A (15.72), T315A+G250E (10.44), E255V (3.73), T315A+E255V+G250E (0.94), G250E (0.84), T315A+E255K (0.70), E255K (0.33)	No hematologic response

LBC-14-01	20/12/2011	IM	1	G250E (~40), E255V (~20)	G250E (34.28), E255V (15.05), E255K (2.34), Y253F (1.10)	G250E (32.91), E255V (14.09), E255K (2.13), Y253F (0.90), G250E+E255V (0.96), G250E+E255K (0.21), G250E+Y253F (0.20)	Progression to lymphoid bast crisis after 32 months on IM
LBC-14-02	08/02/2012	DAS	2	T315I (~30)	T315I (28.92)	T315I (28.92)	Complete hematologic response
LBC-15-01	26/05/2011	IM	1	Y253H (~100)	Y253H (99.88)	Y253H (99.88)	Complete hematologic response, but no cytogenetic response after 12 months on IM
LBC-15-02	05/12/2011	DAS	2	Y253H (~50), F317L (~50)	Y253H (54.90), F317L (54.40)	Y253H+F317L (43.00), Y253H (11.90), F317L (11.40)	Complete hematologic response but, no cytogenetic response
LBC-16-01	14/03/2005	IM	1	E255K (~100)	E255K (98.84)	E255K (98.84)	Progression to lymphoid blast crisis after 9 months on IM; switch to DAS
LBC-16-02	19/04/2005	DAS	2	E255K (~100), T315I (~100)	E255K (99.84), T315I (99.48), L273S (1.30)	E255K+T315I (97.83), E255K (0.71), E255K+T315I+L273S (1.30), T315I (0.35)	No hematologic response
LBC-16-03	23/05/2005	DAS	2	E255K (~100), T315I (~100)	E255K (99.53), T315I (99.53)	E255K+T315I (99.53)	No hematologic response
LBC-17-01	29/11/2010	IM	1	L387M (~30)	L387M (34.12)	L387M (28.18)	Loss of complete hematologic response after 6 months on IM
LBC-17-02	27/02/2012	DAS	2	L387M (~100), T315I (~50), M318V (~50), F317L (~50), Y320N (~50)	L387M (96.33), T315I (51.46), M318V (51.19), F317L (45.21), Y320N (44.79)	L387M+T315I+M318V (47.90), L387M+F317L+Y320N (42.21), L387M (2.63), T315I+M318V (1.82), F317L+Y320N (1.37), L387M+F317L (1.10), L387M+T315I+M318V+Y320N (0.80), L387M+M318V (0.50), L387M+T315I+F317L (0.36), L387M+Y320N (0.25), L387M+T315I (0.25), L387M+T315I+M318V+F317L (0.17), L387M+T315I+Y320N (0.16)	Loss of cytogenetic and hematologic response
LBC-18-01	11/11/2007	IM	1	F359V (~60)	F359V (57.89)	F359V (57.89)	Progression to lymphoid blast crisis after 3 months on IM
LBC-18-02	17/01/2008	DAS	2	None	F317L (8.48), F317I (1.02), F359V (1.02)	F317L (8.48), F317I+F359V (1.02)	Complete hematologic response, partial cytogenetic response
LBC-18-03	12/02/2008	DAS	2	F359V (~100), F317I (~100)	F317I (92.35), F359V (90.11), F317L (4.25)	F359V+F317I (88.01), F317I (4.34), F317L (3.12), F359V+F317L (1.13), F359V (0.97)	Loss of complete hematologic response
ALL-19-01	27/09/2005	IM	1	Y253H (~100)	Y253H (99.79)	Y253H (99.79)	Hematologic relapse after 6 months on IM
ALL-19-02	03/01/2006	DAS	2	None	P465L (2.78), I432I (1.60), T277I (1.56), E352V (1.41), A474A (1.15)	N.A.	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-19-03	02/03/2006	DAS	2	None	A399V (1.58)	A399V (1.58)	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-19-04	15/06/2006	DAS	2	Y253H (~100), T315I (~50)	Y253H (99.70), T315I (40.29), N336S (5.39), W405R (1.89)	N.A.	Hematologic relapse
ALL-20-01	11/01/2005	IM	1	M351T (~100)	M351T (99.91)	M351T (99.91)	Hematologic relapse after 12 months on IM
ALL-20-02	12/03/2005	DAS	2	None	L370L (5.45), I432I (2.65)	N.A.	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-20-03	16/01/2006	DAS	2	M351T (~100), F317L (~50)	M351T (99.77), F317L (53.27)	M351T (46.50), M351T+F317L (53.27)	Hematologic relapse

ALL-20-04	07/04/2006	DAS	2	M351T (~100), F317L (~100)	M351T (95.46), F317L (85.32), A399T (5.64), Y353H (1.00)	M351T+F317L (85.32), M351T+A399T (5.64), M351T (3.50), M351T+F317L+Y353H (1.00)	Progressive disease
ALL-21-01	30/05/2005	DAS	2	F317L (~100)	F317L (99.99), M237I (2.03)	F317L (97.96), F317L+M237I (2.03)	Hematologic relapse after 3 months on DAS
ALL-21-02	27/10/2005	NIL	3	F317L (~100), Y253H (~20)	F317L (98.59), Y253H (17.35)	F317L (81.24), F317L+Y253H (17.35)	Complete hematologic response but no cytogenetic response
ALL-21-03	16/01/2006	NIL	3	F317L (~100), Y253H (~20)	F317L (100.00), Y253H (18.79)	F317L (81.23), F317L+Y253H (18.77)	Hematologic relapse
ALL-21-04	17/03/2006	NIL	3	F317L (~100), Y253H (~100)	F317L (100.00), Y253H (100.00)	F317L+Y253H (100.00)	Progressive disease
ALL-22-01	23/12/2005	IM	1	F359V (~100)	F359V (99.76), M237T (2.11)	N.A.	Hematologic relapse after 26 months on IM
ALL-22-02	29/01/2005	DAS	2	F359V (~100), T315I (~100)	F359V (99.30), T315I (98.77), Y449Y (10.50)	N.A.	Progressive disease
ALL-23-01	14/09/2011	DAS	2	E255K (~30), T315I (~20)	E255K (24.04), T315I (19.37), G303G (1.51)	E255K (20.88), T315I (16.21), E255K+T315I (3.16), G303G (1.51)	Hematologic relapse after 9 months on DAS
ALL-23-02	27/10/2011	PON	3	T315I (~100)	T315I (99.78), Y312C (1.22)	T315I (98.28), T315I+Y312C (1.22)	Complete hematologic response
ALL-23-03	03/11/2011	PON	3	T315I (~100)	T315I (99.83)	T315I (99.83)	Complete hematologic response
ALL-23-04	15/12/2011	POST-SCT, NONE	/	None	None	None	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-23-05	11/01/2012	POST-SCT, IM	4	E255K (~100)	E255K (99.88)	E255K (99.88)	Complete hematologic response but loss of cytogenetic response
ALL-23-06	25/01/2012	PON	5	E255K (~100)	E255K (99.36), S417S (2.21)	N.A.	Hematologic relapse
ALL-23-07	08/02/2012	PON	5	E255K (~30)	E255K (22.49)	E255K (22.49)	Complete hematologic response
ALL-23-08	15/02/2012	PON	5	E255K (~100)	E255K (99.88)	E255K (99.88)	Partial hematologic response
ALL-23-09	12/03/2012	DAS	6	E255K (~70), T315I (~50)	E255K (76.10), T315I (57.35), Q252H (cag>cac)(14.19), Q252H (cag>cat)(7.49), G250E (1.05)	E255K+T315I (51.60), E255K+Q252H (cag>cac) (13.94), E255K+Q252H (cag>cat) (7.34),T315I (5.75), E255K (2.52), E255K+ G250E (0.70), Q252H (cag>cac) (0.25), G250E (0.35), Q252H (cag>cat) (0.15)	Progressive disease
ALL-23-10	28/03/2012	PON	7	E255K (~100), T315I (~100)	E255K (99.64), T315I (98.56)	E255K+T315I (98.20), E255K (1.44), T315I (0.36)	Progressive disease
ALL-24-01	15/06/2011	NIL	1	Y253H (~30)	Y253H (27.35), P465L (1.55)	N.A.	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-	28/07/2011	IM	2	Y253H (~100)	Y253H (99.99), R367L (3.62)	Y253H (99.95), Y253H+R367L (3.62)	Complete hematologic and cytogenetic

24-02							response, but 1-log increase in BCR-ABL transcript levels
ALL-24-03	01/03/2012	DAS	3	None	Y302S (1.62)	Y302S (1.62)	Complete hematologic and cytogenetic response, no molecular assessment performed
ALL-24-04	29/03/2012	DAS	3	Y253H (~100), T315I (~100)	Y253H (99.10), T315I (99.10), L273L (1.09)	Y253H+T315I (98.01); Y253H+T315I+L273L (1.09)	Hematologic relapse
ALL-25-01	02/12/2011	IM	1	Y253H (~20)	Y253H (18.38)	Y253H (18.38)	Complete hematologic and cytogenetic response, molecularly detectable disease after 18 months on IM
ALL-25-02	18/01/2012	DAS	2	Y253H (~100), T315I (~100)	Y253H (87.69), T315I (86.16)	Y253H+T315I (80.53), Y253H (7.16), T315I (5.63)	Hematologic relapse
ALL-26-01	11/01/2012	IM	1	E255K (~60)	E255K (54.75), F359V (13.81), T315I (3.84)	N.A.	Hematologic relapse after 6 months on IM
ALL-26-02	16/06/2012	DAS	2	T315I (~100)	T315I (99.42)	T315I (99.42)	Progressive disease
ALL-27-01	14/01/2011	IM	1	T315I (~100)	T315I (99.87)	T315I (99.87)	Complete hematologic but not cytogenetic response after 3 months on IM
ALL-27-02	31/01/2011	IM	1	T315I (~100)	T315I (99.74)	T315I (99.74)	Stable disease
ALL-27-03	07/02/2011	NIL	2	T315I (~80)	T315I (75.55), E255K (9.70), E255V (1.53)	T315I (70.47), E255K (5.10), T315I+E255K (4.60), E255V (1.05), T315I+E255V (0.48)	Stable disease
ALL-27-04	21/02/2011	NIL	2	T315I (~60), E255K (~30)	T315I (53.80), E255K (25.63), E255V (1.33)	T315I (46.10), E255K (18.26), T315I+E255K (7.37), E255V (1.00), T315I+E255V (0.33)	Hematologic relapse
ALL-28-01	05/12/2007	IM	1	Y253H (~50), E255K (~20)	Y253H (56.01), E255K (14.92), T315I (8.33), Q252H (6.32), A269T (3.57), Y253F (1.37)	Y253H (52.30), E255K (14.70), T315I (5.29), Q252H (3.92), Y253H+T315I (2.93), Q252H+A269T (2.40), Y253F (1.26), Y253H+269T (0.63), A269T (0.54), Y253H+E255K (0.15), Y253F+T315I (0.11)	Hematologic relapse after 9 months on IM
ALL-28-02	26/03/2008	DAS	2	T315I (~100)	T315I (90.96)	T315I (90.96)	Progressive disease
ALL-29-01	06/06/2012	IM POST-SCT	2	T315?	T315M (30.20)	T315M (30.20)	Complete hematologic response, molecularly detectable disease
ALL-29-02	20/06/2012	IM	2	T315?	T315M (22.32)	T315M (22.32)	Hematologic relapse
ALL-29-03	10/07/2012	IM	2	T315?	T315M (30.28)	T315M (30.28)	Progressive disease
ALL-29-04	01/08/2012	IM	2	T315?	T315M (57.28), T315I (1.09)	T315M (57.28), T315I (1.09)	Stable disease
ALL-29-05	22/08/2012	PON	3	T315?, E255V (~20)	T315M (28.61), T315I (22.93), E255V (12.40)	T315M (25.20), T315I (20.01), E255V (5.03), T315M+E255V (3.41), T315I+E255V (2.92)	Progressive disease
ALL-30-01	30/03/2010	NIL	1	E255V (~40)	E255V (34.70)	E255V (34.70)	Complete hematologic response, molecularly detectable disease after 1month on IM

ALL-30-02	12/05/2010	IM	2	E255V (~100)	E255V (91.90), V304A (3.91), L302R (1.61), G303W (1.61)	See Figure 3	Complete hematologic response, molecularly detectable disease with 1 -log increase in BCR-ABL transcript levels
ALL-30-03	19/07/2010	IM	4	E255V (~50), Y253H (~50)	E255V (46.50), Y253H (39.30), E255K (1.02)	See Figure 3	Hematologic relapse
ALL-30-04	07/09/2010	DAS	5	E255V (~100)	E255V (91.60), T315I (2.00), E255K (1.80), K262R (1.00)	See Figure 3	Stable disease
ALL-30-05	05/10/2010	DAS	5	E255V (~50), T315I (~30)	E255V (53.00), T315I (23.00), Q252E (14.30), E255K (2.10)	See Figure 3	Progressive disease
ALL-31-01	04/04/2006	IM	1	F317L (~100)	F317L (99.64)	F317L (99.64)	Hematologic relapse after 5 months on IM
ALL-31-02	06/06/2006	DAS	2	D276G (~50), F317L (~50), T315A (~50)	T315A (51.12), F317L (45.81), D276G (44.86)	F317L (33.45), T315A+D276G (32.00), T315A (17.87), F317L+D276G (11.11), D276G (1.25), T315A+F317L (0.75), T315A+F317L+D276G (0.50)	Hematologic relapse after transient hematologic improvement
ALL-31-03	14/07/2006	IM	3	F317L (~100)	F317L (99.76), R332R (4.17)	F317L (95.59), F317L+R332R (4.17)	Stable disease
ALL-31-04	23/08/2006	IM	3	T315A (~100), G250E (~100)	T315A (100.00), G250E (90.48)	T315A+G250E (90.48), T315A (9.52)	Progressive disease
ALL-31-05	26/09/2006	NIL	4	T315A (~100), G250E (~100)	T315A (99.83), G250E (99.83)	T315A+G250E (99.83)	Progressive disease
ALL-31-06	24/10/2006	NIL	4	T315A (~100), G250E (~60), D276G (~30), Y253H (~30)	T315A (99.96), G250E (67.93), D276G (28.89), Y253H (27.21), Q252E (1.49)	T315A+G250E (65.82), T315A+Y253H+D276G (26.02), T315A (3.76), T315A+G250E+D276G (2.11), T315A+Q252E (0.78), T315A+Y253H (0.48), T315A+Y253H+D276G+Q252E (0.48), T315A+D276G (0.28), T315A+Y253H+Q252E (0.23)	Progressive disease
ALL-32-01	06/01/2012	IM	2	Y253H (~100)	Y253H (99.79)	Y253H (99.79)	Hematologic relapse
ALL-32-02	12/04/2012	DAS	3	None	None	None	Complete hematologic and cytogenetic response, molecularly detectable disease
ALL-32-03	08/08/2012	DAS	3	None	Y253H (2.49), T315I (1.19)	Y253H (1.30), Y253H+T315I (1.19)	Complete hematologic and cytogenetic response, 2-log increase in BCR-ABL transcript levels
ALL-32-04	17/09/2012	DAS	3	Y253H (~100), T315I (~60), F317L(ttc>tta) (~20)	Y253H (100.00), T315I (79.11), F317L(ttc>tta)(15.70), F317L(ttc>ctc)(4.04)	Y253H+T315I ((78.77), Y253H+ F317L(ttc>tta)((15.36), Y253H+ F317L(ttc>ctc)(4.04), Y253H (1.49), Y253H+T315I+F317L(ttc>tta)(0.34)	Hematologic relapse
ALL-33-01	27/03/2005	IM	1	G250E (~100)	G250E (99.50)	G250E (99.50)	Hematologic relapse
ALL-33-02	05/09/2005	DAS	2	G250E (~100), F317L (~70)	N.D.	N.D.	Hematologic relapse after a 5-month complete hematologic and cytogenetic response
ALL-33-03	01/12/2005	NIL	3	G250E (~100), F317L (~50), Y253H (~30)	G250E (99.99), F317L(ttc>tta)(43.63), Y253H (26.36), V299L (4.84), L248R (1.89), F317L(ttc>ctc)(1.01)	G250E (35.54), G250E+F317L(ttc>tta) (31.90), G250E+Y253H (14.86), G250E+F317L(ttc>tta)+Y253H (9.84), G250E+V299L (2.72), G250E+L248R (1.13), G250E+Y253H+V299L (0.99), G250E+F317L(ttc>tta)+V299L (0.77), G250E+F317L(ttc>tta)+L248R (0.76), G250E+F317L(ttc>ctc) (0.70), G250E+F317L(ttc>tta)+V299L+Y253H (0.36), G250E+F317L(ttc>ctc)+Y253H (0.31)	Progressive disease

