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Detection of BCR-ABL kinase mutations in CD34^{+} cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment

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The BCR-ABL kinase inhibitor imatinib mesylate induces complete cytogenetic response (CCR) in a high proportion of chronic myelogenous leukemia (CML) patients. However, patients in CCR usually demonstrate evidence of residual *BCR-ABL*–positive progenitors. The mechanisms underlying persistence of small numbers of malignant progenitors in imatinib-sensitive patients are unclear. *BCR-ABL* kinase domain mutations affecting drug binding can lead to secondary resistance to imatinib. We show here that kinase mutations could be detected in CD34^{+} cells isolated from CML patients in CCR on imatinib. Most mutations seen have not been reported in previous clinical studies. Interestingly, several of the involved amino acid positions have been implicated in an in vitro mutagenesis screen. These *BCR-ABL* mutations were associated with varying levels of imatinib resistance. Two of 5 patients in whom mutations were detected on initial evaluation have relapsed. In addition, 4 patients in whom mutations were not initially detected, but with rising BCR-ABL mRNA levels on quantitative polymerase chain reaction (Q-PCR) analysis, had mutations detected on follow-up evaluation. We conclude that *BCR-ABL* kinase mutations can be detected in CD34^{+} cells from CML patients in CCR on imatinib, may contribute to persistence of small populations of malignant progenitors, and could be a potential source of relapse. (Blood. 2005; 105:2093-2098)

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Introduction

Chronic myeloid leukemia (CML) is a lethal hematopoietic stem cell malignancy characterized by the t(9;22) chromosomal translocation resulting in the formation of the *BCR-ABL* fusion gene.[1,2] The *BCR-ABL* gene is known to be essential to the pathogenesis of CML. The BCR-ABL gene product demonstrates constitutively activated tyrosine kinase activity.[3] CML invariably progresses from an initial chronic phase (CP) to an accelerated phase (AP) and terminal blast crisis (BC).

Imatinib mesylate, an inhibitor of the BCR-ABL tyrosine kinase, has proven highly effective in the treatment of CML.[4-6] Imatinib induces complete cytogenetic response (CCR) in approximately 80% of newly diagnosed CP patients and has emerged as the first-line therapy for CML.[7] Imatinib is effective in inducing hematologic and cytogenetic responses in patients in AP and BC.[8-10] Although relapses are infrequent in CP patients, the long-term durability of response remains unclear because of limited follow-up. The results of sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) analyses indicate that BCR-ABL transcripts are markedly reduced in imatinib-treated patients but that complete molecular remissions are rare.[11] We and others have shown that exposure to imatinib may not completely eliminate leukemic progenitors and that *BCR-ABL*–positive progenitors can be detected in patients in CCR on imatinib treatment.[12-16] The mechanisms underlying incomplete elimination of malignant progenitors in imatinib-treated patients are not clear. There is evidence that quiescent primitive CML progenitors may be resistant to apoptosis following imatinib exposure.[13,14] Primitive progenitors may be resistant to imatinib through mechanisms such as increased drug efflux activity.[17] However, another possibility is that mechanisms known to cause secondary resistance to imatinib could be active in subsets of CML progenitors, allowing their persistence in the setting of overall responsiveness to the drug.

The most common mechanisms of acquired resistance to imatinib are *BCR-ABL* amplification at the genomic or transcript level and point mutations in the kinase domain.[18-20] Several *BCR-ABL* kinase domain mutations have been reported. The Y253, E255, T315, and M351 mutations account for approximately 60% of those detected at the time of relapse. Structural data suggest that these mutations may interfere with imatinib binding to the ABL kinase domain by interrupting critical contact points or by inducing a conformation to which drug binding is reduced.[20-22] Some mutants, such as T315I and E255K, are insensitive to imatinib at clinically achievable doses, whereas others, such as M351T or Y253F, retain intermediate levels of sensitivity to imatinib.[23] The probability of finding a mutation increases with disease duration and with advanced disease stage.[24] Mutations may be detected even prior to initiation of treatment with imatinib in some patients and are hypothesized to result from an inherent genetic instability in

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BCR-ABL transformed cells.[20] Detection of a mutation is associated with an increased risk of relapse, with mutations in the adenosine triphosphate (ATP)–binding loop of the *BCR-ABL* kinase in particular conferring a poor prognosis.[20,24] These studies support the hypothesis that expansion of mutant clones under selective pressure of imatinib leads to clinical relapse.[20]

Patients with CCR on imatinib but with minimal residual disease represent the largest group of CML patients at the present time. In this study we investigated whether mutations in the *BCR-ABL* kinase domain were present in $CD34^+$ progenitor cells from patients in CCR on imatinib treatment. We further investigated whether detected mutations were associated with resistance to kinase inhibition by imatinib and with increased risk of relapse.

