Instability of BCR–ABL Gene in Primary and Cultured Chronic Myeloid Leukemia Stem Cells

Xiaoyan Jiang, Kyi Min Saw, Allen Eaves, Connie Eaves

Background Imatinib mesylate treatment causes remissions in a majority of patients with chronic myeloid leukemia (CML), but relapses are an increasing problem. We hypothesized that imatinib-resistant leukemic cells emerge from CML stem cells that acquire BCR–ABL gene mutations even before exposure to BCR–ABL–targeted agents such as imatinib.

Methods Lineage-negative (i.e., immature) $CD34^+CD38^-$ CML stem cell–enriched populations were isolated from five patients with chronic phase CML samples by fluorescence-activated cell sorting. To identify BCR–ABL gene mutations, complementary DNAs (cDNAs) prepared from purified CML stem cells were subjected to allele-specific amplification using primers corresponding to 16 kinase domain mutations, with normal bone marrow cells serving as negative controls. We also cloned and directly sequenced BCR–ABL cDNAs prepared from freshly isolated CML stem cells and from their progeny generated after 3–5 weeks of culture.

Results In 20%–33% of cDNA preparations from freshly isolated CML stem cell–enriched populations, both allele-specific amplification and direct sequencing methods revealed mutations in sequences corresponding to the BCR–ABL kinase domain. Mutations were not observed in cDNA sequences encoding the c-ABL kinase domain that were obtained from similar types of primitive normal cells. More than 70 different BCR–ABL mutations (including frameshift mutations and premature stop codons) were identified in the progeny of cultured CML stem cells. Analysis of individual clones derived from the cultured cells demonstrated that new BCR–ABL mutations were produced.

Conclusions Primary CML stem cells display instability of the BCR–ABL fusion gene both in vivo and in vitro. Thus, patients may possess leukemic stem cells with BCR–ABL kinase mutations before initiation of BCR–ABL–targeted therapies and would likely be predisposed to develop resistance to these agents.

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Chronic myeloid leukemia (CML) is a clonal, multistep, multilineage myeloproliferative disease that is believed to arise from the deregulated growth and differentiation of a genetically altered hematopoietic stem cell (1,2). The cytogenetic hallmark of the disease is the Philadelphia chromosome (Ph), which forms as a result of a reciprocal translocation between the long arms of chromosomes 9 and 22. At the molecular level, this corresponds to the creation of a BCR–ABL fusion gene. This gene encodes a 210-kDa oncoprotein ($p210^{BCR–ABL}$) that has higher constitutive tyrosine kinase activity than the wild-type ABL kinase protein (3). Many of the target proteins that the BCR–ABL oncoprotein phosphorylates have been described, including itself (4) and CrkL, which has recently been used as a surrogate indicator of $p210^{BCR–ABL}$ kinase activity (5–7).

Imatinib mesylate (imatinib), dasatinib, and nilotinib are small-molecule inhibitors of the BCR–ABL–encoded kinase, but they have different potencies, sensitivities to kinase domain mutations, and cross-reactivity with other kinases (8–11). Clinical trials have shown that imatinib treatment produces statistically significant responses in patients with chronic phase CML (12–14). However, some patients experience early relapses, and imatinib-resistant disease can emerge (15–18). Initial experience with dasatinib and nilotinib treatment of imatinib-resistant CML patients has borne out the predicted broader effectiveness of these newer drugs, which exhibit 30-fold to 300-fold greater potencies than imatinib against both wild-type and many mutant BCR–ABL oncoproteins (9,11,19). Nevertheless, inadequate responses and early relapses have also been encountered in patients treated with these newer agents (20,21).

Affiliations of authors: Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada (XJ, KMS, AE, CE); Departments of Medical Genetics (XJ, CE), Medicine (AE, CE), and Pathology and Laboratory Medicine (AE, CE), University of British Columbia, Vancouver, BC, Canada.

Correspondence to: Xiaoyan Jiang, MD, PhD, Terry Fox Laboratory, 675 West 10th Ave, Vancouver, BC, V5Z 1L3, Canada (e-mail: xjiang@bccrc.ca).

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The development and progression of CML encompasses biologically and clinically distinct phases: a preclinical asymptomatic latent phase; a benign chronic phase in which myeloid differentiation is still normal, albeit deregulated; an accelerated phase, in which clinical control of leukemic cell growth is more difficult; and a final, rapidly fatal acute phase that is characterized by an accumulation of undifferentiated blast cells (1,2,22). Chronic phase CML is propagated by a population of pluripotent hematopoietic cells with many of the characteristics of normal hematopoietic stem cells (1,2,22). Several studies have shown that chronic phase CML stem cell–enriched populations are less sensitive to imatinib than are the more predominant and more mature CML cells (7,23–25). In addition, there is growing evidence that expression of $p210^{BCR-ABL}$ can induce a state of increased genomic instability (26–28). Therefore, we hypothesized that the slowly expanding chronic phase CML stem cell population might serve as a reservoir of cells in which mutations affecting responses to BCR–ABL–targeted therapies would inevitably accumulate, even in the absence of selection imposed by such treatments.