Table 2 – Comparison between mutations detected by SS and mutations detected by UDS and estimated clonal composition of the samples harbouring multiple mutations as assessed by UDS. For SS results, mutation relative abundance was assessed on the basis of variant peak height. In the 'TKI' column, the TKI being administered at the time of analysis is indicated; IM stands for imatinib, DAS for dasatinib, NIL for nilotinib and PON for ponatinib. In the 'line' column, the number of different lines of TKI therapy that had been administered to the patient is indicated. Mutations detected by UDS but not by SS are highlighted in red. Disease status and response at each timepoint are also detailed. In patient ALL-29, 'T315?' denotes that two overlapping peaks at adjacent positions (c/t at 1091 and t/g at 1092) of codon 315 were identified in the SS chromatogram and the resulting amino acid substitution(s) could not be resolved. In patients MBC-11, ALL-23, ALL-32 and ALL-33 the same amino acid changes were found to result from different nucleotide substitutions at the same codons (specified in parentheses). N.A. (not assessable) indicates that clonal analysis could not be possible because of multiple mutations located >450bp apart. N.D. (not done) indicates that amplification with fusion primers was unsuccessful and the sample could not be analyzed with UDS. Other abbreviations: CML, chronic myeloid leukemia; CP, chronic phase; AP, accelerated phase; MBC, myeloid blast crisis; LBC, lymphoid blast crisis; ALL, Ph+ acute lymphoblastic leukemia; SCT, stem cell transplantation. * Cut-off set at variants ≥1%, ** % calculated after manual visual inspection of nucleotide sequences at the specific positions where the variants ≥1% were identified.

Double compound mutants that achieved dominance over single mutants	No. of patients
E255K+T315I	3
M351T+F317L	2
Y253H+T315I	1
Y253H+F317L	1
F359V+T315I	1
Double compound mutants that did not achieve dominance over single mutants	**No. of patients**
E255K+T315I	3
H396R+F317L	1
H396R+T315I	1
T315I+F317L	1
E355G+T315I	1
G250E+F317L	1
E255V+Y253H	1
E255V+T315I	1

Table 3 – Type and recurrence of double compound mutants found by UDS. Some compound mutants were found to achieve dominance over the single mutants; others remained The E255K+T315I compound mutant was found to expand over single mutants in three cases.

PART III

MORE SENSITIVE DETECTION OF DRUG RESISTANCE BCR-ABL MUTATION RELEVANT FOR SECOND-LINE TREATMENT CHOICE BY ULTRA-DEEP SEQUENCING

Background

Second-generation TKIs (2GTKIs) are known to retain a smaller but well defined spectrum of insensitive mutations including Y253H, E255K/V, T315I, F359V/I/C for nilotinib and V299L, T315I/A, F317L/V/I/C for dasatinib and neither is able to overcome the T315I, against which only ponatinib has been reported to be active. (Figure 1) These mutations were found to correlate with poor response rates when already present at the time of imatinib-resistance, before the switch to "2GTKIs" dasatinib, nilotinib or bosutinib. Actually in Piladelphia chromosome-positive leukemia patients who failed imatinib therapy, BCR-ABL KD mutation screening by Sanger sequencing (SS) is recommended since detection of 2GTKIs-resistance mutations influences the selection of the most appropriate second-line therapy. We then wondered what we would find if we analyzed by Ultra-Deep Sequencing (UDS) switchover samples negative for mutation by SS. We thus undertook a retrospective study of Ph+ leukemia patients who were known to have developed 2GTKIs-resistant mutations on second-line therapy to investigate in how many cases these mutations could have been detected at the time of switchover by a sensitive UDS.

Patients and methods

Patients and sample. Between 2006 and 2013, as the Italian and Czech central reference laboratories for BCR-ABL mutation analysis, we have followed 79 imatinib-resistant patients with CML or Ph+ acute lymphoblastic leukemia who failed second-line dasatinib or nilotinib therapy and had evidence of newly acquired mutations at relapse, as assessed by conventional sequencing method. We wondered how many of these mutations could have been detected at the time of switchover using a more sensitive approach. In 60 cases, leftover RNA was available for UDS reanalysis. The main characteristics of these patients are presented in Table 1. All the patients provided written informed consent, in accordance with the Declaration of Helsinki. Institutional Review Boards approval was obtained.

RNA extraction, c-DNA sinthesys and 1st step amplification. RNA extraction, c-DNA sinthesys and 1st step amplification were performed as previously reported.

Amplicon library preparation. Amplicon library preparation was performed as previously reported.

UDS of the BCR-ABL KD. UDS runs were designed for high sensitivity mutation calling with a lower detection limit of 1% of BCR-ABL transcripts

Conventional Sanger Sequencing of the BCR-ABL KD. SS of the BCR-ABL KD was performed as previously reported.

Results

Of the 60 patients who relapsed on a 2nd TKIs included in this study, at the time of switchover, by Sanger Sequencing 28/60 (47%) of the patients had been found to harbor mutations whereas 48/60 (80%) were positive for BCR-ABL mutations by UDS (Figure 2 A). UDS detected all the 30 mutations that had been previously identified by SS, plus 60 low level mutations (>1% but <20%). All these patients received dasatinib or nilotinib based on their mutation status, so no imatinib-resistant patient positive for the T315I mutation by SS was included in this series. Low level mutations were detected in 20 patients with no evidence of mutations by SS and in 15 patients already harboring mutations with ≥20% abundance (Table 2), so that overall, low level mutations additional to those identified by SS were detectable in 58% of the patients at the time of switchover. The UDS provided in addiction a more accurate picture of BCR-ABL KD mutation status and revealed that the number of patients harboring no mutations, 1 mutation, 2 mutations and 3 or more mutations may be different by UDS as against SS (Figure 2 B). As shown in Figure 2C of the 60 low level mutations (>1% but <20%) detected at switchover by UDS 17 (28)% were silent or had an unknown resistance profile, 13 (22)% were known to be resistant to imatinib only, and 30 (50)% were known to confer resistance also to dasatinib or nilotinib. Among the latter, a T315I was identified in 13 (43%) cases, other nilotinib resistant mutations were detected in 12 (40%) cases and other dasatinib-resistant mutations were detected in 5 (17%) cases. The knowledge of the mutations detectable by SS at relapse revealed that low level mutations detected by UDS invariably expanded (after 2 to 9 months) in all the cases (n=26) in which the 2GTKI they were insensitive to happened to be selected (Figure 2 D). We then wondered what we would find if we analyzed by UDS switchover samples of imatinib-resistant patients who did respond to second-line treatment. To explore whether the 1% threshold we chose to adopt might lead to the identification of transient mutants that are not going to undermine TKI effectiveness, switchover samples of 25 randomly selected cases who achieved a stable response to second-line treatment

with dasatinib or nilotinib were also analyzed for comparison. By UDS, a total of 20 mutations were detected including 7 mutations that had already been identified by SS plus 13 low level mutations (Table 2). No low level mutation resistant to the respective 2nd generation TKIs each patient actually received was identified.

Discussion

In Philadelphia chromosome-positive (Ph+) leukemia patients who fail imatinib therapy, BCR-ABL KD mutation screening by Sanger sequencing is recommended since detection of second-generation tyrosine kinase inhibitor-resistant mutations influences the selection of the most appropriate second-line therapy. We retrospectively analyzed 60 Ph+ leukemia patients who developed 2GTKI-resistant mutations to investigate in how many cases these mutations could have been detected at the time of switchover by a more sensitive Ultra-Deep Sequencing (UDS) approach. As compared to SS, UDS detected additional low level sequence variants (>1% but <20%) in 58% of cases, allowing to trace second-line TKI resistant mutations back to the time of switchover in 26/60 (43%) patients. In 25 imatinib-resistant patients who responded to second-line therapy, analysed for comparison, no low level mutation that was resistant to the 2GTKI the patients actually received was detected. Here we present that UDS at the time of switchover may i) better assist physicians in therapeutic decision-making ii) allowed to trace 2nd-line TKI resistant mutations back to the time of switchover in 26/60 (43%) Ph+ leukemia patients, iii). In case of switch to 2nd-line TKI treatment, sensitive BCR-ABL mutation profiling by UDS might allow a more effective therapeutic tailoring. Although safety, tolerability and price are the main components of the decision algorithm, 2GTKIs have Achilles heels in terms of resistant mutations that should also be taken into account. We here provide further evidence that increasing sensitivity in mutational analysis may uncover 2GTKI-resistant mutations at the time of switchover in a greater proportion of patients. This demonstrates that the presence of clinically relevant mutations below the lower detection limit of what nowadays is the gold-standard for BCR-ABL mutation screening is not infrequent in patients who fail first-line TKI therapy. Depending on when the patient is tested, outgrowing mutants might not have achieved a 20% abundance yet or, as opposite, a previously dominant mutant might have temporarily shrunk because of cytoreductive therapy or just because TKI selective pressure is no longer being exerted. Optimizing TKIs choice means optimizing patient outcome, but also optimizing healthcare costs: TKIs, to be administered chronically to a population of patients whose prevalence is steadily increasing, are extremely expensive drugs [160] and a

mutation screening test, even with UDS, is as expensive as a few days of TKI therapy. [161] All this, together with the unique capability of UDS to scan the entire KD – not just specific nucleotide positions – for low level mutations, warrants forthcoming clinical studies aimed to prospectively assess and optimize the integration of UDS in the clinical management of Ph+ leukemia patients.

FIGURES

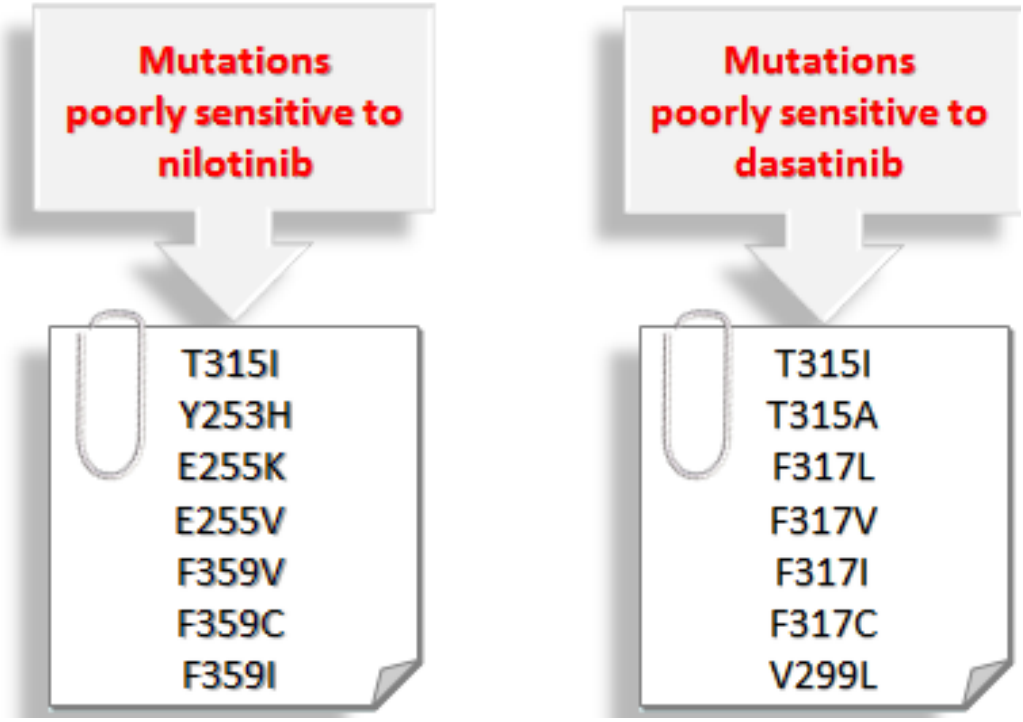


Figure 1 – Summary of the *BCR-ABL* KD amino acid substitutions identified in clinical samples reported to be resistant to the currently approved 2nd generation TKIs Nilotinib and Dasatinib. TKIs. dasatinib and nilotinib are approved both by FDA (Food and Drug Administration) and EMA (European Medicines Agency) for first or subsequent-line use. V299L and F317L/V/I/C retain insensitivity to dasatinib whereas Y253F/H, E255K/V, F359V/I/C retain insensitivity to nilotinib. T315I is a pan-resistant mutation retaining insensitivity to both TKIs.

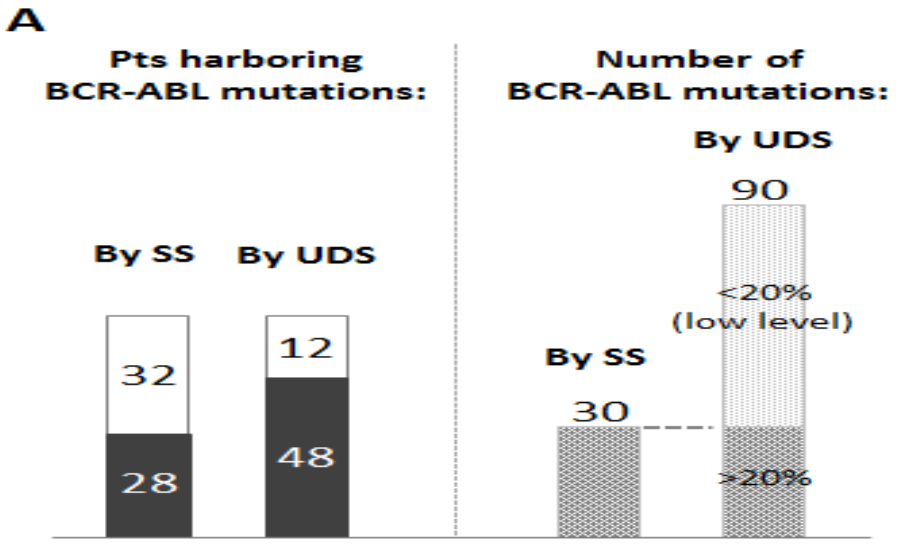


Figure 2 A - Number of patients harboring BCR-ABL mutation by SS and UDS and number of BCR-ABL founded by SS and UDS. Of the 60 patients who later relapsed on a 2GTKI included in this study, 48 were positive for BCR-ABL KD mutations by UDS reanalysis of switchover samples. Only 28 of them had been found to harbor mutations by SS. UDS identified the 30 mutations already detected by SS plus 60 additional, low level mutations.

