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Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy

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Selective inhibition of the BCR-ABL tyrosine kinase by imatinib (STI571, Glivec/Gleevec) is a promising new therapeutic strategy in patients with chronic myelogenous leukemia (CML). Despite significant hematologic and cytogenetic responses, resistance occurs, particularly in patients with advanced disease. We sought to determine the underlying mechanisms. Sixty-six patients with CML in myeloid blast crisis (*n* = 33), lymphoid blast crisis (*n* = 2), accelerated phase (*n* = 16), chronic phase (*n* = 13), and BCR-ABL-positive acute lymphoblastic leukemia (*n* = 2) resistant to imatinib were investigated. Median duration of imatinib therapy was 148 days (range 6–882). Patients were evaluated for genomic amplification of BCR-ABL, overexpression of BCR-ABL transcripts, clonal karyotypic evolution, and mutations of the imatinib binding site in the BCR-ABL tyrosine kinase domain. Results were as follows: (1) Median levels of BCR-ABL transcripts, were not significantly changed at the time of resistance but 7/55 patients showed a >10-fold increase in BCR-ABL levels; (2) genomic amplification of BCR-ABL was found in 2/32 patients evaluated by fluorescence *in situ* hybridization; (3) additional chromosomal aberrations were observed in 19/36 patients; (4) point mutations of the ABL tyrosine kinase domain resulting in reactivation of the BCR-ABL tyrosine kinase were detected in 23/66 patients. In conclusion, although the heterogeneous development of imatinib resistance is challenging, the fact that BCR-ABL is active in many resistant patients suggests that the chimeric oncoprotein remains a good therapeutic target. However, patients with clonal evolution are more likely to have BCR-ABL-independent mechanisms of resistance. The observations warrant trials combining imatinib with other agents.
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Introduction

Chronic myelogenous leukemia (CML) is a clonal neoplastic disorder of hematopoietic stem cells that accounts for 15 to 20% of newly diagnosed cases of adult leukemia. The clinical course of CML is characteristically triphasic, initially comprising a chronic phase (CP) of variable duration, followed by more or less rapid progression through an accelerated phase (AP) to blast crisis (BC).[1]

The causative molecular event in CML is the genetic transposition of ABL and BCR sequences to form a BCR-ABL fusion gene, leading to the expression of a constitutively active, chimeric BCR-ABL protein-tyrosine kinase. Approximately 95% of CML patients have this rearrangement which is identifiable in most cases as Philadelphia (Ph) translocation, t(9;22). Expression of the BCR-ABL gene is sufficient to cause chronic phase CML, while disease progression to AP or BC is thought to depend on additional genetic changes.[2]

Selective inhibition of the BCR-ABL tyrosine kinase by imatinib is a promising new therapeutic strategy in patients with CML. Despite high rates of hematologic and cytogenetic responses, primary refractory disease and secondary resistance have been observed in a proportion of patients on imatinib monotherapy. Clinical studies have demonstrated durable responses in CP patients whereas most responding patients in BC relapse despite continued therapy.[3–7]

In an attempt to model resistance, several groups have generated imatinib-resistant cell lines using BCR-ABL-transformed murine hematopoietic cells and BCR-ABL-positive human cell lines. Mechanisms of imatinib resistance identified from these *in vitro* studies include several-fold increase in the amount of BCR-ABL protein, amplification of the BCR-ABL gene, and overexpression of the multidrug resistance P-glycoprotein.[8–11]

Recently, the crystal structure of the catalytic region of the ABL kinase in complex with an imatinib analogue has been reported.[12] Early investigations proposed that one of the most common mechanisms of relapse is a mutation of amino acid (aa) 315 of the ABL kinase domain,[13] a predicted site of contact between imatinib and the ATP binding site of ABL.[12] However, other investigators found kinase domain mutations at additional sites.[14–16] Parallel studies detected similar mutations in relapsing patients after imatinib therapy for BCR-ABL-positive acute lymphoblastic leukemia (ALL), biphenotypic leukemia, and CML in lymphoid blast crisis.[17,18] In this study, we performed a comprehensive evaluation of mechanisms of primary resistance and relapse in a larger cohort of patients.