To test this hypothesis, we sought to determine whether mutations affecting the BCR–ABL kinase domain could be detected in the leukemic lineage-negative (lin^-) $CD34^+CD38^-$ (i.e., stem) cells (29,30) from chronic phase patients who had never received BCR-ABL–targeted therapy. We also investigated whether new mutations would occur at a detectable frequency when these cells were stimulated to proliferate in vitro for up to 5 weeks. Both questions have been difficult to address because the $lin^-CD34^+CD38^-$ stem cell–enriched population in chronic phase CML patients typically contains a preponderance of normal stem cells, in contrast to the later stages of myeloid differentiation (i.e., the $lin^-CD34^+CD38^+$ as well as the more mature and prevalent lin^+CD34^- cells) where the majority of cells are leukemic (22). In this study, we circumvented this problem by focusing our analyses on cryopreserved cells from patients that we had previously found by functional assays coupled with genotyping analyses to have a preponderance of leukemic cells in the stem cell compartment (29,30).

CONTEXT AND CAVEATS

Prior knowledge

While treatment with imatinib causes remissions in the majority of patients with chronic myeloid leukemia, relapses often occur. The mechanisms by which resistance to imatinib develops are not well characterized.

Study design

Chonic myeloid leukemia stem cells from patients were isolated and genetically characterized for mutations in the oncogene (BCR–ABL) that causes the disease.

Contribution

These chronic myeloid leukemia stem cells were found to be genetically unstable, with a high frequency of mutation, leading to alterations in the BCR–ABL protein, the target of imatinib.

Implications

Resistance to imatinib may ultimately derive from mutations in a population of chronic myeloid leukemia stem cells that occur before exposure to chemotherapy. Further investigation of how mutations develop in these particular cells is needed.

Limitations

More research will be needed to translate these insights into improved therapeutics for patients.

Materials and Methods

Cells

Heparin-treated blood and leukapheresis cells were obtained from five chronic phase CML patients who had elevated white blood cell counts (110×10^9–460×10^9/L) at, or up to 10 years after, the time of their initial diagnosis. None of the five patients had been treated with imatinib, and all lacked any clinical evidence of accelerated disease. Blood from normal adults and from normal adults treated with granulocyte colony-stimulating factor (G-CSF), and bone marrow from normal allogeneic transplant harvests were obtained as leftover material from the Leukemia/Bone Marrow Transplant Program of British Columbia. Bone marrow cells from normal cadaveric donors were obtained from the Northwest Tissue Center (Seattle, WA). Cord blood was obtained from normal cesarian section deliveries. In all cases, informed consent was obtained, and the procedures used were approved by the Research Ethics Board of the University of British Columbia.

Lin^- (>80% $CD34^+$) cells were isolated by means of negative immunomagnetic depletion of lineage marker–positive (lin^+) cells using StemSep columns (StemSep; StemCell Technologies Inc, Vancouver, BC, Canada). The cells were then suspended in a solution of 10% dimethylsulfoxide in fetal calf serum (FCS) and cryopreserved at –135 °C until required (31). Immediately before use, the cells were thawed and stained with antibodies to CD34 directly conjugated to fluorescein isothiocyanate and to CD38 directly conjugated to phycoerythrin (Becton Dickinson Immunocytometry System, San Jose, CA). After staining for 30 minutes at 4 °C, the cells were washed twice in phosphate-buffered saline (PBS) containing 2% FCS (StemCell Technologies Inc) and resuspended in 2 μg/mL propidium iodide (Sigma, St Louis, MO). Subsets of $lin^-CD34^+CD38^-$, $lin^-CD34^+CD38^+$, and lin^+CD34^- cells were collected by fluorescence-activated cell sorting (FACS) using a FACStarPlus or FACSVantage cell sorter (Becton Dickinson) (32).

Fluorescence In Situ Hybridization

Aliquots of approximately 2000 cells in 200 μL of PBS containing 2% FCS were centrifuged at 400*g* for 5 minutes in a Beckman Coulter Allegra 6R Centrifuge (Beckman Coulter Canada Inc, Mississauga, ON, Canada). The cell pellets were then resuspended in hypotonic potassium chloride (75 mM) and placed for 10 minutes on slides pretreated with poly-L-lysine (Sigma). A drop of 20% fixative (3:1 methanol/glacial acetic acid) in hypotonic potassium chloride was added, and after 3 minutes, the slides were immersed in fixative for 15 minutes and dried on wet paper towels placed on a 40 °C slide warmer. The slides were then immersed in 2× saline sodium citrate buffer at 37 °C for 1 hour and then dehydrated in an ethanol series (70%, 85%, and absolute ethanol, 2 minutes at each concentration). Fluorescence in situ hybridization (FISH) analyses were performed with a BCR–ABL dual-color translocation probe according to the manufacturer's (Vysis Inc, Downers Grove, IL)

instructions. Interphase nuclei were evaluated under a ×63 oil immersion lens on a Zeiss Axioplan microscope with a set of 4′,6-diamidino-2-phenylindole (DAPI)/orange/green filters. Images were captured using a color capture device camera and a Genus 3.6 imaging system from Applied Imaging (San Jose, CA).