Materials and methods

Samples

Bone marrow (BM) or peripheral blood stem cell (PBSC) samples were obtained using guidelines approved by the Institutional Review Board of the City of Hope National Medical Center. Inclusion criteria included a diagnosis of CML in CP or AP, CCR after imatinib treatment, and informed consent to donate additional marrow or blood samples for the research studies. Samples were selected for analysis based on availability of adequate material for the planned studies. CCR was defined as the complete absence of t(9;22) on karyotypic analysis or less than normal background limits of *BCR-ABL*–positive cells on FISH analysis. For some patients a second sample was analyzed at a later time point. Patients had not received drugs other than hydroxyurea and interferon.

Bone marrow mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Sigma Diagnostics, St Louis, MO) density gradient separation (specific gravity, 1.077) for 30 minutes at 400*g*. $CD34^+$ cell–enriched populations were selected from BM MNCs or PBSCs using immunomagnetic column separation (Miltenyi Biotec, Auburn, CA).

Mutation analysis

Analysis of mutations was performed using a method modified from Shah et al.[20] RNA was extracted using Triazol reagent (Life Technologies, Long Island, NY). The *BCR-ABL* kinase domain was amplified using a 2-step RT-PCR procedure. Complementary DNA was generated by reverse transcription followed by a first-step PCR reaction to isolate a 1.3-kilobase (kb) cDNA fragment, which included the *BCR-ABL* junction and the *ABL* kinase domain. The primers used for reverse transcription and first-step PCR were CM10 (5′-GAAGCTTCTCCCTGACATCCGT-3′) and 3′ ABL (5′-GCCAGGCTCTCGGGTGCAGTCC-3′). A second-step PCR reaction was performed to isolate the ABL kinase domain using the primers 5′ ABL (5′-GCGCAACAAGCCCACTGTCTATGG-3′) and 3′ ABL (as in the previous sentence). The resulting 0.6-kb fragment was subcloned into the PCRII-TOPO vector (Invitrogen, Carlsbad, CA). Ten to 20 colonies per sample were sequenced in both directions. A mutation was considered to be present in a sample if it was detected on both strands of 2 or more independent clones. The sensitivity and reproducibility of the mutation detection assay was validated using known dilutions of a *BCR/ABL* mutant (L248V) mixed with wild-type *BCR/ABL* plasmids. Mutant *BCR/ABL* genes were reliably detected when they constituted 25% of the mixture, but detection was less consistent when mutant *BCR/ABL* constituted 12.5% of the mixture (mutants detected in 2 of 4 experiments), indicating proximity to the limits of sensitivity of the assay. The assay was specific, because no abnormal clones other than the mutant clone being tested were detected in these experiments.

Site-directed mutagenesis and generation of mutant alleles

Point mutations were introduced into wild-type *BCR-ABL* genes by site-directed mutagenesis using synthetic oligonucleotides with the various mutations and the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). A 0.8 kb *Aat*II/*Kpn*I Abl kinase fragment subcloned into pGEM 7Z (Promega, Madison, WI) was used as template. Successful mutagenesis was confirmed by sequence analysis. *Aat*II/*Kpn*I fragments with point mutations were cloned into full-length $p210^{BCR\text{-}ABL}$, and the final product was cloned into the *Eco*RI site of an MIGR1 retroviral vector.

Retroviral transduction

Infectious virus particles were produced by transient transfection of 293 cells with the retroviral vector plasmid and the pCL-ampho plasmid (kindly provided by Dr Martin Haas, University of California San Diego) as previously described.[25] TF-1 cells were transduced by 2 exposures to retrovirus containing supernatants (multiplicity of infection [MOI], 5) 24 hours apart. Cells were harvested 48 hours after the second virus exposure and green fluorescent protein (GFP)–positive cells collected by flow cytometry sorting using a MoFlo flow cytometer (Cytomation, Fort Collins, CO).