Methods

Patients

Patients were recruited into six multicenter phase II studies and were treated with 400 to 600 mg imatinib p.o. daily. Primary resistant disease was considered as lack of hematologic response after at least 4 weeks of therapy or progressive disease in non-responding patients. In CML, relapse was defined as the occurrence of >10% blasts or >30% blasts plus promyelocytes, or >20% basophils in peripheral blood (PB) or bone marrow (BM) after partial or complete hematologic remission that had lasted at least 4 weeks. In cases of ALL, hematologic relapse was defined as >30% blasts in PB or BM.

All patients expressed BCR-ABL transcripts as determined by multiplex RT-PCR,[19] 16 had b2a2, 44 b3a2, four both b2a2 and b3a2, and two e1a2 transcripts.

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Table 1 PCR primers and digestion products to confirm mutations by restriction digest analysis

ABL codon	Forward primer	Reverse primer	Restriction enzyme	Fragment size (bp)	
				wild type	mutant
253	A4+ (ABL exon 4) TCACCACGCTCCATTATCCA	RSA– (ABL exon 4) TCTTCCACACGCCCTCaTAaACCT	*Rsa*I	138	168
255	A4+	A4– (ABL exon 4) CTTCCACACGCtCTCGTACA	*Mnl*I	136	167
315	A4/5+ (ABL exon 4/5) AAGACCTTGAAGGAGGACACCATG	A6– (ABL exon 6) GTTGCACTCCaTCAaGTAGTCCA	*Dde*I	134	183
351	A6+ (ABL exon 6) CCCGTTCTATATCATCACTGAGTTC	A7–	*Nco*I	218	341

Lower case letters indicate mismatches that were introduced to destroy other restriction sites close to a mutation.

Evaluation of relapse/resistance mechanisms

Prior to imatinib therapy and at the time of resistance or relapse we determined the number of genomic BCR-ABL copies by fluorescence *in situ* hybridization,[20] the expression level of BCR-ABL transcripts in PB leukocytes by quantitative real time RT-PCR,[21] and karyotype evolution by metaphase cytogenetics.[20] Lastly, the BCR-ABL tyrosine kinase domain was sequenced after RT-PCR amplification from PB leukocyte cDNA.[21,22]

To amplify the kinase domain, hemi-nested PCR was performed with the following primers: first step, B2B (BCR exon 13) ACAGCATTCCGCTGACCATCAATAAG plus A7– (ABL exon 7) AGACGTCGGACTTGATGGAGAACT; second step, AN4+ (ABL exon 4) TGGTTCATCATCATTCAACGGTGG plus A7–. This procedure ensured that the normal, unrearranged ABL gene was not analyzed. The 675 bp products encoding the BCR-ABL ATP binding pocket and the activation loop were directly sequenced in both directions (MWG Biotech, Ebersberg, Germany).

Mutation-specific restriction digests were performed to confirm mutations of amino acids 253, 255, 315, and 351 and quantitate the proportion of mutated alleles. Mutations led to loss of *Rsa*I (codon 253), *Mnl*I (codon 255), *Dde*I (codon 315), and *Nco*I (codon 351) restriction sites. Following amplification of the BCR-ABL junction with primers B2B and A7–, fragments flanking each mutation site were reamplified, digested and electrophoresed on 1.8% ethidium bromide-stained agarose gels. Primer combinations and product sizes for each mutation are shown in Table 1. The linearity of the assay was demonstrated using dilutions of plasmids harboring mutations in non-mutated plasmids. Restriction digest revealed mutated plasmids of a concentration of ≥10%.