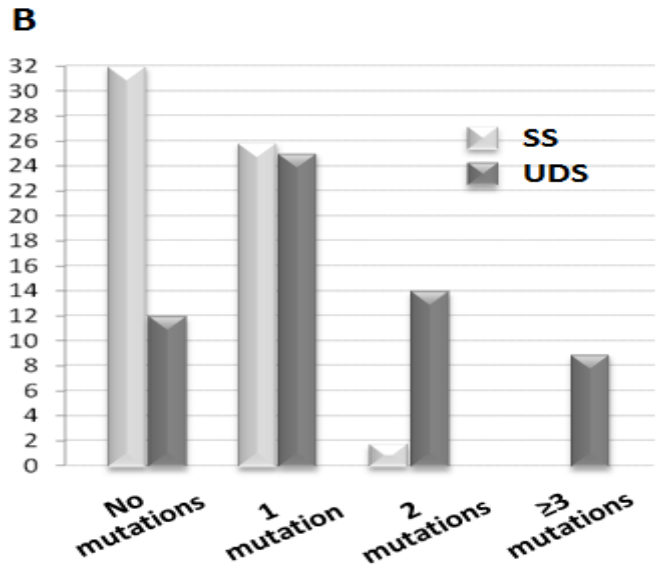


Figure 2 B - Number of patients harboring no mutations, 1 mutation, 2 mutations and 3 or more mutations by UDS as against SS. UDS provided a more accurate picture of BCR-ABL KD mutation status.

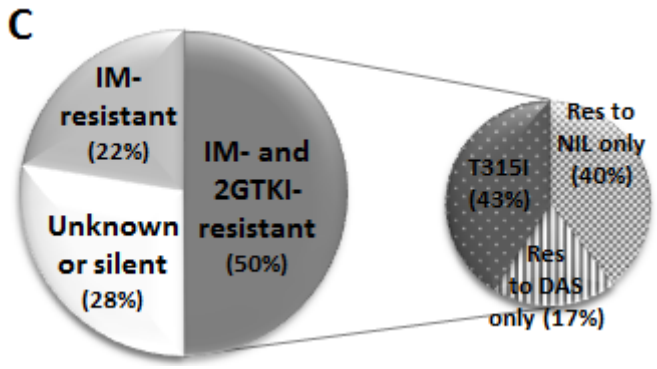


Figure 2 C - Type of mutations that were founded and their clinical correlation. 28% of mutation were silent or had an unknown resistance profile, 22% were known to be resistant to imatinib only, and 50% were known to confer resistance also to dasatinib or nilotinib. Nevertheless no imatinib-resistant patients positive for the T315I mutation by conventional sequencing methods was included in this study the high sensitivity of our approach allowing to identify a low level T315I mutation 13 (43%) cases. Mutations that confer resistance to nilotinib were detected in 12 (40%) whereas dasatinib-resistant mutations were detected in 5 (17%) cases.

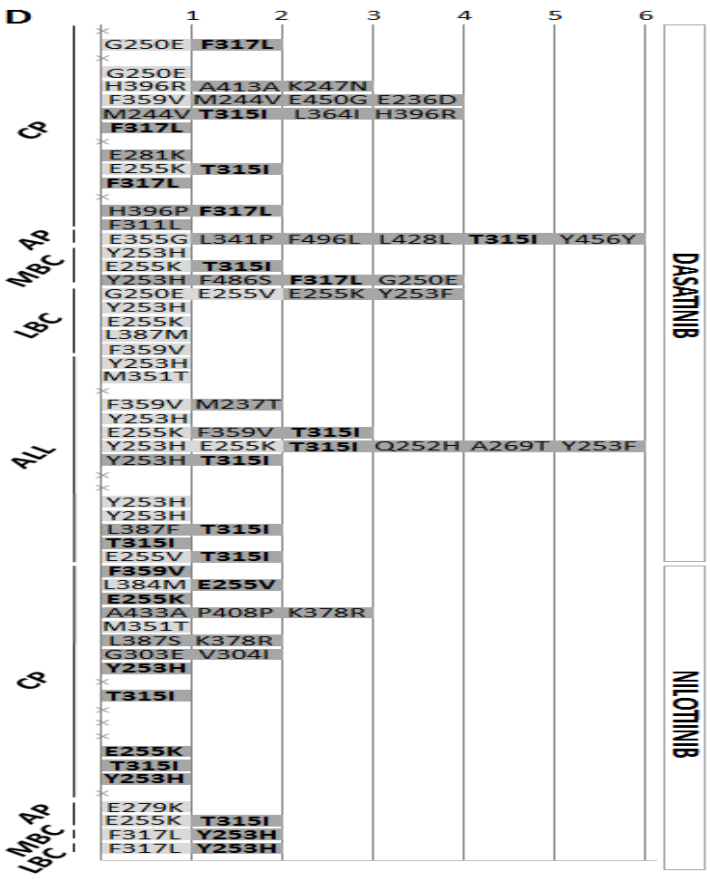


Figure 2 D - Number and type of mutations detected by UDS as against SS detailed for each of the 60 patients, grouped by disease phase/type and by 2GTKI received. Light grey indicates mutations detectable by SS, dark grey indicates mutations detectable by UDS only. The bold font highlights the low level mutations that became detectable by SS at the time of relapse, two to nine months later. In all cases in which the 2GTKI they were insensitive to happened to be selected, low level mutations invariably expanded, alone or in combination with pre-existing mutations. Abbreviations: SS, Sanger Sequencing; UDS, Ultra-Deep Sequencing; pts, patients; Res, resistant; IM, imatinib; DAS, dasatinib, NIL, nilotinib; 2GTKI, second generation tyrosine kinase inhibitor; CP, chronic phase; AP, accelerated phase; MBC, myeloid blast crisis; LBC, lymphoid blast crisis; ALL, acute lymphoblastic leukemia.

TABLES

Pts, total	60
Median age, years (range)	54 (25-78)
Male to female ratio	35:25
Disease phase/type:	
▪ **CML**	45
- chronic phase	32
- accelerated phase	3
- myeloid blast crisis	4
- lymphoid blast crisis	6
▪ **Ph+ ALL**	15
Pts who failed 2nd-line dasatinib	39
Pts who failed 2nd-line nilotinib	21
Median time from diagnosis, months (range)	18 (2-137)

Table 1 - Main features of the 60 patients included in the present study. Abbreviations: Pts, patients; CML, chronic myeloid leukemia; Ph+ ALL, Philadelphia chromosome-positive acute lymphoblastic leukemia

N	Disease Phase	Sex	Age	TKI	Mut by SS	Mut by UDS	Response at the time of analysis
1	CP-CML	F	27	IM	No mutations	No mutations	No CHR after 3 months on IM
				DAS	T315I (~100%)	T315I (94.8%)	Progression to LBC after 2 months on DAS
2	CP-CML	M	69	IM	No mutations	**F359V (16.5%)**	Loss of MMR after 39 months on IM
				NIL	F359V (~60%)	F359V (59.3%)	Loss of complete CyR after 3 months on NIL
3	CP-CML	M	62	IM	G250E (~100%)	G250E (93.7%), **F317L (1.8%)**	Minor CyR after 12 months on IM
				DAS	G250E (~50%), F317L (~40%)	G250E (45.5%), F317L (37.5%), H295H (4.9%), C330C (1.5%)	No CyR after 13 months on DAS
4	CP-CML	F	60	IM	L384M (~100%)	L384M (87.0%), **E255V (15.1%)**	Loss of complete CyR after 24 months on IM
				NIL	E255V (~100%)	E255V (80.7%), L384M (14.4%)	No CHR after 3 months on NIL
5	CP-CML	F	60	IM	No mutations	No mutations	Minor CyR after 12 months on IM
				DAS	F317L (~100%)	F317L (100.0%)	Loss of CHR after 12 months onDAS
6	CP-CML	M	49	IM	No mutations	**E255K (18.4%)**	Loss of complete CyR after 48 months on IM
				NIL	E255K (~100%)	E255K (79.9%)	Minimal CyR after 6 months on NIL
7	CP-CML	F	57	IM	G250E (~100%)	G250E (99.5%)	Loss of complete CyR after 36 months on IM
				DAS	F317L (~100%)	F317L (99.5%)	No CyR after 3 months on DAS
8	CP-CML	F	71	IM	H396R (~100%)	H396R (99.6%), A413A (1.7%), K247N (1.1%)	Partial CyR after 18 months on IM
				DAS	F317L (~20%)	F317L (20.9%), H396R (3.5%)	Minimal CyR after 6 months on DAS
9	CP-CML	M	44	IM	No mutations	A433A (1.5%), P408P (1.4%), K378R (1.3%)	No CyR after 12 months on IM
				NIL	T315I (~70%)	T315I (65.9%), Y253H (16.7%), W235R (3.8%), F497L (3.1%), T406I (2.4%), Q477Q (2.4%), F486S (2.3%), D363N (1.5%)	No CyR after 13 months on NIL
10	CP-CML	M	66	IM	M351T (~100%)	M351T (99.8%)	No CyR after 12 months on IM
				NIL	Y253H(~100%)	Y253H (95.6%), M351T (3.2%)	No CyR after 22 months on NIL
11	CP-CML	F	60	IM	F359V (~80%)	F359V (87.0%), M244V (5.6%), E450G (2.6%), E236D (1.1%)	Loss of MMR after 20 months on IM
				DAS	F317L (~100%)	F317L (100%), F486L (6.8%)	Loss of MMR after 43 months on DAS
12	CP-CML	F	75	IM	No mutations	M224V (3.2%), **T315I (3.1%),** L364I (2.0%), H396R (1.4%)	No complete CyR after 69 months on IM
				DAS	M244V (~100%), T315I (~20%)	M244V (100%), T315I (18.0%)	No CyR after 25 months on DAS
13	CP-CML	M	53	IM	No mutations	L387S (1.6%), K378R (1.0%)	Loss of complete CyR after 20 months on IM

				NIL	T315I (~20%)	T315I (21.1%), Y253H (3.6%)	Loss of complete CyR after 27 months on NIL
14	CP-CML	F	55	IM	No mutations	G303E (1.2%), V304I (1.1%)	No complete CyR after 50 months on IM
				NIL	E255K (~100%)	E255K (89.0%)	No CyR after 6 months on NIL
15	CP-CML	M	43	IM	No mutations	**F317L (11.3%)**	Loss of MMR after 39 months on IM
				DAS	F317L (~50%)	F317L (50.0%), L451P (1.8%), K294E (1.6%)	No CyR after 1 month on DAS
16	CP-CML	M	34	IM	No mutations	No mutations	Loss of complete CyR after 22 months on IM
				DAS	V299L (~100%)	V299L (99.0%), T394A (2.0%)	Loss of MMR after 24 months on DAS
17	CP-CML	M	40	IM	No mutations	E281K (1.3%)	No complete CyR after 18 months on IM
				DAS	T315I (~20%)	T315I (22.0%)	Loss of complete CyR after 12 months on DAS
18	CP-CML	M	64	IM	No mutations	**Y253H (5.1%)**	No CyR after 6 months on IM
				NIL	Y253H (~100%)	Y253H (99.5%)	No CyR after 6 months on NIL
19	CP-CML	M	51	IM	No mutations	No mutations	Loss of MMR after 48 months on IM
				NIL	F359I (~100%)	F359I (99.2%)	Loss of complete CyR after 18 months on NIL
20	CP-CML	M	44	IM	No mutations	**T315I (8.3%)**	Loss of complete CyR after 18 months on IM
				NIL	T315I (~100%)	T315I (99.7%)	Loss of hematologic response after 3 months on NIL
21	CP-CML	M	31	IM	No mutations	No mutations	Loss of MMR after 24 months on IM
				NIL	E255V (~100%)	E255V (99.7%)	Loss of complete CyR after 12 months on NIL
22	CP-CML	F	37	IM	E255K (~25%)	E255K (23.2%), **T315I (16.9%)**	Loss of CHR after 9 months on IM
				DAS	E255K (~100%), T315I (~100%)	E255K (99.6%), T315I (98.6%)	No CHR after 1 month on DAS
23	CP-CML	F	70	IM	No mutations	**F317L (6.5%)**	Loss of complete CyR after 24 months on IM
				DAS	F317L (~100%)	F317L (99.5%)	Progression to AP after 6 months on DAS
24	CP-CML	M	50	IM	No mutations	No mutations	Loss of complete CyR after 36 months on IM
				NIL	T315I (~100%), E255K (~50%), F359I (~30%)	T315I (92.0%), E255K (48.2%), F359I (32.5%)	Progression to MBC after 24 months on NIL
25	CP-CML	F	49	IM	No mutations	No mutations	No complete CyR after 18 months on IM
				NIL	F359V (~100%)	F359V (99.7%)	Loss of CHR after 9 months
26	CP-CML	M	41	IM	No mutations	No mutations	No CyR after 3 months on IM
				DAS	F317L (~100%)	F317L (ttc>tta)(80.7%), F317L (ttc>ctc)(9.73%)	Loss of CHR after 6 months on DAS
27	CP-CML	M	65	IM	No mutations	**E255K (15.4%)**	Loss of complete CyR after 72 months on IM
				NIL	E255K (~100%)	E255K (97.8%)	Loss of CHR after 3 months on NIL
28	CP-CML	M	57	IM	No mutations	**T315I (5.0%)**	Loss of CHR after 24 months on IM