Cell proliferation assay

TF-1 cells (1×10^4) expressing wild-type or mutant BCR-ABL proteins were cultured in triplicate in 96 wells in RPMI medium plus 10% fetal bovine serum (FBS) with or without addition of imatinib mesylate (0.1 to 10 μM) (Novartis Pharmaceuticals, Basel, Switzerland). Viable cell number was assessed 72 hours later using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)–based colorimetric assay using the manufacturer's instructions (Promega, Madison, WI). The mean optical density of the 3-well set for each imatinib dose was expressed as percentage of controls cultured without imatinib. The concentration of drug resulting in 50% of maximal inhibition (IC_{50}) was calculated following curve fitting using GraphPad Prism software (GraphPad Software, San Diego, CA).

Western blot analysis

TF-1 cells expressing wild-type or mutant BCR-ABL proteins were cultured in RPMI medium plus 10% FBS with or without addition of imatinib (0.1 to 10 μM) for 4 hours. Cells were then washed with cold phosphate-buffered saline (PBS) and lysed in buffer containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate and supplemented with protease and phosphatase inhibitors. Protein extracts (150 μg) were resolved by electrophoresis on 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose membranes, blocked with 10% nonfat milk in PBS with 0.1% Tween 20, and incubated with an appropriate dilution of primary antibody (anti-abl [ab-3; Oncogene Science, Cambridge, MA], antiphosphotyrosine [4G10, a kind gift from Dr Brian Druker, Oregon Health Sciences University, Portland]). After washing, blots were incubated with horseradish peroxidase–conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Blots were further washed and antibody detection performed using the Superfemto Chemiluminescent kit (Pierce Biotechnology, Rockford, IL).

Quantitative PCR (Q-PCR) for BCR-ABL

Quantitative PCR (Q-PCR) analysis for BCR-ABL and β_2-microglobulin (B2M) mRNA levels was performed using a real-time TaqMan assay and the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA), with minor modifications of methods described by Radich et al.[26] Different BCR primers were used depending on the BCR-ABL breakpoint (b_2a_2 primers: BCRTM2 [5′-CATTCCGCTGACCATCAATAA3-′] and ABLTM [5′-AACGAGCGGCTTCACTCAGA-3′]; b_3a_2 primers: BCRTM3 [5′-CCACTGGATTTAAGCAGAGTTCA-3′] and ABLTM; and B2M: B2TMF [5′-CATTCGGGCCGAGATGTC-3′] and B2TMR primers [5′CTC-CAGGCCAGAAAGAGAGAGTAG-3′]). RT-PCR was carried out in 50 μL reactions containing TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems), appropriate primers, and template-specific oligonucleotide probes labeled at the 5′ end with 6-carboxy fluorescein and at the 3′ end with 6-carboxy-tetramethyl rhodamine (Synthegen, Houston, TX) (BCR-ABL probe: 5′-AGCGGCCAGTAGCATCTGACTTTGAGC-3′;

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B2M probe: 5′-CCGTGGCCTTAGCTGTGCTCG-C3′). Following an initial RT step, cDNA was subjected to 40 cycles of PCR. A BCR-ABL standard curve was created using serial log dilution of the *BCR-ABL*–positive K562 cell line into BCR-ABL–negative HL-60 cells. A B2M standard curve was obtained by diluting fresh K562 RNA in water and amplifying with B2M primers. Samples were run in duplicate, the amount of BCR-ABL and B2M mRNA was calculated based on the standard curves, and results were expressed as a BCR-ABL/B2M ratio. The assay was capable of linear detection of BCR-ABL mRNA across 5 logs of input RNA, with a detection limit of 10 pg K562 RNA in a 1 μg background of BCR-ABL–negative RNA (1:10^5 ratio).

Statistical analysis

Differences in characteristics between groups were analyzed using Student *t* tests. Relapse-free survival curves were calculated by the method of Kaplan and Meier.[27] Relapse-free survival was defined as survival without morphologic or cytogenetic evidence of recurrent leukemia in either the marrow or blood.