Analysis of BCR-ABL kinase activity

Prior to imatinib therapy, at the time of response and at relapse, BCR-ABL tyrosine kinase activity was determined by assessment of tyrosine phosphorylation of the CRK-oncogene-like protein (CRKL) in leukocytes by gel electrophoresis and immunoblotting with anti-CRKL antiserum.[23–27]

To investigate the biological consequences of mutations ABL autophosphorylation of the wild-type ABL kinase domain consisting of c-ABL aa 220 to 498 and of mutations constructed using PCR amplification were determined as described.[28] Briefly, *in vitro* generated fusion proteins of the ABL kinase domain mutations as well as wild-type ABL were incubated with concentrations of imatinib ranging from 0 to 10 μM, in the presence of γ^{32}P ATP. ABL autophosphorylation signal intensity was quantitated with a phosphoimager.

Results

We investigated mechanisms of resistance in 66 unselected BCR-ABL$^+$ patients resistant to or relapsed on imatinib after a median treatment interval of 148 days (range 6–882). Imatinib resistance developed in 33 patients with CML in myeloid BC, 16 patients in AP, 13 patients in CP, pretreated with hydroxyurea and interferon α ($n=12$) or hydroxyurea ($n=1$), two patients with lymphoid BC and two patients with BCR-ABL-positive ALL. Forty-three patients relapsed after a primary response and 23 showed upfront resistance to imatinib (Table 2). A molecular or cytogenetic mechanism associated with resistance has been found in 7/23 patients with primary resistant disease and in 33/43 patients with relapse. More than one mechanism has been detected in seven patients (Table 3).

Genomic amplification of BCR-ABL

Multiple copies of BCR-ABL were observed in two of 32 patients by FISH. In seven additional patients, two BCR-ABL genes were found and reflect the acquisition of a second Ph chromosome.

Overexpression of BCR-ABL transcripts

The median level of BCR-ABL transcripts expressed as the ratio BCR-ABL/glucose-6-phosphate dehydrogenase (G6PD)

Table 2 Patients resistant to or relapsing on imatinib therapy ($n=66$)

	Relapse	Primary resistance	Total	%
CML myeloid blast crisis	16	17	33	50
CML accelerated phase	11	5	16	24
CML chronic phase	13	—	13	20
CML lymphoid blast crisis	2	—	2	3
BCR-ABL + ALL	1	1	2	3
	43	23	66	

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Table 3 Molecular or cytogenetic mechanisms associated with hematologic resistance to or relapse on imatinib

Mechanism	*Primary resistance n = 23[a]*	*Relapse n = 43[a]*	*Total n = 66[a]*
Genomic amplification of BCR-ABL	2/7	–/25	2/32
Overexpression of BCR-ABL transcripts	3/18	4/37	7/55
Mutation of the ATP binding site	–/23	23/43	23/66
Clonal evolution	5/9	14/27	19/36
No mechanism detectable[b]	15	11	26

[a]Six patients showed two, one patient three mechanisms of resistance.
[b]From 26 patients without detectable mechanism of resistance, FISH analysis was performed in 25, quantitative RT-PCR in 22, and sequencing of the kinase domain in all 26 cases. Paired cytogenetic data (pretherapy/resistance) were available from 10 patients.

was 4.6% (range 0.1–156) prior to imatinib therapy and 6.0% (range 0.07–440) at the time of resistance. Although the median ratio BCR-ABL/G6PD was not significantly changed at the time of resistance as compared to pretherapy, 7/55 patients showed a more than 10-fold increase in BCR-ABL levels (Figure 1). Three of these patients had primary resistance and four relapsed on imatinib therapy.

Clonal cytogenetic evolution

Novel acquired additional chromosomal aberrations were observed in 19/36 patients, eight of whom had multiple changes. Thirteen patients developed aneuploidy, which included a second Ph chromosome in eight patients, trisomy 8 in six patients, trisomy 6, 9, 12 and 18, monosomy 16, and doubling of the genome in one case each. Alteration of the short arm of chromosome 17 leading to the loss of one p53 allele occurred in seven patients and new reciprocal translocations in two cases (t(18;22) and t(6;22) in one patient and t(1;16); t(1;8); t(2;12); t(4;18) in a second patient).