				NIL	T315I (~100%)	T315I (100.0%)	Progression to MBC after 3 months on NIL
29	CP-CML	F	67	IM	No mutations	H396P (14.1%), **F317L (ttc>tta) (1.4%)**	Loss of complete CyR after 24 months on IM
				DAS	F317L (~100%)	F317L (ttc>ctc) (55.2%), F317L (ttc>tta) (44.7%)	Loss of CHR after 3 months on NIL
30	CP-CML	F	59	IM	No mutations	**Y253H (4.4%)**	Loss of MMR after 60 months on IM
				NIL	Y253H (~100%)	Y253H (99.8%)	Loss of CCyR after 3 months on NIL
31	CP-CML	F	62	IM	No mutations	No mutations	No complete CyR after 24 months on IM
				NIL	Y253H (~50%)	Y253H (45.5%)	Loss of complete CyR after 48 months on NIL
32	CP-CML	F	52	IM	No mutations	F311L (5.2%)	No MMR after 24 months on IM
				DAS	T315I (~70%)	T315I (66.5%)	Loss of complete CyR after 36 months on DAS
33	AP-CML	M	49	IM	E355G (~60%)	E355G (43.8%), L341P (19.8%), F496L (19.6%), L428L (11.0%), **T315I (10.2%),** Y456Y (4.8%)	Progression to AP after 9 months on IM
				DAS	T315I (~50%)	T315I (42.6%), F317L (1.3%)	Progression to MBC after 1 month on DAS
34	AP-CML	M	44	IM	E279K (~50%)	E279K (55.4%)	Progression to AP after 6 months on IM
				NIL	E279K (~50%), T315I (~50%)	E279K (48.8%), T315I (49.8%)	Loss of CHR after 6 months on NIL
35	AP-CML	M	39	IM	E255K (~100%)	E255K (98.0%), **T315I (1.7%)**	Progression to AP after 18 months on IM
				NIL	E255K (~100%), T315I (~100%)	E255K (99.0%), T315I (99.0%)	No HR after 1 month on NIL
36	MBC-CML	M	32	IM	Y253H (~100%)	Y253H (100.00%)	Progression to MBC after 22 months on IM
				DAS	F317L (~60%), T315I (~40%)	F317L (55.3%), T315I (35.7%), T315A (7.0%), Y253H (4.1%)	No CHR after 3 months on DAS
37	MBC-CML	M	48	IM	E255K (~100%)	E255K (97.5%), **T315I (3.7%)**	No CHR after 2 months on IM
				DAS	E255K (~100%), T315I (~100%)	E255K (97.7%), T315I (96.8%)	No CHR after 1 month on DAS
38	MBC-CML	F	59	IM	No mutations	Y253H (17.8%), F486S (11.6%), **F317L (9.6%),** G250E (8.2%)	Progression to MBC after 36 months on IM
				DAS	F317L (~100%)	F317L (60.9%)	No CHR after 1 month of DAS
39	MBC-CML	M	43	IM	F317L (~100%)	F317L (99.9%), **Y253H (17.4%)**	Progression to MBC after 44 months on IM
				NIL	F317L (~100%), Y253H (~100%)	F317L (99.9%), Y253H (99.9%)	No CHR after 2 months on NIL
40	LBC-CML	M	56	IM	G250E (~40%), E255V (~20%)	G250E (34.2%), E255V (21.1%), E255K (2.3%), Y253F (1.1%)	Progression to LBC after 32 months on IM
				DAS	T315I (~30%)	T315I (28.9%)	No CHR after 2 months on DAS
41	LBC-CML	F	63	IM	Y253H (~100%)	Y253H (99.9%)	Progression to LBC after 48 months on IM
				DAS	Y253H (~50), F317L (~50)	Y253H (54.9%), F317L (54.4%)	Loss of CHR after 6 months on DAS
42	LBC-CML	F	26	IM	E255K (~100%)	E255K (98.8%)	Progression to LBC after 9 months on IM
				DAS	E255K (~100%), T315I (~100%)	E255K (99.8%), T315I (99.4%), L273S (1.4%)	No CHR after 1 month on DAS

43	LBC-CML	M	78	IM	L387M (~30%)	L387M (34.1%)	Loss of CHR after 6 months on IM
				DAS	L387M (~100%), T315I (~50%), M318V (~50%), F317L (~50%), Y320N (~50%)	L387M (96.3%), T315I (51.5%), M318V (51.2%), F317L (45.2%), Y320N (44.8%)	Loss of complete CyR and CHR after 14 months on DAS
44	LBC-CML	M	28	IM	F359V (~60%)	F359V (57.9%)	Progression to LBC after 3 months on IM
				DAS	F359V (~100%), F317I (~100%)	F317I (92.4%), F359V (90.1%), F317L (4.3%)	Loss of CHR after 9 months on DAS
45	LBC-CML	M	55	IM	F317L (~100%)	F317L (99.5%), **Y253H (1.11%)**	Progression to LBC after 32 months on IM
				NIL	F317L (~100%), Y253H (~100%)	Y253H (98.9%), F317L (98.1%)	No CHR after 1 month on NIL
46	ALL	M	26	IM	Y253H (~100%)	Y253H (99.8%)	Hematologic relapse after 6 months on IM
				DAS	Y253H (~100%), T315I (~50%)	Y253H (99.7%), T315I (40.3%), N336S (5.4%), W405R (1.9%)	Hematologic relapse after 9 months on DAS
47	ALL	M	64	IM	M351T (~100%)	M351T (99.9%)	Hematologic relapse after 12 months on IM
				DAS	M351T (~100%), F317L (~50%)	M351T (99.8%), F317L (53.3%)	Hematologic relapse after 12 months on DAS
48	ALL	M	37	IM	No mutations	No mutations	Hematologic relapse after 15 months on IM
				DAS	F317L (~100%)	F317L (100.0%)	Hematologic relapse after 6 months on DAS
49	ALL	M	36	IM	F359V (~100%)	F359V (99.8%), M237T (2.1%)	Hematologic relapse after 26 months on IM
				DAS	F359V (~100%), T315I (~100%)	F359V (99.3%), T315I (98.8%), Y449Y (10.5%)	Progressive disease after 1 month on DAS
50	ALL	F	50	IM	Y253H (~30%)	Y253H (28.4%)	Molecular relapse after 12 months on IM
				DAS	Y253H (~100%), T315I (~100%)	Y253H (87.7%), T315I (86.2%)	Hematologic relapse after 2 months on DAS
51	ALL	F	33	IM	E255K (~60%)	E255K (54.8%), F359V (13.8%), **T315I (3.8%)**	Hematologic relapse after 6 months on IM
				DAS	T315I (~100%)	T315I (99.4%)	Progressive disease after 5 months on DAS
52	ALL	M	25	IM	Y253H (~50%), E255K (~20%)	Y253H (56.0%), E255K (19.9%), T315I (8.3%), Q252H (6.3%), A269T (3.6%), Y253F (1.4%)	Hematologic relapse after 9 months on IM
				DAS	T315I (~100%)	T315I (91.0%)	Progressive disease after 3 months on DAS
53	ALL	M	74	IM	No mutations	Y253H (2.5%), **T315I (2.5%)**	Hematologic relapse after 6 months on IM
				DAS	Y253H (~50%), T315I (~50%), F317L(ttc>tta) (~20%)	Y253H (49.0%), T315I (49.0%), F317L(ttc>tta)(20.0%), F317L(ttc>ctc)(5.0%)	Progressive disease after 2 months on DAS
54	ALL	F	78	IM	No mutations	No mutations	Hematologic relapse after 12 months on IM
				DAS	T315I (~100%)	T315I (99.5%)	Hematologic relapse after 7 months on DAS
55	ALL	F	60	IM	No mutations	No mutations	Hematologic relapse after 5 months on IM
				DAS	T315I (~100%)	T315I (92.5%)	Hematologic relapse after 3 months on DAS
56	ALL	F	64	IM	Y253H (~100%)	Y253H (100.00%)	Molecular relapse after 15 months on IM

				DAS	T315I (~100%)	T315I (100.00%), V289A (3.0%)	Hematologic relapse after 12 months on DAS
57	ALL	F	68	IM	Y253H (~100%)	Y253H (98.1%)	Hematologic relapse after 6 months on IM
				DAS	T315I (~100%), Y253H (~60%)	T315I (99.6%), Y253H (63.3%)	Hematologic relapse after 3 months on DAS
58	ALL	M	59	IM	No mutations	L387F (1.4%), **T315I (1.4%)**	Hematologic relapse after 9 months on IM
				DAS	L387F (~100.0%), T315I (~100.0%)	L387F (100.0%), T315I (91.2%)	Hematologic relapse after 2 months on DAS
59	ALL	M	44	IM	No mutations	**T315I (2.4%)**	Hematologic relapse after 15 months on IM
				DAS	T315I (~80%)	T315I (70.7%)	Hematologic relapse after 3 months on DAS
60	ALL	F	66	IM	E255V (~100%)	E255V (92.8%), **T315I (2.1%)**	Hematologic relapse after 18 months on IM
				DAS	E255V (~50%), T315I (~30%)	E255V (50.4%), T315I (24.3%)	Progressive disease after 1 month on DAS

Table 2 – SS and UDS results at the time of switchover and at the time of subsequent resistance to second-line dasatinib or nilotinib in the 60 imatinib-resistant patients herein analyzed. Mutation burden is indicated in brackets; estimation was based on relative mutated to wild-type peak height in the sequencing output chromatogram or on the ratio between the number of reads harbouring the substitution and the total reads covering the corresponding nucleotide position for SS and UDS, respectively. Dasatinib- or nilotinib-resistant mutations detected by UDS at switchover and by SS at the time of resistance to second-line therapy are in bold. When the same F317L mutation was found to result from different nucleotide sustitutions in distinct clones, codon change is specified in brackets. Abbreviations: CP, chronic phase; MBC, myeloid blast crisis; LBC, lymphoid blast crisis; CyR, cytogenetic response; MMR, major molecular response; CHR, complete hematologic response; IM, imatinib; DAS, dasatinib; NIL, nilotinib.

N	Disease Phase	Sex	Age	Mutation status by SS	Mutation status by UDS	Response at switchover	2nd-line TKI
1	CP-CML	M	39	No mutations	Y320H (1.5%), H295R (1.5%)	Loss of complete CyR after 36 months on IM	NIL
2	CP-CML	M	47	No mutations	No mutations	Loss of MMR after 48 months on IM	NIL
3	CP-CML	F	72	No mutations	No mutations	No complete CyR after 24 months on IM	DAS
4	CP-CML	M	66	H396P (~70%)	H396P (65.2%)	Loss of complete CyR after 44 months on IM	NIL
5	CP-CML	F	55	No mutations	L298R (1.4%)	No MMR after 24 months on IM	NIL
6	CP-CML	F	51	No mutations	No mutations	Loss of MMR after 38 months on IM	DAS
7	CP-CML	F	31	No mutations	S438F (2.8%)	Loss of MMR after 48 months on IM	DAS
8	CP-CML	M	69	No mutations	No mutations	No MMR after 18 months on IM	DAS
9	CP-CML	M	45	No mutations	No mutations	No MMR after 24 months on IM	NIL
10	CP-CML	M	52	No mutations	Y253C (2.0%), I432V (2.0%)	No complete CyR after 18 months on IM	DAS
11	CP-CML	F	73	No mutations	F317L (3.6%)	Loss of complete CyR after 60 months on IM	NIL
12	CP-CML	M	37	Y253H (~100%)	Y253H (98.5%)	Loss of complete CyR after 37 months on IM	DAS
13	CP-CML	F	44	No mutations	H396P (14.7%), F317L (1.4%)	Loss of complete CyR after 36 months on IM	NIL
14	CP-CML	M	65	No mutations	No mutations	No MMR after 26 months on IM	NIL
15	CP-CML	F	61	No mutations	No mutations	No CyR after 6 months on IM	NIL
16	CP-CML	M	53	No mutations	No mutations	Minor CyR after 12 months on IM	NIL
17	CP-CML	M	49	No mutations	F497L (18.1%)	No complete CyR after 24 months on IM	DAS
18	MBC-CML	M	64	L387M (~30%)	L387M (32.2%)	Progression to MBC after 89 months on IM	DAS
19	MBC-CML	F	70	Y253H (~30%)	Y253H (27.6%), P465L (1.6%)	Progression to MBC after 22 months on IM	DAS
20	MBC-CML	M	52	No mutations	No mutations	Progression to MBC after 89 months on IM	DAS
21	LBC-CML	M	53	Y253F (~50%)	Y253F (45.6%)	Progression to LBC after 17 months on IM	DAS
22	ALL	F	71	No mutations	P439S (7.8%), A474A (6.2%)	Molecular relapse after 14 months on IM	DAS
23	ALL	M	69	M351T (~100%)	M351T (100.0%)	Molecular relapse after 18 months on IM	DAS
24	ALL	M	66	No mutations	Y253H (2.1%)	Molecular relapse after 21 months on IM	DAS
25	ALL	M	59	M244V (~40%)	M244V (37.1%)	Loss of hematologic response after 12 months on IM	DAS

Table 3 – UDS results in the 25 imatinib-resistant patients who had an optimal response to second-line dasatinib/nilotinib therapy, analyzed for comparison. Definition of optimal response as per 2013 ELN recommendations. The main features (phase/type of disease, male to female ratio, median age, type of resistance to first-line imatinib therapy, type of 2GTKI administered, median time from diagnosis) do not differ significantly from the 60 patients this study focused on. Median follow-up on second-line therapy, 16 months (range, 9-36). Abbreviations: CP, chronic phase; MBC, myeloid blast crisis; LBC, lymphoid blast crisis; CyR, cytogenetic response; MMR, major molecular response; IM, imatinib; DAS, dasatinib; NIL, nilotinib.

PART IV

DYNAMICS OF EXPANSION OF TYROSINE KINASE INHIBITOR-RESISTANT MUTANTS IN PHILADELPHIA CHROMOSOME-POSTIVE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS AS ASSESSED BY ULTRA DEEP SEQUENCING OF THE BCR-ABL KINASE DOMAIN: IMPLICATIONS FOR ROUTINE MUTATION TESTING.

Background

Although in chronic myeloid leukemia patients the introduction of tyrosine kinase inhibitors targeting BCR-ABL has translated into an excellent response rates and an excellent relapse free survival and overall survival, in Ph+ acute lymphoblastic leukemia (ALL) patients clinical results have been more frustrating, since the long-term stability of responses, obtained in the induction phase, is frequently undermined by the selection of drug-resistant BCR-ABL KD mutants that can survive TKI therapy and trigger relapse. [162,163] We decided to take advantage of a UDS-based BCR-ABL kinase domain mutation screening approach in order to i) study the dynamics of expansion of BCR-ABL KD mutations in Ph+ acute lymphoblastic leukemia developing resistance to TKI-based therapies; and ii) test the ability of Ultra-Deep Sequencing (UDS) to highlight emerging clones harboring TKI-resistant mutations earlier than conventional sequencing (SS).

Patients and methods

Patients and samples. A total of 113 bone marrow and/or peripheral blood samples from 35 Ph+ ALL patients who relapsed during TKI-based therapy (imatinib, dasatinib, nilotinib) with acquisition of BCR-ABL KD mutations (as assessed by conventional SS) were retrospectively analyzed. In order to reconstruct the dynamics of mutation emergence, longitudinal re-analysis of samples from the time of hematologic relapse backwards time of diagnosis was performed on a Roche GS Junior instrument. Two to seven samples were analyzed for each patient with maximum interval of sampling from four to fifteen week-intervals . All the patients had been referred to our laboratory for minimal residual disease (MRD) follow-up monitoring by real-time reverse transcription (RT)-polymerase chain reaction (PCR) and for BCR-ABL KD mutation analysis by SS in case of MRD positivity. At the time of mutation appearance, ten patients with de novo Ph+ ALL were receiving first-line TKI therapy. Two patients were receiving imatinib plus chemotherapy; six patients were receiving nilotinib 400 mg twice daily and imatinib 300 mg twice daily in rotation, in six week

cycles, in the framework of the GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) LAL1408 clinical trial (www.clinicaltrials.gov identifier NCT01025505); two patients were receiving dasatinib 140 mg once daily in the framework of the of the GIMEMA LAL1509 clinical trial (www.clinicaltrials.gov identifier NCT01361438). The remaining 25 patients, with advanced Ph+ ALL, developed mutations while receiving dasatinib or nilotinib for recurrent disease. Baseline and clinical features are summarized in Table 1. This study was approved by the Review Board of the S.Orsola-Malpighi Hospital. All patients provided written informed consent in accordance with the Declaration of Helsinki and later amendments.