Suspension Cultures

FACS-sorted lin$^-$CD34$^+$CD38$^-$ cells from CML patients were collected into microfuge tubes containing serum-free medium, which consisted of Iscove's medium supplemented with 10 mg/mL bovine serum albumin, 10 μg/mL insulin, 200 μg/mL transferrin (StemCell Technologies Inc), 40 μg/mL low-density lipoproteins, and 10^{-4} M 2-mercaptoethanol (both from Sigma). Aliquots (of 1 mL) containing 5×10^4 cells were then added to each well of a 24-well plate with or without imatinib (0.1–10 μM, Novartis, Basel, Switzerland) and with or without the following combination of human recombinant growth factors: interleukin (IL)-3 (20 ng/mL; Novartis), IL-6 (20 ng/mL; Cangene, Mississauga, ON, Canada), G-CSF (20 ng/mL, StemCell Technologies Inc), flt3-ligand (100 ng/mL; Immunex Corp, Seattle, WA), and Steel factor (100 ng/mL, StemCell Technologies Inc). The media were replaced every week with fresh media with or without the same imatinib and growth factor supplements. At various time points, viable cell numbers were determined by hemocytometer counts of trypan blue–excluding cells.

Progenitor Assays

Assays for quantification of colony-forming cells (CFCs) present in FACS-purified lin$^-$CD34$^+$CD38$^-$ cells and their progeny generated from 3-week suspension cultures from CML patients were performed in FCS-containing methylcellulose medium (H4535, StemCell Technologies Inc) in the absence or presence of imatinib (1–5 μM). Erythroid, granulocyte–macrophage, and mixed-lineage colonies were scored visually using an inverted microscope and a scoring grid after 14 days of incubation as previously described (33). Five to ten individual CFC-derived colonies from each treatment condition studied in the 3-week suspension culture experiments were analyzed cytogenetically for the presence of the Ph. Results were based in each case on an examination of at least two Giemsa-banded metaphases per individually processed CFC-derived colony (34).

Quantitative Reverse Transcription–Polymerase Chain Reaction Analyses

Total RNA was extracted from aliquots of FACS-purified CML cells and their progeny from both suspension cultures and the colonies the cultured cells formed in the methylcellulose assays using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) or the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA). DNase I was added during the RNA isolation step according to the manufacturer's instructions to minimize contamination with genomic DNA. RNA (0.1–0.25 μg) was reverse transcribed in a 20-μL reaction volume using random hexamers as primers and SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada) (35). Next, 25 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was combined with 1 μL of a 20 μM solution of the specific primers, 1–2 μL complementary DNA (cDNA), and water to a final volume of 50 μL. Optimal amplification of BCR–ABL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs was achieved with the following primers: 5′-CATTCCGCTGACCATCAATAAG-3′ and 5′-GATGCTACTGGCCGCTGAAG-3′ (BCR–ABL) and 5′-CCCATCACCATCTTCCAGGAG-3′ and 5′-CTTCTCCATGGTGGTGAAGACG-3′ (GAPDH). An initial AmpErase UNG (uracil-N-glycosylate) activation step (50 °C for 2 minutes), followed by a denaturation step (95 °C for 10 minutes) and 40 cycles of amplification (95 °C for 15 seconds and 60 °C for 60 seconds) were carried out with a single fluorescence measurement per cycle. Real-time polymerase chain reaction (PCR) products were subjected to melting curve analysis to verify that target amplicons were amplified and that primer dimers were not present (36,37). Reactions and data analysis were performed using an Applied Biosystems 7500 Real-Time PCR system and SDS V1.4 Software (Applied Biosystems).

To compare BCR–ABL transcript levels in different cell populations, the threshold cycle (Ct) values of all the samples were first normalized to the Ct value of an endogenous housekeeping gene (GAPDH) in the same sample and then this normalized value was compared to the normalized value of the reference sample (e.g., the day 0 sample) using the following formula: relative expression of BCR–ABL in the sample of interest normalized to that of the reference sample = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is ΔCt, sample $-\Delta Ct$, reference. ΔCt, sample is the Ct value for any sample normalized to GAPDH, and ΔCt, reference is the Ct value for the calibrator (e.g., day 0 sample) also normalized to the Ct value for GAPDH. The efficiency of amplification of both GAPDH and BCR–ABL sequences was confirmed to be close to 100% from the observed linear relationship obtained between the value for ΔCt and the template cDNA dilution. Samples from three patients were used to compare the transcript levels of BCR–ABL from freshly isolated lin$^-$CD34$^+$CD38$^-$ cells, from progeny cells harvested 3 weeks later from cultures, and from individual CFC-derived colonies grown in the presence and absence of imatinib. The BCR–ABL transcript levels in suspension-cultured cells were determined from three independent experiments each combining samples from the same three patients. For determining transcript levels in CFC-derived colonies, colonies grown from a total of eight nonmutant and 12 mutant CFCs pooled from all three patients were used.