Mutations

A variety of point mutations in the ABL tyrosine kinase domain were detected in 23 out of 66 patients at the time of relapse.

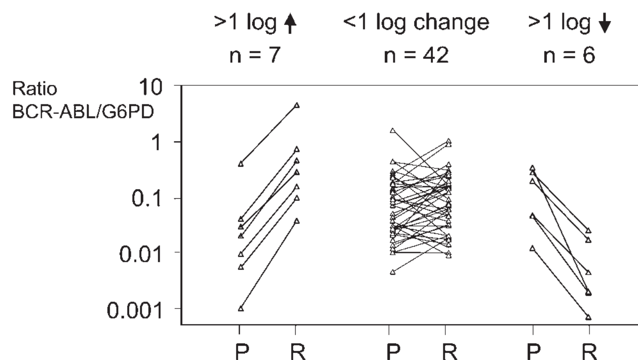

Figure 1 Ratios of BCR-ABL to glucose-6-phosphate dehydrogenase transcripts prior to imatinib therapy (P) and at the time of resistance (R). Seven of patients showed more than a 10-fold increase in BCR-ABL levels.

Table 4 Point mutations of the BCR-ABL tyrosine kinase domain in 23 of 66 patients who relapsed on imatinib therapy

Genomic mutation	*Amino acid*	*n*	*IC₅₀ (μM)*
A 58768 G	M 244 V	1	
G 58794 C	Q 252 H	1	
T 58795 C	Y 253 H	4	3.7
A 58796 T	Y 253 F	1	1.8
G 58801 A	E 255 K	3	>5.0
A 58802 T	E 255 V	1	>5.0
C 68721 T	T 315 I	6	>5.0
T 68829 C	M 351 T	4	[a]
A 68841 G	E 355 G	1	
A 70794 G	H 396 R	1	
Wild-type ABL			0.025

[a]M351T resulted in a >80% reduction of the kinase activity.
Amino acid substitutions from the corresponding mutations are listed along with an *in vitro* analysis of the median inhibitory concentration (IC_{50}) of imatinib. ABL nucleotide positions refer to locus U97563.1 and amino acid positions refer to protein sequence AAB60394.

These mutations could not be detected in samples processed prior to imatinib therapy.

Mutations led to amino acid substitutions at the ATP binding site (Table 4, Figure 2). These substitutions included changes at codon 244 from ATG, coding for methionine to GTG, coding for valine ($n = 1$); codon 252 from CAG, coding for glutamine to CAC, coding for histidine ($n = 1$); codon 253 from TAC, coding for tyrosine to CAC (histidine, $n = 4$), or to TTC (phenylalanine, $n = 1$). A substitution at codon 255 changed GAG (glutamic acid) to AAG (lysine, $n = 3$), or to GTG (valine, $n = 1$). Substitutions at codon 315 changed ACT (threonine) to ATT (isoleucine, $n = 6$). A mutation was observed at codon 351 changing ATG (methionine) to ACG (threonine, $n = 4$), and at codon 355 changing GAG (glutamic acid) to GGG (glycine) ($n = 1$). The activation loop was