RNA extraction, c-DNA sinthesys and 1st step amplification. RNA extraction, c-DNA synthesis and 1st step amplification were performed as previously described.

Amplicon Library preparation. Amplicon Library preparation was performed as previously described.

UDS of the BCR-ABL KD. The UDS approach used in this study has previously been described. Amplicon Variant Analyzer ver4.0 (454-Life Sciences) and Sequence Pilot ver4.0.1 (JSI-Medical Systems, Kippenheim, Germany) were used to align reads to the reference ABL1 sequence (GenBank accession no.X16416.1) and calculate variant frequencies. Reads from both strand orientations and from overlapping amplicons were combined into a single alignment and primer regions were automatically trimmed to avoid artefacts deriving from nucleotide synthesis errors. The presence of all relevant variants was also manually verified by inspection of individual flowgrams at the corresponding nucleotide positions. DS runs were designed to enable highly sensitive mutation calling, with a target sequence coverage up to 14,337 independent reads for each nucleotide position.

Conventional sequencing of the BCR-ABL KD. Direct sequencing of the BCR-ABL KD was performed as previously described.

Real-time quantitative RT-PCR for BCR-ABL1 transcript levels. Molecular monitoring of MRD was routinely performed by real-time RT-PCR on an ABI PRISM 7900 instrument, with standardized Europe Against Cancer (EAC) primers and probe sets and reaction conditions [164-166]

Results

Dynamics of mutation emergence in de novo Ph+ ALL patients who relapsed on first-line TKI therapy. Table 2 details the evolution of BCR-ABL KD mutation status as assessed by SS and by

UDS in the ten patients with de novo Ph+ acute lymphoblastic leukemia patients who developed resistance to first-line TKI therapy (imatinib plus chemotherapy in two cases, nilotinib and imatinib in rotation in six cases, dasatinib in two cases). All the patients were analyzed in parallel by SS and UDS at each timepoint from relapse back to diagnosis, dating one to ten months before – for a total of 37 samples analyzed (but in two samples with undetectable BCR-ABL transcripts as assessed by real-time RT-PCR, amplification of the KD was unsuccessful). The time lapse between samples was often four-five weeks, and never exceeded fifteen weeks – which allowed a close and thorough monitoring of the dynamics of mutations over time and in relation to total BCR-ABL transcript levels. Comparison between SS and UDS results showed 100% concordance in the detection and quantitation of nucleotide substitutions with a relative abundance of 20% or greater. However, in 14/35 samples SS had misclassified or underestimated BCR-ABL KD mutation status since low-level mutations were identified. In particular, 11 samples displayed no mutations by SS but one or more low-level mutations by UDS. Further, 3 more samples, with mutations detectable by SS, turned out to harbour one or more additional low level mutations by UDS. The higher complexity in mutation status uncovered by UDS was particularly evident at diagnosis, when 0/10 as against 7/10 patients were found to be positive for mutations by SS and UDS, respectively (Table 2). All but one of these low level mutations were either silent or apparently not TKI-resistant. In patient no. 01, an emerging T315I mutation with an abundance of 2.58% was detected by UDS four weeks before hematologic relapse and only after four weeks this mutation became detectable by SS. In one case, (patient no. 02), the imatinib resistant mutation Y253H, found in virtually 100% of BCR-ABL transcripts at relapse with an abundance of 1.82%, could be identified by UDS two months before at the time of diagnosis. In patient no. 03, a low level imatinib-resistant (but nilotinib-sensitive) mutation E355G was transiently detected by UDS (11.93%) but disappeared in the subsequent sample, collected six weeks later when the patient had switched from imatinib to nilotinib, as per protocol. In three patients UDS could pick emerging TKI-resistant mutations: in patient no. 05 was identified an emerging E255K mutation with an abundance of 9.68% six weeks before it became detectable by conventional sequencing, in patient no. 06 was highlighted an emerging Y253H mutation six weeks in advance, once again when the patient had minimal residual disease molecularly detectable but not yet evidence of hematologic relapse. In an attempt to gain further insights into the kinetics of mutation emergence, sequence reads and corresponding flowgrams at relevant nucleotide positions were visually inspected – but except for the cases described above, not a single read was found to harbour the mutation(s)

detectable at later timepoints, despite the high sequencing coverage (Figure 1). Even in patients no. 05 and 07, who were positive for the T315I mutation in 98-100% of BCR-ABL1 transcripts after only six weeks of nilotinib treatment, not a single sequence read out of 14337 and 6692, respectively, was found to carry the C to T nucleotide substitution at position c.1091 in the sample collected at diagnosis. Similarly, in patient 03, zero out of 5124 reads at diagnosis as against 6656 out of 6631 reads just six weeks later were found to carry the c.910G>A nucleotide substitution corresponding to the nilotinib- and imatinib-resistant E255K mutation. At the time of relapse, only 2/10 patients (no. 05 and no. 08) had multiple TKI-resistant mutations and only one, who never achieved a molecular response, had a complex, polyclonal mixture of single and compound mutants. Patient 10 displayed two nucleotide changes at two adjacent residues, with the same T315I amino acid substitution carried by two clearly distinct clones. One clone had the 'usual' 'act' to 'att' codon shift resulting from the c.1091C>T nucleotide change; the other one displayed the c.1091C>T but also an adjacent c.1092T>C nucleotide change, thus leading to an 'act' to 'atc' codon shift still corresponding to the threonine to isoleucine amino acid substitution. In the remaining seven cases, resistance was rather sustained by a single mutated clone.

Dynamics of mutation emergence in advanced Ph+ ALL patients who relapsed on TKI therapy administered for recurrent disease. Table 3 details BCR-ABL KD mutation status as assessed by SS and by UDS in the 25 patients who relapsed on dasatinib or nilotinib therapy administered for recurrent disease. Once again, all the patients were analyzed in parallel at each timepoint from relapse backwards in an attempt to trace mutation emergence; a total of 76 samples were retrospectively analyzed – although in four samples with undetectable BCR-ABL transcripts as assessed by real-time RT-PCR, amplification of the KD did not yield any product. Again, time lapse between samples was often four/five weeks and never exceeded fifteen weeks. At the time of second or subsequent relapse, dasatinib-resistant (T315I, F317L, F317I, V299L) or nilotinib-resistant (Y253H) mutations were found to have emerged in all patients – in the majority of the cases, in addition to the pre-existing mutation(s). Multiple mutations were detected, by SS and/or UDS, in 15/25 patients (as against 2/10 patients who had relapsed on first line therapy). Although, in this setting, several cases had two (in one case, even three) mutations detectable by SS, in patients no. 20, 24, 29, 32 and 34 the mutation landscape turned out to be even more complex when DS was applied. Twenty-four out of 72 samples were found to harbour low level mutations. In addition, clonal analysis, whenever feasible – all samples where multiple mutations mapped within a region of 450 bp – showed a complex texture of single and compound mutants (Table 3).

As in the former group, mutations conferring 251 resistance to the TKI being administered arose and were selected very rapidly and only the tight sample collection schedule allowed UDS to detect emerging mutations ahead of SS in ten patients (no. 13, 14, 20, 24, 25, 28, 30, 31, 34, 35) (Table 3 and Figure 2).

Relationship between total BCR-ABL1 transcript levels and BCR-ABL KD mutation status. Tables 2 and 3 also report BCR-ABL transcript levels, expressed as BCR-ABL1/ABL1%, measured as part of the routine MRD assessments. In cases with low BCR-ABL transcript levels, detection of emerging TKI-resistant mutations by UDS was frequently accompanied by transcript increase from half a logarithm to two logarithms, or preceded by a shift from undetectable to detectable BCR-ABL1 transcripts. Mutation (re)appearance, as assessed by UDS, could quickly occur and even low levels of residual disease (but always >0.1%) were found to hide emerging TKI-resistant mutations. In the subgroup of fifteen patients with advanced Ph+ ALL who failed dasatinib or nilotinib and could be reassessed by UDS back to baseline, three main scenarios could be observed (exemplified in Figure 3A, B, C). In patients no. 11, 12, 20 and 24, the switch to dasatinib or nilotinib resulted in a 2-3 log reduction in BCR-ABL transcript levels (with patients no. 11, 12 and 20 having undetectable BCR-ABL1 transcripts at one or more timepoints), paralleled by the baseline mutation becoming undetectable by both SS and UDS. Four to nine months later, however, the baseline mutations reappeared in all four cases, paired with dasatinib-resistant mutations in compound mutants, which was accompanied by total BCR-ABL transcript increase. In patients no.13, 17, 21, 22, 32, 34 and 35, no relevant reduction in BCR-ABL transcript levels was detected after the switch to dasatinib or nilotinib – and no regression of the baseline mutation was observed. Newly acquired dasatinib- or nilotinib-resistant (patient no. 13) mutations were found to rapidly appear and to outgrow in parallel with transcript level increase. In the majority of the cases, these mutations were found both alone and paired with 276 the baseline mutation in compound mutants. In patients no. 14, 15, 18 and 32, no molecular response was observed, the baseline mutations regressed and were quickly replaced by dasatinib-resistant ones –either because the latter were acquired by unmutated cells, that were then able to outcompete the imatinib-resistant ones (like in patient no. 15), or because the dasatinib resistant mutations were acquired both by mutated and by unmutated cells, but the fitness of the single mutant was, for some reason, greater than that of the compound mutant (like in patient no. 18).

Discussion

Having recently implemented and optimized a strategy for BCR-ABL1 KD mutation screening based on the Roche-454 NGS technology [142,153] we decided to take advantage of its high-sensitivity approach in order i) to study the dynamics of expansion of BCR-ABL KD mutations ii) to characterize the complexity and the clonal relationships of major and minor mutated populations and iii) to assess whether emerging mutations could be detected earlier during disease course than with sanger sequencing in 35 Philadelphia-positive ALL patients with persisting molecularly detectable residual disease. We focused on Ph+ ALL patients, where BCR-ABL KD mutations may so frequently and so quickly arise and undermine the long-term efficacy of TKI-based therapies. A retrospective, longitudinal re-analysis of 113 follow-up samples from relapse backwards (sampling intervals, 4 to 15 weeks) was performed on a Roche GS Junior instrument. Our results show that UDS may actually provide a more accurate picture of BCR-ABL KD mutation status, both in terms of presence/absence of mutations and in terms of clonal complexity. In 13/35 patients UDS would have allowed to detect emerging mutations earlier than SS. No mutations were detected by SS in the 10 TKI-resistant Ph+ ALL cases that could be analyzed at diagnosis – confirming the limited clinical value of conventional mutation analysis in de novo patients. [163,167] The great majority of such low level mutations were either silent or (apparently) innocuous, but in one case (patient no. 08 in Table 2) an imatinib- (and nilotinib-) resistant Y253H mutation in a sample collected at diagnosis was detected from a patient who relapsed eight weeks later. The patient never achieved a reduction in BCR-ABL1 transcript level and after two months, when imatinib was discontinued because of progressive disease, the Y253H-mutated BCR-ABL had gained full dominance, as assessed both by UDS and by SS. This case is a proof-of-principle that a more sensitive screening of the BCR-ABL1 KD for mutations at diagnosis might be, in some cases, clinically informative. Silent or innocuous low level mutations present at diagnosis are rapidly cleared by TKI treatment, and only rarely and transiently such type of mutations can be detected (we define this 'passenger' mutations). In 13 cases, the higher sensitivity of UDS allowed to 'pick up' emerging T315I, F317L, F317I, E255K, V299L or Y253H mutations. This was possible, however, only because MRD monitoring (and mutation analysis in case of MRD positivity) could be very tight in the patients herein reported – the frequency of sampling being often of five/six weeks and in any case not longer than eighteen weeks. Relapse kinetics in Ph+ ALL is known to be fast, with a median time between MRD elevation and relapse of only 2-3 months. [168] Similarly, the kinetics of emergence of mutations, at least for the T315I and the other highly resistant mutations that could be herein

studied (F317L, V299L, Y253H, E255V and E255K are by far the most frequent ones arising under imatinib, dasatinib or nilotinib therapy in Ph+ ALL) was found to be strikingly rapid. This suggests that chances to anticipate and possibly prevent hematological relapse by timely therapeutic switch could be maximized if sensitive mutation screening was performed monthly in patients with persistence or reappearance of residual disease at the molecular level. However, even when visually inspecting individual reads for sequence variations at key positions (1091 for codon 315, 1096 or 1098 for codon 317, etc) we found only one case in which the mutation could have been present below the threshold we fixed (patient no. 12) – and this despite the fact that our sequencing runs were designed to achieve high coverage. This further indicates that highly resistant mutants expand very rapidly and can be captured in the initial phases of their clonal selection only with frequent monitoring. It remains unclear whether mutants with lower 'fitness' (e.g. lower IC50), may have more indolent kinetics of expansion and whether different TKIs (e.g. imatinib versus the more potent dasatinib and nilotinib) may exert differently strong selective pressures. s, it was quite clear that. Patients with advanced disease who received two or even three lines of TKI therapy generally displayed greater complexity and polyclonal mixtures of single and compound mutants are very frequent in Ph+ ALL patients. In patient no. 05, who received first-line treatment with nilotinib and imatinib with a six week rotation schedule and never experienced a significant reduction of the disease burden, we observed high complexity. This complexity resembles the recently described intra-clonal heterogeneity and branching evolution of multiple myeloma. [169,170] In the great majority of cases (Figure 3)– but not in all – compound mutants were found to achieve dominance over single mutants. It is difficult to assess whether different compound mutants (e.g., with the T315I in combination with different partner mutations) may have different levels of TKI insensitivity, whether the type and number of coexisting mutants may influence their fitness, or whether this observation simply reflects the fact that we are comparing mutational 'snaphots' taken at different timepoints in TKI therapy –would compound mutants always achieve dominance if selective pressure by the same TKI was exerted for a sufficiently long period of time? Whatever the case, detection of compound mutants (that SS does not allow, unless preceded by cloning) is becoming a relevant issue, since recent evidences suggest that while the T315I is sensitive, some specific compound mutants that include the T315I are resistant also to the third generation, pan-BCR-ABL1 inhibitor ponatinib.[171] In conclusion, our data further strengthen the notion that Ph+ ALL is a genetically unstable disease and that BCR-ABL1 KD is highly prone to accumulate complex mutation patterns especially when subjected to

the changing selective pressures exerted by multiple lines of therapy. At diagnosis, rare patients might already have TKI-resistant mutations detectable in a minority of BCR-ABL transcripts. During therapy, even low levels of residual disease or shifts from undetectable to low level BCR-ABL1 transcripts may hide emerging mutations. The value of MRD monitoring in Ph+ ALL for the early assessment of an impending relapse is well established. MRD elevations or reconversion to MRD positivity should trigger sensitive mutation screening, in order to timely allow tailored therapeutic intervention. At the time of resistance, multiple mutations and polyclonal mixtures of single and compound mutants can often be detected and their comprehensive characterization might help understanding how to prevent them through a better therapeutic tailoring.