Results

Thirteen patients with CML (12 in CP and 1 in AP prior to starting imatinib treatment) who achieved CCR on imatinib treatment were studied. Patient characteristics are shown in Table 1. The median time from diagnosis to study was 15 months (range, 6-60 months), from diagnosis to initiation of imatinib was 9 months (range, 1-57 months), and from initiation of imatinib treatment to study was 6 months (range, 3-25 months). Six patients received prior treatment with interferon-α. BM cells were studied from 11 patients, whereas granulocyte colony-stimulating factor (G-CSF)–mobilized PBSCs were studied from 2 patients. $CD34^+$ cells were selected for study in all patients save one, in whom BM MNCs were studied. All patients were treated with standard doses (400 mg/d) of imatinib. BCR-ABL transcripts were detected using RT-PCR techniques in MNCs from all patients.

As shown in Table 1, point mutations resulting in amino acid changes in the *BCR-ABL* kinase domain were identified in 5 of the 13 patients studied. Seven different mutations were detected. The L248V and D276G mutations have been previously reported.[24,28] The other mutations, to the best of our knowledge, have not been previously described in imatinib-treated CML patients. However, 3 mutations (G321E, E352G, and E373G) are at locations that have been previously identified to be associated with imatinib resistance using a mutational screen.[29] The frequency of mutant subclones varied between samples, and multiple clonal abnormalities were observed in some samples. We did not observe significant differences in patient age, prior therapy with interferon, time from diagnosis to study, time from diagnosis to imatinib treatment, and duration of imatinib treatment. No significant difference in levels of BCR-ABL mRNA levels as determined by Q-PCR was seen between MNCs from patients with or without mutations.

Because most of the observed mutations were novel, sensitivity to inhibition by imatinib was tested. The M237I, L248V, D276G, G321E, E352G, Y353G, and E373G mutations were introduced into wild-type *BCR-ABL* genes by site-directed mutagenesis followed by expression in TF-1 cells. The effects of increasing concentrations of imatinib on BCR-ABL phosphorylation and cell growth were evaluated. Varying degrees of resistance to imatinib-induced inhibition of BCR-ABL phosphorylation (Figure 1A,C) and overall tyrosine phosphorylation (data not shown) were observed in cell lines expressing mutant *BCR-ABL* genes. The effect of imatinib on growth of cells expressing mutant and wild-type *BCR-ABL* genes was evaluated using an MTS cell proliferation assay (Figure 1B, Table 2). The mutant BCR-ABL kinases generally showed intermediate levels of imatinib resistance, with the L248V mutant demonstrating the greatest degree of resistance. However, the Y353H mutant demonstrated similar sensitivity to imatinib as wild-type BCR-ABL.

No evidence of cytogenetic or hematologic relapse was observed in the 8 patients in whom kinase mutations were not detected, with a median follow-up of 27 months (range, 12-34 months) (Table 1). Detection of a kinase domain mutation was associated with a trend toward increased risk of relapse ($P = .058$). Of 5 patients in whom kinase domain mutations were detected, 2 subsequently relapsed (Table 1). One patient who relapsed (no. 4) was detected to have the L248V mutation when studied in CCR 24 months after initiation of imatinib. This mutation, located in the ATP-binding loop (P-loop) of the ABL kinase domain, is associated with a relatively high degree of imatinib resistance. This patient developed cytogenetic evidence of relapse 9 months from the time this sample was collected and subsequently developed hematologic relapse. The L248V mutation was observed at high frequency in blood samples collected following relapse. The second patient who relapsed (no. 3) was detected to have 2 mutant clones (E352G, Y353H) when studied in CCR 4 months after initiation of imatinib. This patient relapsed in BC 2 months after this sample was obtained. A new mutation (D276G) was seen at the time of relapse, but the 2 mutations originally seen were no longer detected. As expected, increased BCR-ABL mRNA levels on Q-PCR analysis were observed in these 2 patients. Three other patients in whom kinase domain mutations were detected continued to be in CCR with follow-up of 21, 27, and 30 months. Follow-up samples were available for analysis from 2 patients. In one patient (no. 1), the M237I mutation detected at the first assessment was detected once again on reevaluation 7 months later. Three additional mutations (E352G, V304A, M351T) that had not been previously detected were also seen. Q-PCR analysis showed increased BCR-ABL levels in this sample. In the other patient (no. 2), 3 mutations originally detected were not seen on reevaluation 4 months later, but 2 new mutations (E352G, T389A) were detected. Q-PCR analysis did not reveal a consistent rise in BCR-ABL levels in follow-up samples from this patient. For patients without *BCR-ABL* mutations, Q-PCR analysis revealed increased BCR-ABL levels in follow-up samples from 5 patients, confirmed in 2 or more follow-up samples in 3 patients, and unchanged or reduced levels in 3 patients. Patients with rising levels of BCR/ABL on Q-PCR were analyzed for kinase mutations. Mutations were detected in follow-up samples from 4 of the 5 patients with rising BCR/ABL levels (Table 1). Taken together, mutations were detected on either initial or follow-up evaluation in 7 of 8 patients with rising BCR/ABL levels on Q-PCR analysis.