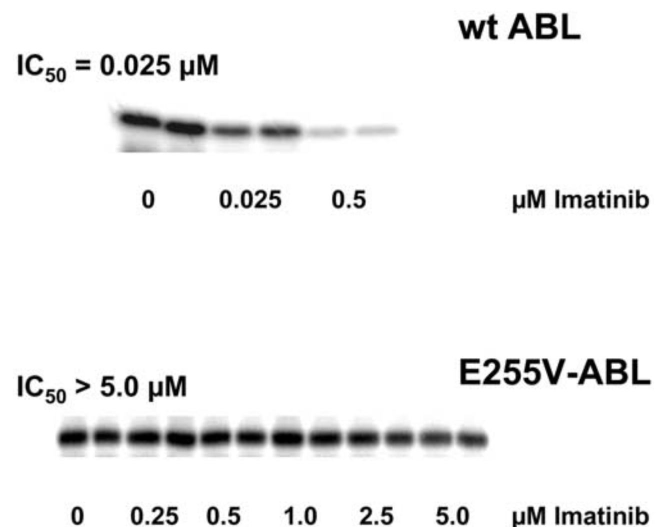

Figure 2 ABL autophosphorylation assay. Wild-type ABL showed a median inhibitory imatinib concentration of 0.025 μM. As an example, this figure shows that mutation of amino acid 255 from glutamic acid to valine leads to virtual insensitivity to imatinib with an IC_{50} of >5 μM.

mutated at position 396 changing CAT (histidine) to CGT (arginine, $n = 1$).

The biological significance of these amino acid substitutions was determined by the ability of imatinib to inhibit the kinase activity of mutant ABL *in vitro*.[29] Imatinib inhibited wild-type ABL at a median inhibitory concentration (IC_{50}) of 0.025 μM; mutations E255K, E255V and T315I led to virtual insensitivity to imatinib with an IC_{50} of $>5\mu$M. The substitutions Y253F and Y253H resulted in an IC_{50} of 1.8 μM and 3.7 μM, respectively. The mutation M351T caused a significant reduction of the ABL kinase activity, such that an IC_{50} could not be determined (Table 4).

Restriction analysis of cDNA and genomic DNA was used to confirm the presence of the mutation and to track it during the course of treatment. Mutations were not detected in pretherapy samples. However, in one case, chimerism of mutated and unmutated alleles was observed 3 months prior to relapse (Figure 3).

All patients with mutations had relapsed after an initial response to imatinib. Relapses occurred after a median of 237 days (range 65–882) in patients with mutations as compared to 251 days (range 55–770) in non-mutated patients (NS, Figure 4).

In vivo activity of BCR-ABL

Reactivation of BCR-ABL kinase activity was confirmed by immunoblotting of the BCR-ABL substrate CRKL in patients with point mutations as seen by an increase in the proportion of phosphorylated CRKL indicating reactivation of BCR-ABL (Figures 5 and 6). The median proportion of phosphorylated CRKL in patients prior to imatinib ($n = 25$) was 50% (range 32–77%), and significantly reduced in responding patients at the time of remission ($n = 17$, median 23%, range 0–44%; $P < 0.0001$). Whilst BCR-ABL was reactivated in all but one patient with point mutations of BCR-ABL ($n = 12$, median 66%, range 12–98%), BCR-ABL was still inhibited in a proportion of resistant patients lacking mutations ($n = 23$, median 45%, range 9–76%) (Figure 6).

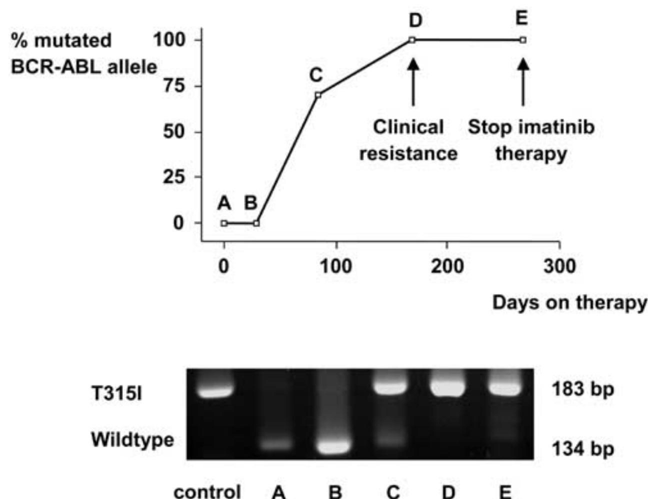

Figure 3 Proportion of T315I mutated BCR-ABL during the course of imatinib therapy as determined by restriction digest analysis. The mutation results in loss of a *Dde*I restriction site. Three months prior to hematological resistance (time-point C) the proportion of undigested fragment (183 bp) reflecting the mutated allele was 70%.