FIGURES

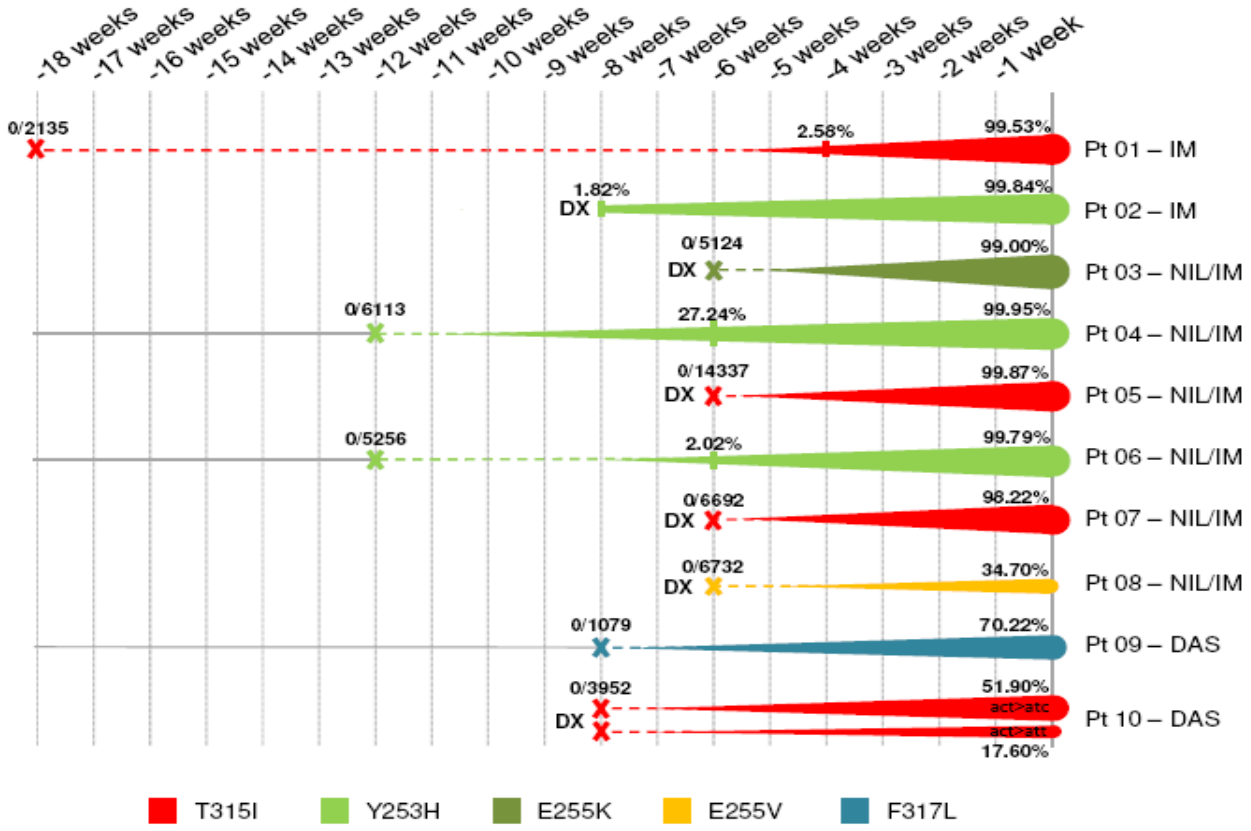


Figure 1 – Time course of the relative abundance of sequence reads harbouring the imatinib-, dasatinib- or nilotinib-resistant mutation(s) that were selected and gained dominance in the ten *de novo* Ph+ ALL patients who relapsed on first-line TKI therapy. Earlier identification of these key mutations (T315I, red; Y253H, light green; E255K, dark green; E255V, yellow; F317L, light blue) might have allowed to counteract relapse. The TKI being administered is specified for each patient. Patient IDs are the same as in Table 2. In patients who developed multiple mutations (no. 05 and 08) the focus is on the first TKI-resistant mutation that emerged and became detectable by SS. The percentages indicate the relative abundance of sequence reads carrying the corresponding nucleotide substitutions at that specific timepoint, as assessed by DS. The 'X' indicates that no sequence reads harbouring that nucleotide substitutions could be detected; in such cases, coverage (number of reads covering that base position) is indicated as the denominator. In patient no. 10, two distinct groups of reads – one harbouring the c.1091C>T single nucleotide substitution and one harbouring a CT to TC dinucleotide substitution at positions c.1091-1092 – both leading to the T315I were identified, but at diagnosis no mutated reads at either positions could be detected; whether these two clones arose independently from each other or whether the second represents a subclone of the first, cannot be established so they are presented separately in the graph. Abbreviations: IM, imatinib; NIL/IM, nilotinib and imatinib in rotation; DAS, dasatinib; DX, diagnosis.

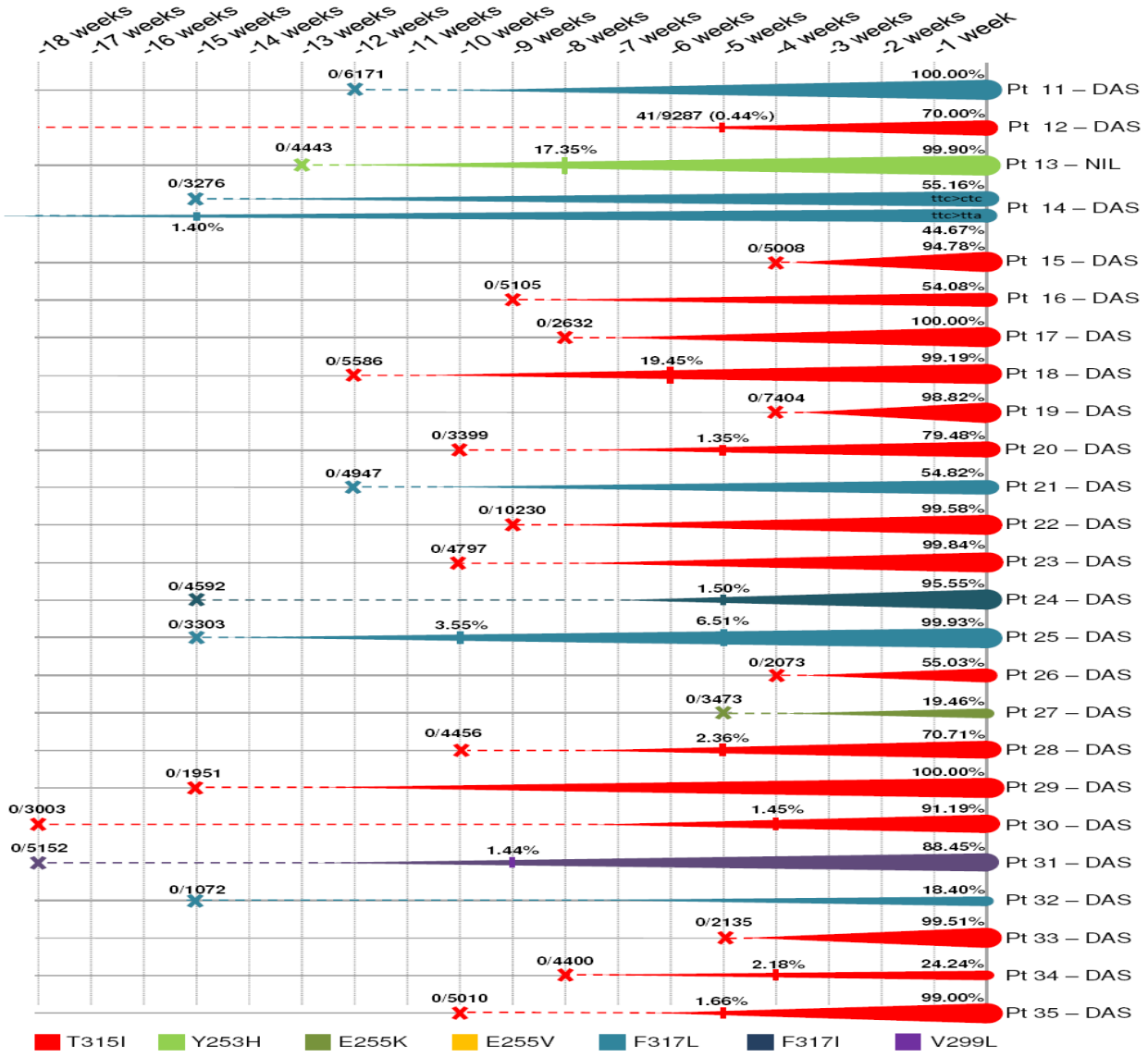
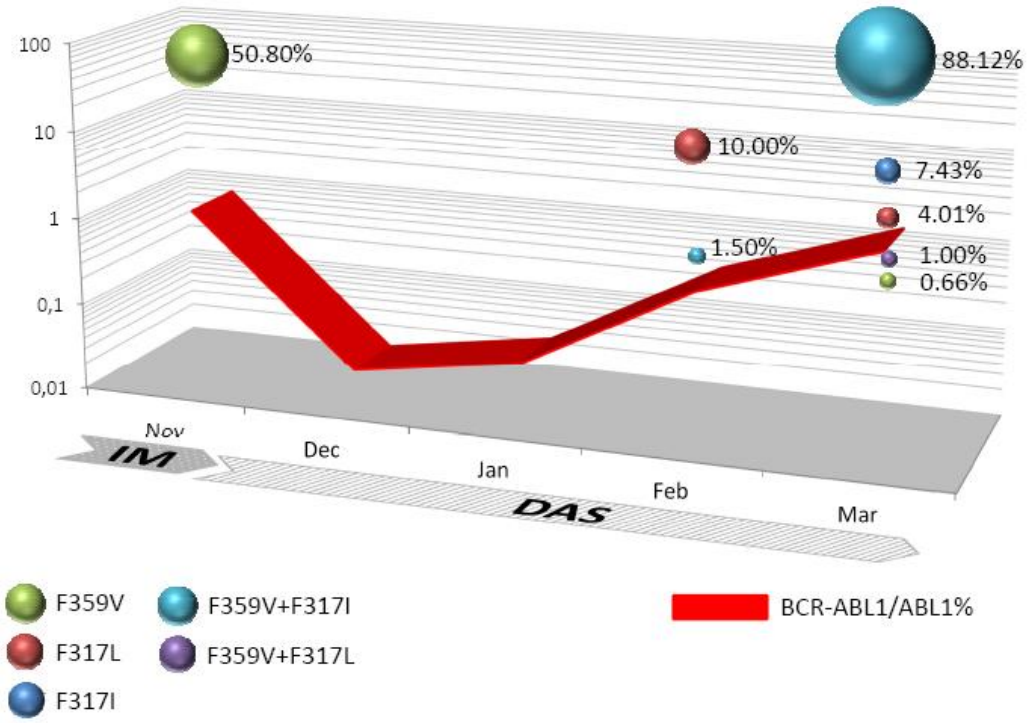


Figure 2 – Time course of the relative abundance of sequence reads harbouring the newly acquired dasatinib- or nilotinib-resistant mutation(s) that took over in the twenty-five patients with advanced Ph+ ALL who relapsed on dasatinib or nilotinib administered for recurrent disease. Earlier identification of these key mutations (T315I, red; Y253H, light green; E255K, dark green; E255V, yellow; F317L, light blue; F317I, dark blue; V299L, purple) might have allowed to counteract relapse. The TKI being administered is specified for each patient. Patient IDs are the same as in Table 3. The percentages indicate the relative abundance of sequence reads carrying the corresponding nucleotide substitutions at that specific timepoint, as assessed by DS. Percentages <1% are also given, with the number of reads found to carry the relevant nucleotide substitution/coverage indicated in parentheses. The 'X' denotes that no sequence reads harbouring that nucleotide substitutions could be detected; in such cases, coverage (number of reads covering that base position) is indicated as the denominator. In patients who showed more than one newly acquired mutations, the focus is, for simplicity, on the mutation that was more likely to be the driver (e.g., the T315I rather than the Y253H in patients no. 19 and 20) or on the

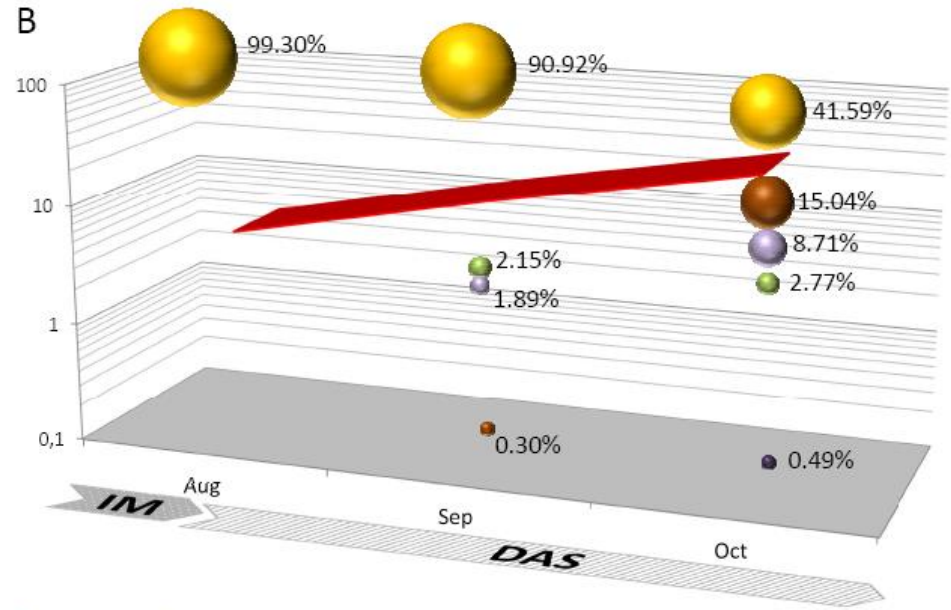
mutation that later became dominant (e.g., the F317I rather than the F317L in patient no. 24). In patient no. 14, the dynamics of two distinct groups of reads harbouring two distinct nucleotide substitutions (c.1096T>C and c.1098C>A), both leading to the dasatinib-resistant F317L, are shown. The second clone, however, originated before the first one, since fifteen weeks before 1.4% of the reads were positive for the c.1098C>A substitution whereas no reads had evidence of the c.1096T>C substitution. Abbreviations: NIL, nilotinib; DAS, dasatinib.