Mutation Analysis

Mutations in sequences encoding the BCR–ABL kinase were identified in CML cells using a previously described method (38) with some modifications. Total RNA was extracted from FACS-purified subsets of normal and CML cells and their progeny generated in suspension culture or in CFC assays using the Absolutely RNA Nanoprep Kit (Stratagene) or the PicoPure RNA Isolation Kit (Arcturus). The sequence corresponding to the BCR–ABL kinase domain was amplified using a two-step reverse transcription (RT)–PCR method (38). The cDNA thus obtained was then amplified with the Platinum Taq DNA polymerase High Fidelity (Invitrogen). In the first step of the PCR, a 1.3-kilobase (kb) BCR–ABL fusion fragment was amplified using the primer BCR-ATPB1 (5′-GAAGCTTCTCCCTGACATCCGTG-3′, nucleotide position

3205–3227, GenBank accession number NM_004327) and ABL-ATPB2 (5′-GCCAGGCTCTCGGGTGCAGTCC-3′, nucleotide position 1578–1600, and GenBank accession number M 14752) as primers and then purified by agarose gel electrophoresis. A second PCR was then used to amplify the ABL kinase domain using ABL-ATPB1 (5′-GCGCAACAAGCCCACTGTCTATGG-3′) and ABL-ATPB2 as primers. The 0.6-kb product was purified and cloned into the pCR2.1-TOPO vector (Invitrogen). In control experiments, BCR–ABL PCR products were not obtained from extracts of normal bone marrow cells or other leukemic cell lines (Hut 78 and Hut 102 from American Type Tissue Collection, Manassas, VA) that do not contain a BCR–ABL fusion gene. At least five bacterial colonies obtained by transformation with the cDNA prepared from each CFC-derived colony or 10–20 bacterial colonies from the cDNA prepared from fresh or cultured cells were inoculated into media. Plasmid DNA was then isolated from the cultured bacterial cells by standard methods and sequenced using a 3730XL DNA Analyzer System from Applied Biosystems. A mutation was considered to be present only if it was detected in both strands in two or more independent bacterial clones transformed with the cDNA from the suspension culture cells or CFC-derived colonies. Sequence analysis of a 0.7-kb fragment of BCR–ABL immediately 5′ to the kinase domain was performed by amplifying the 1.3-kb BCR–ABL fusion fragment described above using BCR-ATPB1 and ABL-ATPB3 (5′-CCA-TAGACAGTGGGCTTGTTGCGC-3′) and cloning it into the pCR2.1-TOPO vector. A 0.6-kb fragment derived from the wild-type ABL kinase domain in extracts obtained from normal bone marrow cells was amplified using the ABL-ATPB1 and ABL-ATPB2 primers, and then cloned and sequenced.

Allele-Specific Real-Time Polymerase Chain Reaction Analysis

RNA extraction and cDNA synthesis for allele-specific real-time PCR analyses were carried out as described above. PCR conditions for the allele-specific PCR amplification of each of the 16 previously described BCR–ABL kinase domain mutations studied were first individually optimized for maximal sensitivity and specificity using mixtures of wild-type and mutant plasmids in varying ratios from $1:10^5$ to 1:1. Primer sequences, PCR conditions, and the sensitivity of this assay are given in Supplementary Table 1 (available online). Optimal PCR conditions for the amplification of each kinase domain mutation were further verified in various sources of normal bone marrow, cord blood cells, and mobilized peripheral blood samples. The specificity with which each separate mutation was detected in CML cells by PCR amplification was determined both by melting curve analysis (to confirm true target amplicons and the absence of primer–dimer artifacts or contaminants) (Supplementary Fig. 1, available online) and by electrophoresis to confirm the presence of a PCR fragment of the predicted length on a 1.5% ethidium bromide–stained agarose gel, as shown in Fig. 1, A.