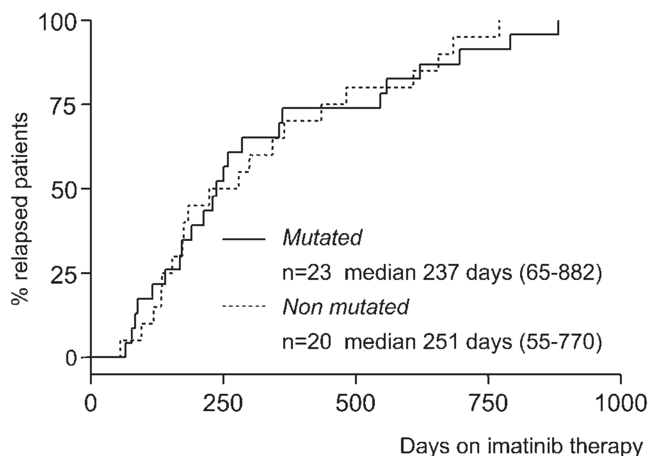

Figure 4 Comparison of the time to relapse in patients with mutations of the ATP binding site ($n = 23$, median 237 days, range 65–882) and patients without mutations ($n = 20$, median 251 days, range 55–770, NS).

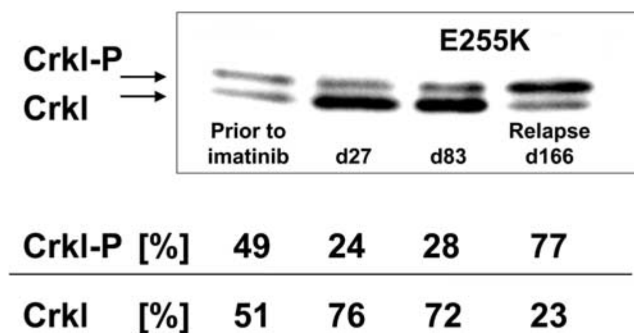

Figure 5 Reactivation of BCR-ABL at time of relapse in a patient with an E255K mutation. CRKL that is phosphorylated by BCR-ABL migrates more slowly than the unphosphorylated form. The relative proportion of phosphorylated CRKL (reflecting active BCR-ABL) was 49% before imatinib therapy, 24% at day 27, 28% at day 83, and 77% at the time of clinical relapse at day 166.

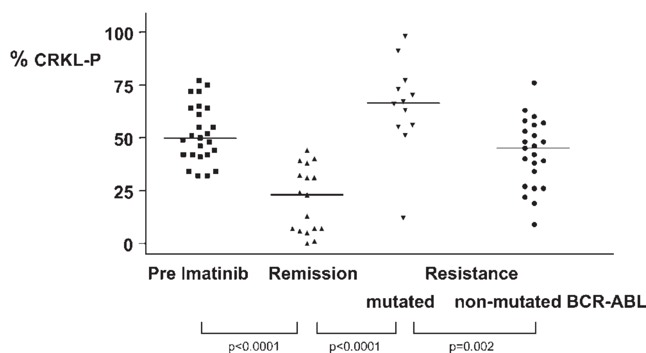

Figure 6 CRKL P/CRKL mobility shift assay. Proportion of phosphorylated CRKL prior to imatinib ($n = 25$, median 50%, range 32–77), at the time of remission ($n = 17$, median 23%, range 0–44), and relapse in patients with mutations of the ATP binding site ($n = 12$, median 66%, range 12–98), and relapse or refractory disease in patients without such mutations ($n = 23$, median 45%, range 9–76%). Paired samples (pretherapy/refractory or resistant disease) were available in 23 patients.