A


100
10
1
0,1
0,01
50.80%
88.12%
10.00%
7.43%
4.01%
1.50%
1.00%
0.66%
Nov
Dec
Jan
Feb
Mar
IM
DAS

F359V
F359V+F317I
BCR-ABL1/ABL1%
F317L
F359V+F317L
F317I

B


100
10
1
0,1
99.30%
90.92%
41.59%
15.04%
8.71%
2.77%
2.15%
1.89%
0.30%
0.49%
Aug
Sep
Oct
IM
DAS


E255V
E255V+T315I
BCR-ABL1/ABL1%
T315I
E255K+T315I
E255K

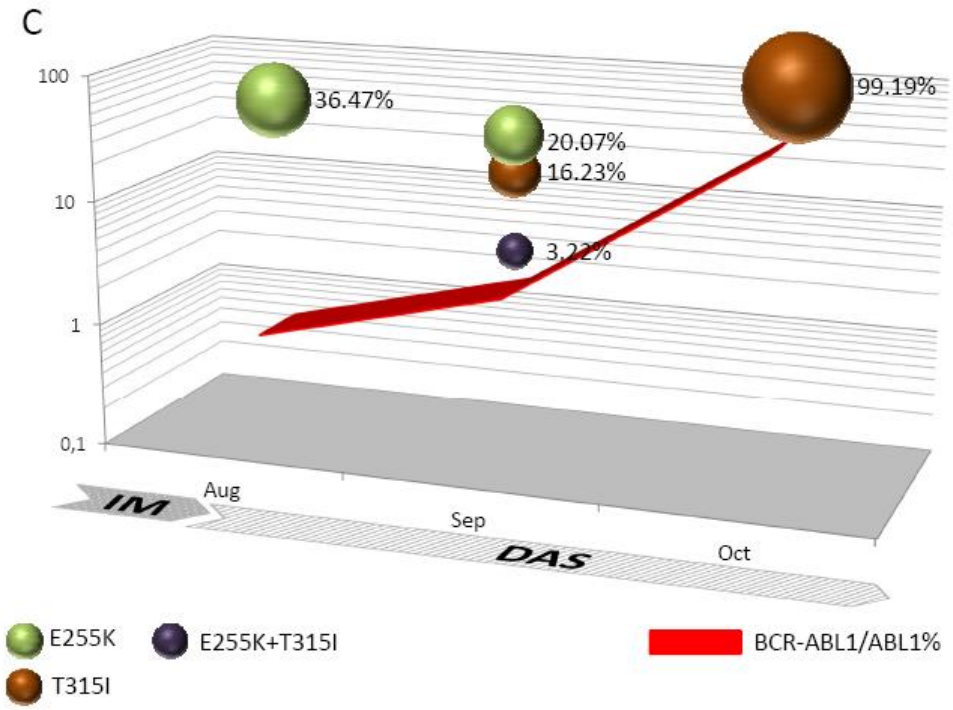


Figure 3 – Representative examples of BCR-ABL1 transcript level and mutated clones dynamics. Relative clone abundance, represented by dots of different size, and BCR-ABL1/ABL1 transcript levels, represented by the red ribbon, are both expressed on a logarithmic scale. **(A)** - First scenario, patient no. 24: after one month of dasatinib therapy, a 1-log decrease in BCR-ABL1 transcript levels was observed and the imatinib-resistant F359V mutation became undetectable both by SS and DS. Two months later, however, two mutated clones – one harbouring an F317L and one harbouring an F317I paired with the original F359V mutation became detectable by DS (with 10% and 1.5% abundance, respectively). The latter clone outgrew rapidly and, at the subsequent evaluation one month later, was detected with 88% abundance. The identification of minor mutant clones harbouring the F317I alone and the F317L and F359V alone and in combination suggests that the two dasatinib-resistant mutations were acquired in parallel by unmutated cells and by F359V-mutated cells. (B) - second scenario, patient no. 34: the switch from imatinib to dasatinib did not result in any relevant reduction in disease burden, as testified by BCR-ABL1 transcript levels. After only one month, an E255K, a T315I and a compound mutant with E255V and T315I together became detectable by DS. After two months, the E255V-positive clone had decreased in abundance from 91% to 41%, whereas the compound mutant E255V+T315I and T315I-positive clone had increased to 15% and 8%, respectively. Also an E255K mutant was found to have acquired a T315I. The patient stopped dasatinib for therapeutic inefficacy and deceased soon after. (C) – third scenario, patient no. 18: after one month of dasatinib therapy, two new mutated clones (one harbouring the T315I mutation alone, one harboring both the E255K and the T315I) became detectable. After two months, the imatinib-resistant E255K clone had disappeared, replaced by the T315I. BCR-ABL1 transcript levels steadily increased. Although in the majority of the cases the compound mutants showed greater fitness than the single mutants, in this patient

the E255K+T315I double mutant was only transiently detected before the T315I single mutant acquired full dominance.

TABLES

Total pts, n	35
Males/females, n	18/17
Median age, years (range)	59 (19-73)
p190-positive, n	24
p210-positive, n	11
De novo Ph+ ALL pts receiving first-line TKI therapy, n	10
Advanced Ph+ ALL pts receiving TKIs for recurrent disease, n	25
Median time from diagnosis to last hematologic relapse, months (range)	18 (4-36)

Table 1 – Patient demographics. p190 and p210 refer to the two Bcr-Abl protein isoforms resulting from different breakpoints in the BCR gene.

Code	Sex	Age	Date	TKI	Mutations by DS	Estimated clonal composition by DS	Mutations by SS	BCR-ABL/ ABL%	Transcript
01-01	**F**	**59**	**Oct-08**	**IM**	**c.1091C>T: p.T315I (99.53%)**	**T315I (99.53%)**	**c.1091C>T: p.T315I (~100%)**	**2.2**	**p190**
01-02			Sep-08	IM	c.1091C>T: p.T315I (2.58%)	T315I (2.58%)	none	0.07	
01-03			Jun-08	IM	none	none	none	0.012	
01-04			Mar-08	dx	c.1346A>G: p.K400X (2.44%) c.1364C>T: c.T406I (2.22%)	K400X (2.44%) T406I (2.22%)	none	11.15	
02-01	**M**	**61**	**Sep-08**	**IM**	**c.904T>C: p.Y253H (99.84%)**	**Y253H (99.84%)**	**c.904T>C: p.Y253H (~100%)**	**89.2**	**p210**
02-02			Jul-08	dx	c.1555G>A: p.E470K (5.87%) c.1454G>A: p.G436D (4.24%) c.927G>A: p.V260V (3.28%) c.912G>A: p.E255E (1.87%) c.904T>C: p.Y253H (1.82%)	n.a.	none	61.2	
03-01	**F**	**34**	**Jul-11**	**NIL**	**c.910G>A: p.E255K (99.00%)**	**E255K (99.00%)**	**c.910G>A: p.E255K (~100%)**	**2.2**	**p190**
03-02			Jun-11	dx	c.1287T>C: A380A (2.36%) c.1002A>G: p.K285K (2.03%)	n.a.	none	1.2	
04-01	**F**	**63**	**Jul-11**	**IM**	**c.904T>C: p.Y253H (99.95%)**	**Y253H (99.95%)**	**c.904T>C: p.Y253H (~100%)**	**4.9**	**p190**
04-02			Jun-11	NIL	c.904T>C: p.Y253H (27.24%)	Y253H (27.24%)	c.904T>C: p.Y253H (~30%)	0.006	
04-03			May-11	IM	c.1211A>G: p.E355G (11.93%)	E355G (11.93%)	none	0.02	
04-04			Feb-11	NIL	no amp	no amp	no amp	undetectable	
04-05			Dec-10	IM	no amp	no amp	no amp	undetectable	
04-06			Nov-10	NIL	none	none	none	0.02	
04-07			Sep-10	dx	c.1401C>T: p.I418I (4.77%) c.1174A>G: M343V (3.35%) c.1015A>G: p.M290V (1.48%)	n.a.	none	146.68	
05-01	**F**	**68**	**Mar-11**	**NIL**	**c.1091C>T: p.T315I (53.43%)** **c.910G>A: p.E255K (25.57%)** **c.911A>T: p.E255V (1.33%)**	**T315I (45.75%)** **E255K (18.22%)** **T315I+E255K (7.35%)** **E255V (1.00%)** **T315I+E255V (0.33%)**	**c.1091C>T: p.T315I (~50%)** **c.910G>A: p.E255K (~30%)**	**56.99**	**p190**
05-02			Feb-11	IM	c.1091C>T: p.T315I (76.12%)	T315I (71.02%)	c.1091C>T: p.T315I (~70%)	n.d.	

				c.910G>A: p.E255K (9.68%)	E255K (5.08%)			
				c.911A>T: p.E255V (1.22%)	T315I+E255K (4.60%)			
					E255V (0.72%)			
					T315I+E255V (0.50%)			
05-03			Jan-11	NIL	c.1091C>T: p.T315I (99.87%)	T315I (99.87%)	c.1091C>T: p.T315I (~100%)	61.35	
05-04			Nov-10	dx	none	none	none	55.44	
06-01	**M**	**73**	**Jul-11**	**NIL**	**c.904T>C: p.Y253H (99.79%)**	**Y253H (99.79%)**	**c.904T>C: p.Y253H (~100%)**	**2.12**	**p190**
06-02			May-11	IM	c.904T>C: p.Y253H (2.02%)	Y253H (2.02%)	none	0.05	
06-03			Apr-11	NIL	c.1030C>T: p.H295Y (4.36%)	H295Y (4.36%)	none	0.03	
06-04			Mar-11	IM	none	none	none	0.04	
06-05			Jan-11	NIL	none	none	none	0.09	
06-06			Dec-10	dx	c.914T>C: p.V256A (4.45%)	V256A (4.45%)	none	54.09	
07-01	**M**	**69**	**Aug-10**	**NIL**	**c.1091C>T: p.T315I (98.22%)**	**T315I (98.22%)**	**c.1091C>T: p.T315I (~100%)**	**n.d.**	**p190**
07-02			Jul-10	dx	c.861A>G: p.E238E (5.34%)	n.a.	none	n.d.	
					c.1446T>C: p.A433A (2.76%)				
08-01	**F**	**70**	**Jul-10**	**IM**	**c.911A>T: p.E255V (45.97%)**	**E255V (45.97%)**	**c.911A>T: p.E255V (~50%)**	**51.75**	**p190**
					c.904T>C: p.Y253H (41.41%)	**Y253H (41.41%)**	**c.904T>C: p.Y253H (~50%)**		
08-02			May-10	IM	c.911A>T: p.E255V (99.98%)	E255V (92.76%)	c.911A>T: p.E255V (~100%)	0.43	
					c.1058T>C: p.V304A (5.17%)	E255V+V304A (5.17%)			
					c.956T>C: p.V270A (2.05%)	E255V+V270A (2.05%)			
08-03			Apr-10	NIL	c.911A>T: p.E255V (34.70%)	E255V (34.70%)	c.911A>T: p.E255V (~40%)	0.04	
08-04			Feb-10	dx	none	none	none	4	
09-01	**F**	**47**	**Mar-13**	**DAS**	**c.1096T>C: p.F317L (70.22%)**	**F317L (70.22%)**	**c.1096T>C: p.F317L (~70%)**	**37.5**	**p210**
09-02			Jan-13	DAS	none	none	none	0.33	
09-03			Oct-12	DAS	none	none	none	55.76	
09-04			Jul-12	dx	c.886A>G: p.K247E (2.07%)	K247E (2.07%)	none	274.65	
10-01	**M**	**19**	**Sep-13**	**DAS**	**c.1091C>T: p.T315I (69.50%)**	**T315I (act>atc)(51.90%)**	**c.1091C>T: p.T315? (~70%)**	**1.22**	**p190**
					c.1091-1092CT>TC: p.T315I (51.90%)	**T315I (act>att)(17.60%)**	**c.1092T>C: p.T315? (~50%)**		
10-02			Jun-13	dx	none	none	none	44.15	

Table 2 – BCR-ABL1 KD mutation status as assessed by DS as against SS in the ten patients who developed resistance to first-line TKI therapy. For each sample analyzed, the TKI being administered and the BCR-ABL1 transcript levels as assessed by real-time RT-PCR are also indicated. Variations in the BCR-ABL1 KD sequence are expressed as both nucleotide and amino acid change according to the standard HGVS (Human Genome Variation Society) nomenclature, using the GenBank reference sequence X16416.1. Relative abundance of each mutation is indicated in parentheses and was estimated based on the proportion of mutant sequence reads or the mutant peak height in the SS sequencing chromatogram. Emerging mutations are highlighted in red. Relapse samples are in bold. When multiple mutations fell in a region ≤400bp and could thus be covered by a single amplicon, the mutations or mutations combinations estimated to be present in each clone together with relative clone abundance were estimated based on manual visual inspection of sequence reads at the specific positions where the variants were identified. When the same mutation resulted from two different codon changes in two distinct clones, the codon change was detailed in parentheses. The question marks in SS results of sample 10-01 indicates that the amino acid change(s) could not be inferred from the sequencing chromatogram. Patient 10 indeed displayed two nucleotide substitutions at two adjacent residues – that were detectable by SS but that only DS could resolve – with the same T315I amino acid substitution carried by two clearly distinct clones. One clone had the 'usual' 'act' to 'att' codon shift resulting from the c.1091C>T nucleotide change; the other one displayed the c.1091C>T but also an adjacent c.1092T>C nucleotide change, thus leading to an 'act' to 'atc' codon shift still corresponding to the threonine to isoleucine amino acid substitution. Abbreviations: 'n.a.' (not assessable) indicates that clonal analysis could not be possible; 'n.d.' (not done) indicates that real-time RT-PCR was not performed; 'no amp' (no amplification) means that amplicon generation was unsuccessful because of too low BCR-ABL1 transcript levels and the sample could thus not be analyzed either by SS or by DS; dx, diagnosis; IM, imatinib; DAS, dasatinib; NIL, nilotinib.