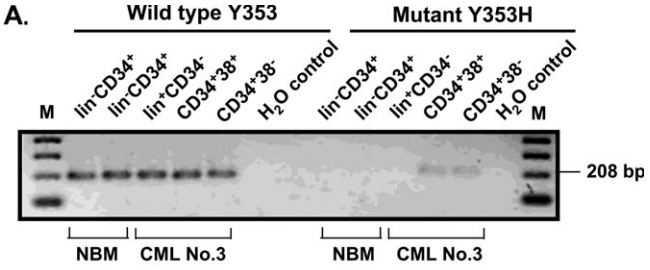
Immunoblot Analyses

1×10^5–2×10^5 cells (cultured lin$^-$CD34$^+$CD38$^-$ cells isolated from CML patients and K562 cells, a cell line derived from a CML patient in blast crisis) were lysed in phosphorylation solubilization buffer (50 mM Hepes buffer, 100 mM NaF, 10 nM sodium pyrophosphate, 2 mM Na_3VO_4, and 4 mM EDTA) containing 0.5% Nonidet P-40 and protein inhibitor cocktail (Sigma) as previously described (35). Proteins in cell lysates were separated by polyacrylamide gel electrophoresis (NuPage BisTris 4%–12% gel, Invitrogen) followed by electroblotting on a polyvinyl difluoride membrane (Millipore, Billerica, MA). Each membrane was then blocked with 5% skim milk, incubated with primary antibody, washed, and then incubated with a horseradish peroxidase–conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). Antibody binding was detected by incubating the membrane with Western lighting chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Inc; Boston, MA) and exposing it to Kodak X-Omat film (Eastman Kodak, Rochester, NY). Primary antibodies used in this study included a monoclonal anti-abl antibody (8E9, 1:500 dilution, Pharmingen, Mississauga, ON, Canada), an anti-phosphotyrosine antibody (4G10, 1:2000 dilution, Upstate Biotechnology, Lake Placid, NY), and anti-phospho-CrkL and anti-CrkL antibodies (1:500 dilution for each, Cell Signaling Technology, Beverly, MA). In some experiments, membranes were stripped with strippingbuffer (2.5 mM Tris-Cl [pH 6.8], 2% sodium dodecyl sulfate, 100 mM 2-mercaptoethanol) at 50 °C for 30 minutes before being reblocked, washed, and reprobed.

Table 1. Frequency and amino acid substitutions encoded by BCR–ABL kinase domain mutations detected by sequencing cloned transcripts present in extracts of lin$^-$CD34$^+$CD38$^-$ CML cells or their progeny grown in vitro in the absence or presence of imatinib*

	Fresh lin$^-$CD34$^+$CD38$^-$ cells		Cultured lin$^-$CD34$^+$CD38$^-$ cells			
			No. of mutant transcripts/No. of cDNA clones sequenced (%)		Amino acid substitutions	
Patient	No. of mutant transcripts/No. of cDNA clones sequenced (%)	Amino acid substitutions	–Imatinib	+Imatinib	–Imatinib	+Imatinib
1	3/9 (33)	V268M E316G, N331S F359L	2/8 (25)	2/8 (25)	V228A S265C	K262R K357E
2	2/10 (20)	T272A I360T, D391G	2/8 (25)	2/8 (25)	Q333L, V377A E334G	L323P L323P
3	3/10 (30)	Y232H Y232H, K247E, S265G G254R, K400E	2/8 (25)	2/6 (33)	D276G D276G, H375R	Y353H Y353H

* PCR-amplified cDNAs from BCR–ABL transcripts present in RNA extracts of the cells indicated were cloned and amplified in bacteria, and the cloned sequences were individually sequenced as described in the Methods. CML = chronic myelogenous leukemia; cDNA = complementary DNA; PCR = polymerase chain reaction.



B. **Detection of known BCR-ABL TK mutations in different CML subsets using allele-specific RT-PCR**

Mutations	34^+38^-	34^+38^+
No.1		
K247stop	-	-
F274E	-	-
T315A	-	-
F359L*	+	-
M388V	+	+
Y393C	-	-
	2/6	1/6
No.3		
G250R	-	-
D276G	-	-
E281stop	-	-
V304A	-	-
E352K	-	-
Y353H	+	+
F359S	-	-
F382S	-	-
K400E*	+	-
K400R	+	-
	3/10	1/10

Fig. 1. BCR–ABL tyrosine kinase (TK) domain mutations identified by allele-specific reverse transcription–polymerase chain reaction (RT–PCR) in freshly isolated chronic myeloid leukemia (CML) cells, including lin$^-$CD34$^+$CD38$^-$ and lin$^-$CD34$^+$CD38$^+$ cells, from two chronic phase CML samples by fluorescence-activated cell sorting. **A**) Detection of the Y353H mutation in lin$^-$CD34$^+$CD38$^-$ and lin$^-$CD34$^+$CD38$^+$ cells from patient 3. A PCR fragment (208 base pair [bp]) amplified by primers for the wild-type sequence was detected in agarose gels for all samples examined, including lin$^-$CD34$^+$ bone marrow cells from two normal individuals and lin$^-$CD34$^+$CD38$^-$, lin$^-$CD34$^+$CD38$^+$, and lin$^+$CD34$^-$ cells from CML patient 3. In contrast, the mutant sequence was amplified only in the lin$^-$CD34$^+$CD38$^-$ and lin$^-$CD34$^+$CD38$^+$ cells from the same CML patient. **B**) Results of analyzing the lin$^-$CD34$^+$CD38$^-$ and lin$^-$CD34$^+$CD38$^+$ cells from two CML patients for the presence of 16 specific BCR–ABL kinase domain mutations (six found in patient 1 and 10 in patient 3). Identical analysis of the lin$^+$CD34$^-$ cells from the same two patients failed to detect any of the 16 mutations. **Asterisks** indicate the kinase domain substitutions that were detected by direct sequencing of cloned cDNAs from lin$^-$CD34$^+$CD38$^-$ cells freshly isolated from the same patients (see Table 1).