Discussion

Although most CML patients treated with imatinib achieve CCR, complete molecular remissions are rare.[11] The mechanisms underlying resistance of a subset of malignant progenitors to elimination by imatinib is not clear. Here we investigated whether *BCR-ABL* kinase domain mutations could contribute to the persistence of small numbers of malignant progenitors in patients in CCR on imatinib treatment.

To detect *BCR-ABL* kinase mutations in residual malignant progenitors in the setting of CCR where only a very small

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Table 1. Clinical characteristics of patients studied

Patient no.	Age at study, y	Sex	BM versus PBSC	Stage	$CD34^+$ cells versus MNC	Prior inter-feron	Months from diagnosis to study	Months from diagnosis to imatinib	Months on imatinib	*BCR/ABL*-positive $CD34^+$ cells detected on FISH*	Mutations detected on initial evaluation (clones containing mutation/clones sequenced)	Months from initial to follow-up evaluation	Mutations detected on follow-up evaluation (clones containing mutation/clones sequenced)†	Status at follow-up
1	64	M	BM	CP	$CD34^+$	Y	31	25	6	Yes	M2371 (8/10)	7	M2371 (6/13) E352G (4/13) V304G (3/13) M351T (3/13)§	CCR‡
2	70	M	BM	CP	$CD34^+$	N	15	2	13	Yes	G321E (9/15) E373G (2/15) D276G (2/15)	15	E352G (5/14) T389A (2/14)	CCR
3	37	F	BM	CP	MNC	N	6	2	4	NA	Y353H (2/20) E352G (2/20)	2	D276G (5/17)	Relapse-BC‡
4	54	M	PBSC	AP	$CD34^+$	Y	38	13	25	NA	L248V (6/10)	18	L248V (13/13)	Relapse-AP‡
5	66	M	PBSC	CP	$CD34^+$	N	12	1	11	Yes	G321E (6/10)	NA	NA	NA
6	52	F	BM	CP	$CD34^+$	Y	45	26	19	Yes	ND (0/14)	30	L248V (3/10) V371A (2/10)	CCR‡
7	67	F	BM	CP	$CD34^+$	Y	60	57	3	Yes	ND (0/10)	25	G321E (11/14)	CCR‡
8	41	M	BM	CP	$CD34^+$	N	11	1	10	Yes	ND (0/10)	NA	NA	CCR
9	57	F	BM	CP	$CD34^+$	Y	60	56	4	No	ND (0/28)	NA	NA	CCR
10	58	F	BM	CP	$CD34^+$	N	15	9	6	NA	ND (0/22)	18	ND (0/13)	CCR‡
11	54	F	BM	CP	$CD34^+$	Y	57	51	6	Yes	ND (0/10)	15	D276G (2/14)	CCR‡
12	54	F	BM	CP	$CD34^+$	N	7	1	6	NA	ND (0/12)	16	Y353H (10/10)	CCR‡
13	54	M	BM	CP	$CD34^+$	N	10	3	7	NA	ND (0/10)	NA	NA	CCR

BM indicates bone marrow; PBSC, peripheral blood stem cells; MNC, mononuclear cells; FISH, fluorescence in situ hybridization; CP, chronic phase; CCR, complete cytogenetic response; NA, not available; BC, blast crisis; AP, accelerated phase; ND, not detected.

*FISH results for these patients were previously reported by Bhatia et al.[12] Eight patients were common to the 2 studies. Of these, 5 had mutations detected in the present study, and all had *BCR/ABL*-positive cells detected by FISH in the previous study. *BCR/ABL*-positive $CD34^+$ cells were detected by FISH in 2 of the 3 patients in whom mutations were not detected in this study.