Code	Sex	Age	Date	Months since dx	TKI	Mutations by DS	Estimated clonal composition by DS	Mutations by SS	BCR-ABL/ ABL%	Trans
11-01	**M**	**66**	**Jul-06**	**27**	**DAS**	**c.1098C>G: p.F317L (100.00%)**	**F317L+M351T (99.57%)**	**c.1098C>G: p.F317L (~100%)**	**1.11**	**p190**
						c.1199T>C: p.M351T (99.57%)	**F317L (0.43%)**	**c.1199T>C: p.M351T (~100%)**		
11-02			Apr-06		DAS	none	none	none	0.004	
11-03			Jan-06		DAS	no amp	no amp	no amp	undetectable	
11-04			Oct-05		IM	c.1199T>C: p.M351T (99.99%)	M351T (99.99%)	c.1199T>C: p.M351T (~100%)	1.01	
12-01	**M**	**26**	**Apr-08**	**19**	**DAS**	**c.904T>C: p.Y253H (99.10%)**	**Y253H+T315I (70.00%)**	**c.904T>C: p.Y253H (~100%)**	**0.9**	**p210**
						c.1091C>T: p.T315I (70.00%)	**Y253H (29.10%)**	**c.1091C>T: p.T315I (~70%)**		
12-02			Mar-08		DAS	none	n.a.	none	0.005	
12-03			Feb-08		DAS	no amp	no amp	no amp	undetectable	
12-04			Jan-08		DAS	no amp	no amp	no amp	undetectable	
12-05			Dec-07		DAS	no amp	no amp	no amp	undetectable	
12-06			Nov-07		DAS	none	none	none	0.031	
12-07			Oct-07		IM	c.904T>C: p.Y253H (99.00%)	Y253H (99.00%)	c.904T>C: p.Y253H (~100%)	4.48	
13-01	**M**	**33**	**Mar-07**	**21**	**NIL**	**c.1098C>A: p.F317L (100.00%)**	**F317L+Y253H (99.90%)**	**c.1098C>A: p.F317L (~100%)**	**21.88**	**p210**
						c.904T>C: p.Y253H (99.90%)	**F317L (0.10%)**	**c.904T>C: p.Y253H (~100%)**		
13-02			Jan-07		NIL	c.1098C>A: p.F317L (99.89%)	F317L (82.54%)	c.1098C>A: p.F317L (~100%)	6.26	
						c.904T>C: p.Y253H (17.35%)	F317L+Y253H (17.35%)			
13-03			Dec-06		DAS	c.1098C>A: p.F317L (100.00%)	F317L (100.00%)	c.1098C>A: p.F317L (~100%)	5.05	
14-01	**F**	**82**	**Jul-10**	**16**	**DAS**	**c.1096T>C: p.F317L (55.16%)**	**F317L(ttc>ctc)(55.16%)**	**c.1096T>C: p.F317? (~50%)**	**55.11**	**p190**
						c.1098C>A: p.F317L (44.67%)	**F317L(ttc>tta)(44.67%)**	**c.1098C>A: p.F317? (~50%)**		
						c.1386C>T: p.Y413Y (4.22%)				
14-02			Apr-10		DAS	c.1334A>C: p.H396P (14.07%)	n.a.	none	1.8	
						c.1622C>T: p.A442V (2.18%)				
						c.1098C>A: p.F317L (1.40%)				
14-03			Feb-10		DAS	c.1334A>C: p.H396P (45.89%)	n.a.	c.1334A>C: p.H396P (~50%)	10.83	
						c.1522G>A: p.E459K (7.92%)				
14-04			Jan-10		IM/NIL	c.1334A>C: p.H396P (54.57%)	n.a.	c.1334A>C: p.H396P (~50%)	1.05	
						c.1522G>A: p.E459K (43.33%)		c.1522G>A: p.E459K (~50%)		

15-01	**F**	**24**	**Sep-07**	**4**	**DAS**	**c.1091C>T: p.T315I (94.78%)**	**T315I (94.78%)**	**c.1091C>T: p.T315I (~100%)**	**22.76**	**p210**
15-02			Aug-07		IM	c.1222T>G: p.F359V (17.24%)	F359V (17.24%)	c.1222T>G: p.F359V (~20%)	13.18	
16-01	**M**	**32**	**Mar-08**	**17**	**DAS**	**c.1091C>T: p.T315I (54.08%)**	**T315I (54.08%)**	**c.1091C>T: p.T315I (~50%)**	**3.23**	**p210**
16-02			Jan-08		IM	none	none	none	0.3	
17-01	**M**	**30**	**Feb-06**	**6**	**DAS**	**c.1091C>T: p.T315I (100.00%)**	**T315I+F359V (100.00%)**	**c.1091C>T: p.T315I (~100%)**	**3.15**	**p190**
						c.1222T>G: p.F359V (100.00%)		**c.1222T>G: p.F359V (~100%)**		
						c.1644A>G: p.E499E (99.92%)		**c.1644A>G: p.E499E (~100%)**		
						c. 1494T>C: p.Y449Y (10.11%)				
17-02			Dec-05		IM	c.1222T>G: p.F359V (99.87%)	F359V (99.87%)	c.1222T>G: p.F359V (~100%)	1.11	
						c.1644A>G: p.E499E (99.85%)		c.1644A>G: p.E499E (~100%)		
18-01	**M**	**25**	**Oct-11**	**23**	**DAS**	**c.1091C>T: p.T315I (99.19%)**	**T315I (99.19%)**	**c.1091C>T: p.T315I (~100%)**	**39.86**	**p210**
18-02			Sep-11		DAS	c.910G>A: p.E255K (23.92%)	E255K (20.07%)	c.910G>A: p.E255K (~30%)	3.11	
						c.1091C>T: p.T315I (19.45%)	T315I (16.23%)	c.1091C>T: p.T315I (~20%)		
							E255K+T315I (3.22%)			
18-03			Aug-11		IM	c.910G>A: p.E255K (36.47%)	E255K (36.47%)	c.910G>A: p.E255K (~50%)	0.67	
						c.1165C>T:p.L340L (12.41%)				
19-01	**F**	**64**	**Mar-12**	**18**	**DAS**	**c.904T>C: p.Y253H (98.84%)**	**Y253H+T315I (98.82%)**	**c.904T>C: p.Y253H (~100%)**	**1.99**	**p190**
						c.1091C>T: p.T315I (98.82%)		**c.1091C>T: p.T315I (~100%)**		
19-02			Feb-12		DAS	none	none	none	n.d.	
20-01	**M**	**74**	**Sep-12**	**21**	**DAS**	**c.904T>C: p.Y253H (100.00%)**	**Y253H+T315I (79.48%)**	**c.904T>C: p.Y253H (~100%)**	**2.85**	**p190**
						c.1091C>T: p.T315I (79.48%)	**Y253H+F317L(ttc>tta)(15.36%)**	**c.1091C>T: p.T315I (~70%)**		
						c.1098C>A: p.F317L (15.70%)	**Y253H+F317L(ttc>ctc)(4.04%)**	**c.1098C>A: p.F317L (~20%)**		
						c.1096T>C: p.F317L (4.04%)	**Y253H+T315I+F317L(ttc>tta)(0.34%)**			
20-02			Aug-12		DAS	c.904T>C: p.Y253H (2.50%)	Y253H+T315I (1.35%)	none	0.87	
						c.1091C>T: p.T315I (1.35%)	Y253H (1.15%)			
20-03			Jul-12		DAS	none	none	none	0.05	
20-04			May-12		DAS	no amp	no amp	no amp	undetectable	
20-05			Feb-12		IM	c.904T>C: p.Y253H (99.00%)	Y253H (99.00%)	c.904T>C: p.Y253H (~100%)	0.91	
21-01	**F**	**61**	**Dec-10**	**29**	**DAS**	**c.904T>C: p.Y253H (55.06%)**	**Y253H+F317L (43.46%)**	**c.904T>C: p.Y253H (~50%)**	**9.48**	**p210**
						c.1098C>A: p.F317L (54.82%)	**Y253H (11.60%)**	**c.1098C>A: p.F317L (~50%)**		

						F317L (11.36%)				
21-02			Sep-10		IM	c.904T>C: p.Y253H (100.00%)	Y253H (100.00%)	c.904T>C: p.Y253H (~100%)	7.77	
22-01	**F**	**26**	**Oct-06**	**36**	**DAS**	**c.910G>A: p.E255K (99.59%)**	**E255K+T315I (99.58%)**	**c.910G>A: p.E255K (~100%)**	**4.43**	**p190**
						c.1091C>T: p.T315I (99.58%)		**c.1091C>T: p.T315I (~100%)**		
22-02			Aug-06		IM	c.910G>A: p.E255K (98.28%)	E255K (98.28%)	c.910G>A: p.E255K (~100%)	1.18	
23-01	**M**	**64**	**Jul-06**	**23**	**DAS**	**c.1091C>T: p.T315I (99.84%)**	**T315I (99.84%)**	**c.1091C>T: p.T315I (~100%)**	**0.29**	**p190**
23-02			May-06		DAS	none	none	none	0.009	
24-01	**M**	**22**	**Mar-10**	**17**	**DAS**	**c.1096T>A: p.F317I (95.55%)**	**F359V+F317I (88.12%)**	**c.1096T>A: p.F317I (~100%)**	**1.75**	**p210**
						c.1222T>G: p.F359V (89.78%)	**F317I (7.43%)**	**c.1222T>G: p.F359V (~100%)**		
						c.1098C>A: p.F317L (5.01%)	**F317L (4.01%)**			
							F359V+F317L (1.00%)			
							F359V (0.66%)			
24-02			Feb-10		DAS	c.1098C>A: p.F317L (10.00%)	F317L (10.00%)	none	0.42	
						c.1096T>A: p.F317I (1.50%)	F359V+F317I (1.50%)			
						c.1222T>G: p.F359V (1.50%)				
24-03			Jan-10		DAS	none	none	none	0.04	
24-04			Dec-09		DAS	none	none	none	0.02	
24-05			Nov-09		IM	c.1222T>G: p.F359V (50.80%)	F359V (50.80%)	c.1222T>G: p.F359V (~50%)	0.99	
25-01	**M**	**34**	**Apr-12**	**17**	**DAS**	**c.1098C>A: p.F317L (99.93%)**	**F317L (99.93%)**	**c.1098C>A: p.F317L (~100%)**	**16.72**	**p190**
25-02			Mar-12		DAS	c.1098C>A: p.F317L (6.51%)	F317L (6.51%)	none	0.42	
25-03			Feb-12		DAS	c.1098C>A: p.F317L (3.55%)	F317L (3.55%)	none	0.69	
25-04			Jan-12		DAS	none	none	none	0.14	
25-05			Dec-11		IM	none	none	none	0.008	
26-01	**M**	**35**	**Oct-12**	**29**	**DAS**	**c.1091C>T: p.T315I (55.03%)**	**T315I (55.03%)**	**c.1091C>T: p.T315I (~50%)**	**87.58**	**p190**
26-02			Sep-12		DAS	c.1018A>G: p.Y291E (1.99%)	Y291E (1.99%)	none	18.94	
27-01	**F**	**63**	**Dec-13**	**35**	**NIL**	**c.910G>A:p.E255K (19.46%)**	**E255K (19.46%)**	**c.910G>A:p.E255K (~20%)**	**77.52**	**p190**
27-02			Nov-13		NIL	c.1298T>C:p.L384P (1.14%)	L384P (1.14%)	none	0.82	
27-03			Oct-13		NIL	none	none	none	0.01	
28-01	**M**	**42**	**Oct-13**	**16**	**DAS**	**c.1091C>T: p.T315I (70.71%)**	**T315I (70.71%)**	**c.1091C>T: p.T315I (~70%)**	**57.54**	**p190**
28-02			Sep-13		DAS	c.1091C>T: p.T315I (2.36%)	T315I (2.36%)	none	0.9	

28-03			Aug-13		DAS	none	none	none	0.05	
29-01	**M**	**32**	**Apr-13**	**12**	**DAS**	**c.1091C>T: p.T315I (100.00%)**	**T315I (78.87%)**	**c.1091C>T: p.T315I (~100%)**	**1.19**	**p210**
						c.957G>T: p.V270V (13.73%)	**T315I+V270V (13.73%)**			
						c.994T>C: p.F283L (4.40%)	**T315I+F283L (4.40%)**			
						c.1375A>G:p.S410G (3.00%)	**T315I+S410G (3.00%)**			
29-02			Jan-13		DAS	c.1636T>C: p.F497L (18.31%)	F497L (18.31%)	none	1.61	
30-01	**M**	**37**	**Dec-13**	**33**	**DAS**	**c.1091C>T: p.T315I (91.19%)**	**L387F+T315I (91.19%)**	**c.1091C>T: p.T315I (~100%)**	**9.02**	**p190**
						c.1308G>C: p.L387F (99.85%)	**L387F (8.64%)**	**c.1308G>C: p.L387F (~100%)**		
						c.1644A>G: p.E499E (99.77%)				
30-02			Nov-13		DAS	c.1091C>T: p.T315I (1.45%)	T315I+L387F (1.11%)		0.96	
						c.1308G>C: p.L387F (1.43%)	L387F (0.32%)			
						c.1644A>G: p.E499E (99.77%)	T315I (0.34%)			
30-03			Aug -13		DAS	none	none		0.0012	
31-01	**F**	**33**	**Jan-14**	**9**	**DAS**	**c.1042G>C: p.V299L (88.45%)**	**V299L (88.45%)**	**c.1042G>C: p.V299L (~100%)**	**11.77**	**p190**
31-02			Nov-13		DAS	c.1042G>C: p.V299L (1.44%)	V299L (1.44%)	none	0.7	
31-03			Sep-13		DAS	none	none	none	0.003	
32-01	**F**	**66**	**May-06**	**13**	**DAS**	**c.1306T>A: p.L387M (99.89%)**	**L387M (66.42%)**	**c.1306T>A: p.L387M (~100%)**	**8.88**	**p190**
						c.1098C>A: p.F317L (18.40%)	**L387M+F317L (18.40%)**	**c.1098C>A: p.F317L (~20%)**		
						c.1319G>A: p.D371N (15.07%)	**L387M+D371N (15.07%)**			
32-02			Feb-06		IM	c.1522G>A: p.E459K (91.70%)	n.a.	c.1522G>A: p.E459K (~100%)	4.23	
						c.1306T>A: p.L387M (4.97%)		none		
33-01	**F**	**60**	**Aug-13**	**16**	**DAS**	**c.1091C>T: p.T315I (99.51%)**	**T315I (99.51%)**	**c.1091C>T: p.T315I (~100%)**	**0.16**	**p190**
33-02			Jul-13		DAS	none	none	none	0.05	
34-01	**F**	**61**	**Oct-11**	**9**	**DAS**	**c.911A>T: p.E255V (50.30%)**	**E255V (41.59%)**	**c.911A>T: p.E255V (~50%)**	**34.56**	**p190**
						c.1091C>T: p.T315I (24.24%)	**T315I (15.04%)**	**c.1091C>T: p.T315I (~30%)**		
						c.910G>A: p.E255K (3.26%)	**E255V+T315I (8.71%)**			
							E255K (2.77%)			
							E255K+T315I (0.49%)			
34-02			Sep-11		DAS	c.911A>T: p.E255V (92.81%)	E255V (90.92%)	c.911A>T: p.E255V (~100%)	14.26	
						c.1091C>T: p.T315I (2.19%)	E255K (2.15%)			

						c.910G>A: p.E255K (2.15%)	E255V+T315I (1.89%) T315I (0.30%)			
34-03			Aug-11		IM	c.911A>T: p.E255V (99.30%)	E255V (99.30%)	c.911A>T: p.E255V (~100%)	5.28	
35-01	**F**	**64**	**Jul-10**	**14**	**DAS**	**c.910G>A: p.E255K (99.44%)**	**E255K+T315I (97.60%)**	**c.1091C>T: p.T315I (~100%)**	**1.99**	**p210**
						c.1091C>T: p.T315I (99.00%)	**E255K (1.84%)** **T315I (1.40%)**	**c.910G>A: p.E255K (~100%)**		
35-02			Jun-10		DAS	c.910G>A: p.E255K (97.97%) c.1091C>T: p.T315I (1.66%) c.1240G>A: p.A365T (1.31%)	n.a.	c.910G>A: p.E255K (~100%)	0.93	
35-03			May-10		IM	c.910G>A: p.E255K (99.18%)	E255K (99.18%)	c.910G>A: p.E255K (~100%)	0.74	

Table 3 – BCR-ABL1 KD mutation status as assessed by DS as against SS in the twenty-five patients who developed resistance to TKI therapy administered for recurrent disease.

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ACKNOWLEDGMENTS

Molecular Biology Unit, Institute of Hematology and Medical Oncology "L. e A. Seràgnoli"

Simona Soverini, Luca Zazzeroni, Manuela Mancini, Ilaria Iacobucci, Claudia Venturi, Maria Teresa Bochicchio

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Katerina Machova-Polakova, Adela Brouckova

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Roche Applied Science, Monza, Italy;

Michele Iacono, Francesca Dal Pero

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