The intensity of chemiluminesence was measured using a STORM 860 phosphoimager and ImageQuant software from Molecular Dynamics (Sunnyvale, CA, USA). The extent of phosphorylation of $p210^{BCR-ABL}$ and CrkL was then determined by comparing the chemiluminescence derived from the binding of antibodies specific for phosphotyrosine or phospho-CrkL to the chemoluminescence derived from the binding of antibodies raised to $p210^{BCR-ABL}$ or CrkL.

Statistical Analysis

Results are shown as the mean ± 95% confidence intervals of values obtained in independent experiments. Differences between groups were assessed using the two-sided Student's *t* test.

Results

Accumulation of BCR–ABL Mutations in Chronic Myeloid Leukemia Stem Cells In Vivo

To obtain an enriched population of CML stem cells, we used FACS to isolate the lin$^-$CD34$^+$CD38$^-$ subset of cells from three patients' samples in which these cells had been previously found to be 55%–100% Ph-positive (Ph$^+$) by FISH (data not shown). We then generated BCR–ABL cDNA by reverse transcription of RNA extracted from these cells and subcloned the PCR-amplified kinase domain sequence into a plasmid vector. Sequencing of the cloned fragments revealed that 33%, 20%, and 30% of the cloned cDNAs (nine or 10 from each patient) from patients 1, 2, and 3, respectively, contained mutations (Table 1). In contrast, we did not detect any kinase domain mutation among 30 cloned ABL cDNAs generated from extracts of lin$^-$CD34$^+$CD38$^-$ cells that were isolated from three different normal adult human bone marrow samples.

We also used a highly sensitive allele-specific real-time PCR method to look for specific point mutations in BCR–ABL cDNAs from two of the same three CML patients' samples studied by sequencing. Sixteen point mutations (Supplementary Table 1, available online) were examined, six in patient 1 and 10 in patient 3. Using this method, we analyzed extracts of the patients' lin$^-$CD34$^+$CD38$^-$ (stem) cells, as well as extracts of two more mature subsets of FACS-purified cells (lin$^-$CD34$^+$CD38$^+$ and lin$^+$CD34$^-$ cells) from the same patients. We identified five different mutant sequences in the leukemic stem cells from these patients (Fig. 1, B), including two (one encoding F359L in patient 1 and one encoding K400E in patient 3) that had also been found by direct sequencing. None of the mutations surveyed were detected in the more mature (lin$^-$CD34$^+$) cells (data not shown), but it is possible that the lower levels of BCR–ABL transcripts present in these cells (7,39) would have reduced the sensitivity of the allele-specific real-time PCR method for detection of mutations.

To investigate the specificity of the allele-specific real-time PCR methodology for detecting pre-existing mutations in cDNAs prepared from leukemic cells (as opposed to artifactual changes in transcript sequence acquired during the previous PCR steps), extracts from nine different samples of normal control cells were subjected to the same analysis as had been applied to the leukemic cells. Six of these control samples were lin$^-$CD34$^+$ cells from various sources (two from normal adult bone marrow samples, two from large pools of cord blood cells, and two from G-CSF–mobilized peripheral blood progenitor cells harvested from normal adults). The other three were normal granulocytes isolated by density centrifugation of normal human blood. None of the 16 BCR–ABL kinase domain mutations surveyed were detected in any of the control samples, as evidenced by allele-specific real-time PCR and the absence of amplified fragments in agarose gels stained with ethidium bromide. Figure 1, A shows an example of the specificity analysis. Using primers for the wild-type ABL sequence, a