†Mutation analysis on follow-up samples was performed using RNA extracted from $CD34^+$ cells for patients 1, 2, and 3 and from MNC for patients 4, 6, 7, 10, 11, and 12.

‡These patients had rising BCR/ABL mRNA levels on Q-PCR analysis.

§The V304G and M351T mutations were detected simultaneously in 3 clones.

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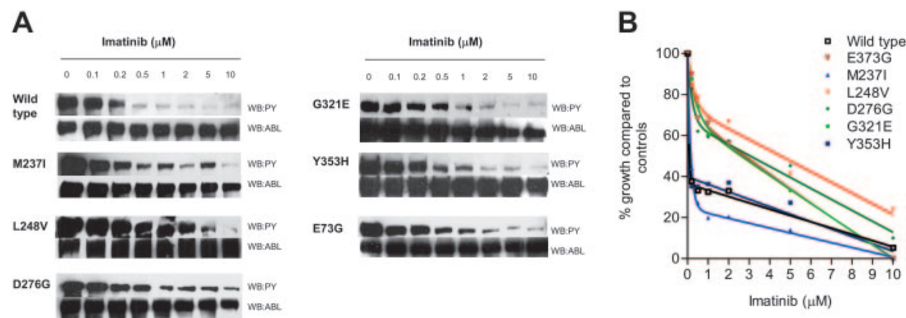

Figure 1. Effect of imatinib on BCR-ABL kinase activity and growth of cells expressing *BCR-ABL* kinase domain mutants. (A) TF-1 cells expressing BCR-ABL kinase domain mutations and wild-type BCR-ABL were exposed to imatinib (0 to 10 μM) for 4 hours. Whole-cell lysates were analyzed by Western blotting using antiphosphotyrosine (PY) and anti-abl (ABL) antibodies. WB indicates Western blot. (B) TF-1 cells expressing mutant *BCR-ABL* genes were cultured in the presence of imatinib (0 to 10 μM) for 3 days. Viable cells were quantified using an MTS assay. The resulting optical density (OD) values were expressed as percentage of controls cultured simultaneously without imatinib. □ indicates wild type; ▽, E373G; ▲, M237I; ▼, L248V; ◆, D276G; ●, G321E; and ■, Y353H. Each data point represents the mean of 3 experiments (except wild-type [n = 2]), with each experiment based on 3 replicates. Curve fitting was performed using GraphPad Prism software.

fraction of progenitors are malignant, $CD34^+$ cells were selected to enrich for the progenitor subfraction, and a first-step RT-PCR reaction was performed to selectively amplify BCR-ABL but not c-ABL transcripts. The BCR-ABL kinase domain was then amplified in a second-step PCR reaction. Multiple subclones of the resulting PCR product from each sample were analyzed, in preference to direct sequencing of the PCR product. Using this approach, *BCR-ABL* kinase mutations were detected on initial examination of $CD34^+$ progenitor cells from 5 of 13 CML patients in CCR on imatinib treatment.

Two patients in whom mutations were initially detected subsequently relapsed. In one patient the L248V P-loop mutation was present in $CD34^+$ cells when the patient was evaluated in CCR and was also detected at high frequency in peripheral blood cells at the time of clinical relapse, suggesting that relapse was likely related to selective outgrowth of cells bearing this mutation. This mutation resulted in significant resistance to kinase inhibition by imatinib. The second patient who relapsed rapidly evolved to blast crisis. The originally observed clone was not seen at relapse, but 2 new mutations were seen. It is possible that underlying genetic instability could have contributed to rapid evolution to blast crisis in this patient. The 8 patients in whom mutations were not detected have stayed in continued CCR. However, some of these patients have also shown evidence of rising BCR-ABL levels on Q-PCR analysis. Although rising BCR-ABL levels are associated with increased risk of relapse in CML patients after allogeneic hematopoietic cell transplantation, the clinical significance of increasing BCR-ABL levels in imatinib-treated patients in CCR has not been definitively proven at this time.[26] It appears logical that patients with a rising burden of leukemic cells may be at increased risk for relapse. Significantly, kinase mutations were detected in follow-up samples from 4 of 5 patients with rising Q-PCR levels, in whom mutations were not originally detected. Consistent with these results, Branford et al have recently reported that rising BCR-ABL mRNA levels in imatinib-treated patients, including patients in CCR, were associated with a high incidence of kinase mutations.[30] Because our study included a relatively small number of patients, one cannot exclude the possibility that the population studied may be skewed toward a more high-risk population of CML patients who achieve CCR. Larger, population-based studies with extended follow-up will be required to estimate the true incidence of kinase mutations in this patient population, to confirm the association between mutations and clinical relapse, and determine whether screening for mutations may be helpful for risk stratification and prognostication in patients responsive to imatinib treatment.