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Patient outcome

Forty-six of the 66 imatinib resistant/relapsed patients died from progressive disease and four patients underwent allogeneic stem cell transplantation. Sixteen non-transplanted patients are alive, three of whom still on imatinib therapy. Of the 23 patients with mutations of the ATP binding site, 10 died from progressive disease, one died after allogeneic stem cell transplantation, two are in molecular remission after chemotherapy and allogeneic stem cell transplantation, and 10 patients switched from imatinib therapy to hydroxyurea ($n=5$), hydroxyurea plus ara-C ($n=2$), or hydroxyurea plus mercaptopurin ($n=3$). Investigation of the proportion of mutated BCR-ABL by restriction digest analysis in a patient with a Y253H mutation in hematologic remission after stopping imatinib and commencing hydroxyurea plus mercaptopurin showed the proportion of the mutated BCR-ABL allele decreasing from 100% at the time of hematologic relapse to <10% within 5 months after stopping imatinib therapy.

Discussion

The advent of molecular targeted therapies is a significant advance in cancer medicine. Hematologic responses to imatinib have been observed in 95% of patients in CP CML after failure of interferon-α therapy, 71% of AP patients, and 31% of patients in myeloid BC.[3,4,7] However, drug resistance has been observed, particularly in advanced phase CML. In AP, 29% of patients failed to reach hematologic remission and 91 of 181 patients (50%) have relapsed after an initial response to imatinib monotherapy.[3] In myeloid BC, 69% failed to achieve hematologic remission and 43/70 (61%) relapsed after initial response.[4] In contrast, less than 10% of CP patients have relapsed with a median duration follow-up of 18 months.[7]

Our data show that mechanisms of imatinib resistance are heterogeneous. BCR-ABL amplification at the genomic and transcript levels occurs in some patients. This may confer a growth advantage of cells that overexpress BCR-ABL.

Sequencing of the region encoding the aa that bind ATP and imatinib revealed acquired mutations which lead to substitutions of amino acids which are important for specific binding of imatinib. These mutations were clustered in four regions (1) the P-loop, a highly conserved region responsible for phosphate binding (aa 248–256, motif LGGGQYGEV).[12,30] (2) at T315, a non-conserved residue that is in part responsible for the selective inhibition of ABL by imatinib.[12] (3) M351 and E355, (4) mutations of the activation loop, resulting in an activated conformation of ABL insensitive to imatinib.[12] In vitro analysis of the capacity of imatinib to inhibit mutated ABL protein demonstrated variable consequences of these mutations. The substitutions of E255 and T315 resulted in virtual insensitivity to imatinib with an IC_{50} >5.0 μM whilst mutation of Y253 led to a significant but lower increase in the IC_{50} to 1.8–3.7μM. As the ABL kinase was still active, this demonstrates that ATP binding in these mutants remains intact. In contrast, M351T led to a significant decrease in kinase activity. The functional data are consistent with findings investigating the proliferation of Ba/F3 cells expressing wild-type and mutant BCR-ABL.[18]

The decreased sensitivities of the mutations at residues 253, 255 and 315 to imatinib relative to wild-type ABL are consistent with predictions from the crystal structure. A hydrogen bond between ABL and an imatinib-related compound was predicted with T315, hydrophobic interactions were predicted with Y253 and V256.[12]

Mutations affecting imatinib binding have been described by several groups within the last year in CML[13–16,18,31,32] and Ph+ ALL[17,18] patients relapsing on therapy. Many of the residues are similar to those reported here. In addition, the frequency of mutations has been variable with one of the original reports demonstrating a high rate of mutation at residue 315. The differences in frequency of mutations may be result of patient selection (primary resistant *vs* relapsing cases; lymphoid *vs* myeloid disease), or of technical factors (allele specific *vs* nonspecific PCR, sequencing strategies). Regardless, it is now clear that mutations are relatively common in patients who initially respond to imatinib and relapse later on and that these mutations are widely scattered throughout the ABL kinase domain.