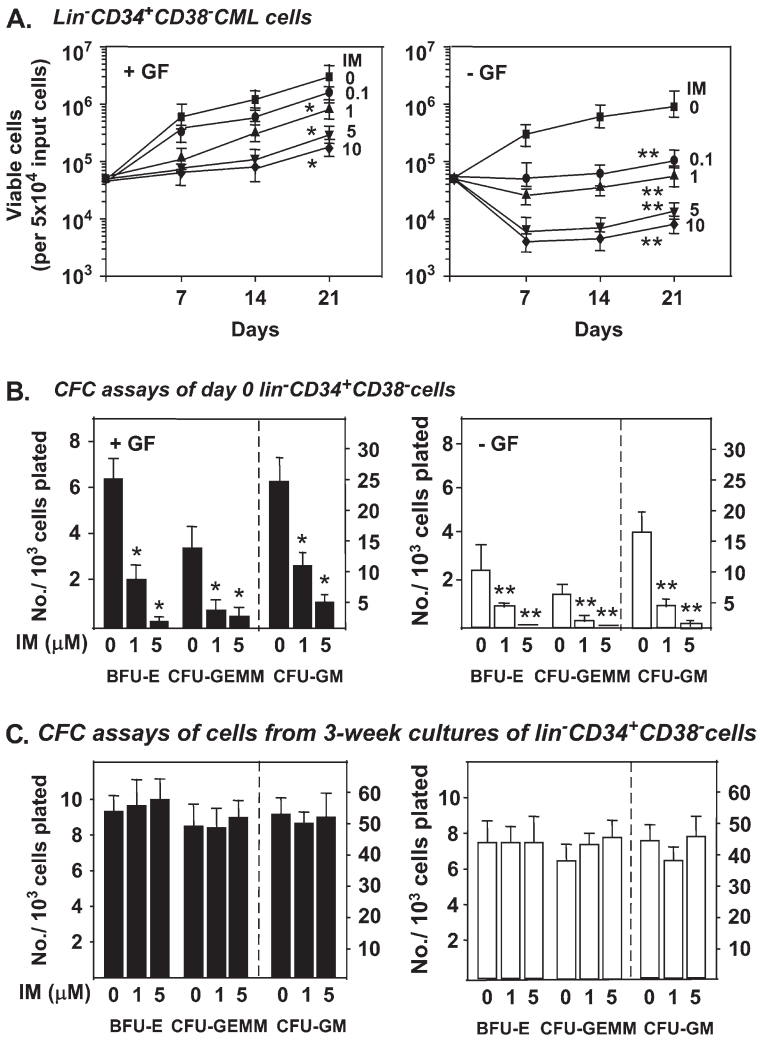


Fig. 2. Effects of imatinib on the production of cells from chronic myeloid leukemia (CML) stem cells and their colony-forming cell (CFC) progeny in vitro. **A**) Time course of changes in viable cells in serum-free cultures initiated with lin$^-$CD34$^+$CD38$^-$ (stem) cells (5 × 10^4 per mL) from five chronic-phase CML patients in whom the majority of the cells were positive for the Philadelphia chromosome. Suspension cultures were set up with fluorescence-activated cell sorting–purified cells and maintained for 3 weeks in the presence (**left panel**) or absence (**right panel**) of growth factors (GFs). Imatinib was added on day 0 and was replenished weekly at concentrations ranging from 0 to 10 μM, as indicated in the graphs. Viable cell yields were determined by hemocytometer counts of trypan blue–excluding cells from triplicate cultures and then expressed as the mean fold-change relative to the input number (with their 95% confidence intervals). *P = .015 to .01 as compared to the control without imatinib in the presence of GFs at day 21; 95% confidence intervals were 5 × 10^5 to 1 × 10^6, 1 × 10^5 to 5 × 10^5, and 5 × 10^4 to 2 × 10^5 with 1, 5, and 10 μM imatinib, respectively. **P<.001 as compared to the control without imatinib in the absence of GFs (95% confidence intervals were 1.6 × 10^5 to 3.8 × 10^5, 2.5 × 10^4 to 3 × 10^5, 2 × 10^4 to 2.5 × 10^5, and 2.5 × 10^3 to 2.5 × 10^4 with 0.1, 1, 5, and 10 μM imatinib, respectively). **B** and **C**) The effect of different doses of imatinib on the number of CFCs in freshly isolated lin$^-$CD34$^+$CD38$^-$ CML cells used to establish the suspension cultures (**B**) or in the 3-week suspension cultures (**C**) that contained 0 or 5 μM imatinib with (**left panels**) or without (**right panels**) added GFs. Both the fresh and cultured cells were assayed in standard 1 mL methylcellulose assays at 10^3 cells per culture containing 0, 1, or 5 μM imatinib. The colonies seen 2 weeks later were then classified and counted separately as derived from burst-forming units-erythroid (BFU-Es), colony-forming units-granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMMs), or colony-forming units-granulocyte-macrophage (CFU-GMs). The values shown are the means determined from data obtained from samples from five different chronic phase CML patients. *P≤.003 as compared to the control without imatinib in the presence of GFs (using the Student's t test for paired samples) (95% confidence intervals = 0.2 to 3.5 [BFU-Es], 0.3 to 1.2 [CFU-GEMMs], and 3 to 14 [CFU-GMs]). **P≤.0035 as compared to the control without imatinib in the absence of GFs (95% confidence intervals were 0.1 to 1.1 [BFU-Es], 0.07 to 0.6 [CFU-GEMMs], and 0.1 to 1.2 [CFU-GMs]). The y-axis on the **left side of each panel** indicates the scales used for BFU-E and CFU-GEMM values, and the y-axis on the **right side of each panel** indicates the scale used for the matching CFU-GM values.

208–base pair (bp) PCR fragment was detected using cDNAs obtained from lin$^-$CD34$^+$ bone marrow cells from either of two normal individuals or from lin$^+$CD34$^-$, lin$^-$CD34$^+$CD38$^+$, or lin$^-$CD34$^+$CD38$^-$ cells from CML patient 3. In contrast, using primers for the mutant sequence encoding Y353H, a 208-bp PCR-amplified fragment was detected only in the lin$^-$CD34$^+$CD38$^+$ and lin$^-$CD34$^+$CD38$^-$ cells from patient 3.