Table 2. Summary of *BCR-ABL* kinase domain mutations

	IC_{50} cell growth, μM imatinib	IC_{50} BCR/ABL phosphorylation	Location
Wild-type	0.08	0.11	NA
M237I	0.17	0.25	Adjacent to P-loop
L248V	4.62	1.87	P-loop
D276G	2.97	0.60	C-helix
G321E	2.61	0.39	Imatinib binding site
Y353H	0.02	0.2	Adjacent to activation loop
E373G	2.78	0.71	Activation loop

NA indicates not applicable.

Multiple factors may affect the propensity of specific *BCR-ABL* kinase-domain mutations to cause relapse, including the grade of imatinib resistance and differences in biologic effects of different mutants. The mutations commonly seen in patients with clinical resistance to imatinib were not detected in $CD34^+$ cells from patients in CCR. To the best of our knowledge, the mutations detected in this study, with the exception of the L248V and D276G mutations, have not been reported in previous clinical studies. Interestingly, mutations at the same amino acid positions as 3 other mutations observed here were identified by Azam et al in an in vitro screen for mutations conferring imatinib resistance using randomly mutagenized *BCR-ABL*.[29] Although the specific amino acid substitutions described by Azam et al differ from those observed here, in both studies mutations at these amino acid positions were associated with similarly modest levels of resistance to imatinib. Previous clinical studies focusing on patients with clinical resistance to imatinib may have failed to detect similar mutations because the level of resulting imatinib resistance may not be sufficient by itself to promote selective outgrowth of mutant clones and relapse in the presence of clinically achieved levels of imatinib. However, the modest reduction in imatinib sensitivity may be sufficient to prevent complete elimination of primitive progenitors. Increased drug efflux activity in primitive progenitors resulting in reduced intracellular levels of imatinib could contribute to incomplete elimination of these cells. Follow-up evaluations indicated that mutant clones did not always persist and that new mutations sometimes emerged over time. However, this phenomenon has been previously observed.[24] Mutations may not persist if they do not result in a significant clonal growth advantage. Some mutations may occur in committed progenitors without long-term hematopoietic capacity. In addition, mutations present at low frequency, close to the limits of sensitivity of the assay, may not be consistently detected.

Because most of the mutations detected here resulted in reduced in vitro sensitivity to imatinib, it is likely that the presence of such mutations contributed at least in part to disease persistence in these patients. It is also possible that the presence of mutations could

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reflect an underlying genetic instability and that patients bearing mutations may be at increased risk for acquiring additional genetic abnormalities contributing to disease persistence or progression. However, it is likely that additional factors besides kinase domain mutations may also contribute to the persistence of subsets of malignant progenitors in imatinib-responsive CML patients. First, kinase domain mutations could not be detected in several patients. Second, even for patients in whom mutations were detected, kinase mutations were not seen in a large proportion of subclones. However, we cannot exclude the possibility of mutations outside the kinase domain that might affect imatinib sensitivity, as was reported in an in vitro screen.[29] Other potential mechanisms that could contribute to the persistence of primitive malignant progenitors include insensitivity of quiescent primitive progenitors to imatinib-induced apoptosis,[13,14] transmission of survival or proliferation signals in response to microenvironmental stimuli,[31] and acquisition of genetic abnormalities in genes other than *BCR/ABL*.[32]

In summary, we have found that *BCR-ABL* kinase domain mutations can be detected in CML patients in CCR on imatinib and may contribute to persistence of small subsets of malignant progenitors in some patients. Larger studies to further investigate the relationship of kinase domain mutations with risk of subsequent relapse in these patients are warranted.

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