Mutations in the ABL tyrosine kinase domain may lead to various biological consequences: (1) disturbed function of BCR-ABL that would lead to death of the individual cell and would not be detectable; (2) impaired binding of imatinib but retained binding of ATP, resulting in restoration of BCR-ABL function and clonal selection of mutated cells; (3) impaired binding of imatinib and ATP, resulting in reduced kinase activity that is sufficient to allow cellular survival with imatinib resistance; (4) mutations of the activation loop, which may result in an activated conformation that is insensitive to inhibition by imatinib, as suggested by Ref. 18.

Mutations occurred only in relapsing patients, but not in patients with primary resistance. For certain, imatinib therapy led to clonal selection of resistant cells. One patient was found to have a mutation present 3 months prior to relapse with the proportion of cells harboring this mutation increasing until clinical relapse occurred. Another patient in AP had a gradual decrease in the proportion of the mutated BCR-ABL allele after imatinib was replaced by hydroxyurea and mercaptopurin. Whether the BCR-ABL mutations are present at the time of treatment initiation resulting from therapy is less clear. Although we could only detect mutations in one patient prior to relapse, the sensitivity of sequencing and restriction digest analysis is limited to about 10% (data not shown). More sensitive assays may reveal mutated clones prior to clinical relapse. It is certainly possible that BCR-ABL kinase mutations could be present at low levels prior to imatinib therapy. In this scenario, the mutant BCR-ABL would not confer a growth advantage or a significant growth disadvantage to the cell, but upon exposure to imatinib, cells expressing the mutant would then have a selective advantage.

Novel cytogenetic aberrations were detected in 19 patients. Aneuploidy was observed in 13 cases and is associated with chromosomal instability.[33] The proportion of phosphorylated CRKL at the time of resistance was <30% in six resistant patients without BCR-ABL mutations, two of them showing clonal evolution. In these cases BCR-ABL was certainly not reactivated. This indicates that resistance may occur despite continuous inhibition of BCR-ABL by imatinib. However, the genetic basis of additional cytogenetic changes that occur is frequently unknown, and presumably many of the molecular alterations responsible for disease progression are not discernable from cytogenetic analysis.[34] Comprehensive gene expression analysis may help to detect molecular aberrations leading to resistance.[35]

In order to circumvent resistance, the use of subtherapeutic dosages of imatinib (<300 mg/day) should be avoided. The optimal therapy of resistant patients is still unclear. Options are dose increases up to 2×400 mg/day,[3,4,18] combination

with synergistically acting drugs, ie low-dose ara-C, or switch to an alternative cytostatic therapy. As trough levels of imatinib are approximately 1 μM at 300 mg daily, it is possible that dosage increase in patients with mutations at aa 253 could recapture a response, but with the virtual insensitivity to imatinib of mutations at aa 255 and 315, it would be predicted that dose increases in these patients would not be a useful strategy.

Our data show that imatinib resistance may occur on the basis of clonal selection of cells which reactivate BCR-ABL despite continuation of imatinib therapy. The reactivation may be accompanied by genomic amplification, overexpression of BCR-ABL or spontaneous mutations of the tyrosine kinase domain of BCR-ABL resulting in insensitivity to imatinib. Furthermore, resistance may be due to evolution of the disease with the occurrence of novel numeric or structural cytogenetic aberrations which lead to BCR-ABL-independent proliferation of leukemic cells.

Although the development of imatinib resistance presents new therapeutic challenges, the fact that BCR-ABL is active in many imatinib-resistant patients suggests that the chimeric oncoprotein remains a rational drug target. Since mutations are heterogeneous, it may be unlikely to find a common new inhibitor with broad utility to overcome resistance, which argues for the combination of imatinib with other agents.[36] Knowledge of the mutations should permit the development of assays to detect drug-resistant clones before clinical relapse.

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