Taken together, the results of both the direct sequencing of cloned BCR–ABL cDNAs and the search for mutations by allele-specific real-time PCR indicate a high degree of instability of the BCR–ABL gene in primitive chronic phase CML cells during their growth in vivo in the absence of selective drug treatment.

Generation of Imatinib-Resistant Colony-Forming Cells From Chronic Myeloid Leukemia Stem Cells Incubated In Vitro

We then sought to determine whether additional mutations in BCR–ABL accumulated in the progeny of chronic phase CML stem cells stimulated to proliferate in vitro. Accordingly, we isolated the lin$^-$CD34$^+$CD38$^-$ fraction of cells by FACS from the three patients with predominantly leukemic cells in this compartment as well as from two other patients and then used these cells to establish serum-free suspension cultures. The cultures contained either no imatinib (controls) or from 0.1 to 10 μM imatinib. Half of the cultures contained a supportive combination of growth factors (20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 100 ng/mL flt3-ligand, and 100 ng/mL Steel factor) and the other half contained no added growth factors. In both cases, the medium was replaced at weekly intervals. Time course studies showed that the yield of viable cells decreased as a function of imatinib dose, with the most pronounced decrease seen in cultures to which no growth factors were added (Fig. 2, A).

The lin$^-$CD34$^+$CD38$^-$ cells used to establish 3-week suspension cultures as well as the cells harvested from the 3-week suspension cultures were also assayed for CFCs in methylcellulose cultures. Burst-forming units-erythroid (BFU-Es), colony-forming units-granulocyte–macrophage (CFU-GMs), and colony-forming units-granulocyte–erythrocyte–monocyte–megakaryocyte (CFU-GEMMs) were detectable at the same numerical frequencies in both populations (Fig. 2, B versus Fig. 2, C), even though, after 3 weeks in suspension culture, the majority of the cells grown from the input lin$^-$CD34$^+$CD38$^-$

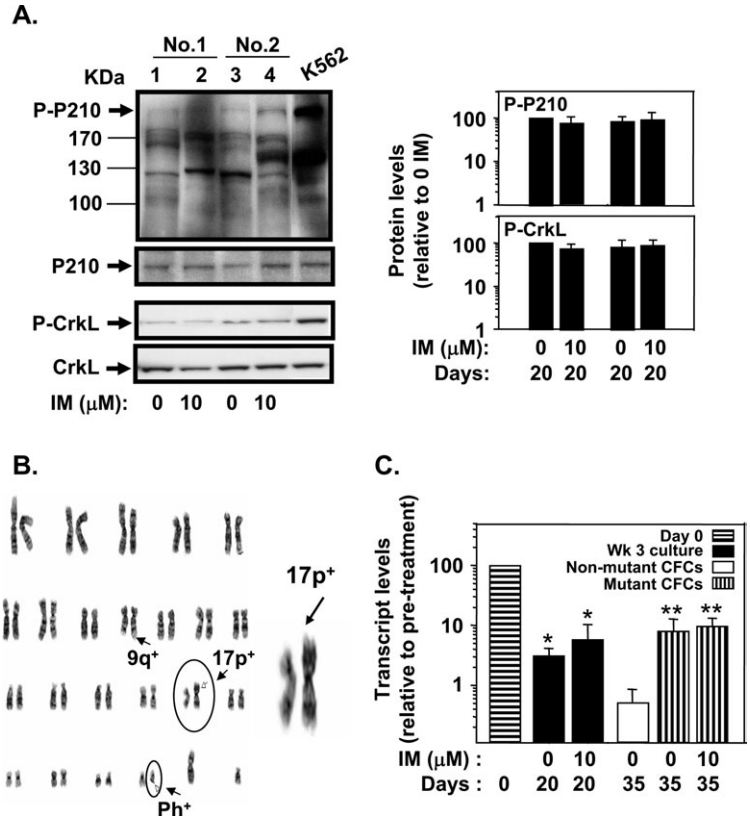


Fig. 3. Selection of previously undetected mutant Philadelphia chromosome (Ph^+)/BCR–ABL^+ cells after exposure of primitive chronic myeloid leukemia (CML) cells to imatinib (IM) in vitro. **A**) Comparison of the levels and activity of $p210^{BCR-ABL}$ protein in $lin^-CD34^+CD38^-$ cells grown in the presence or absence of IM. $Lin^-CD34^+CD38^-$ cells were isolated from two patients and maintained in culture for 20 days in the presence (**lanes 2 and 4**) or absence (**lanes 1 and 3**) of 10 μM IM. The electrophoresed cell lysates were then transferred to two filters; one filter was probed with an anti-phosphotyrosine (4G10) antibody (**top panel**), then stripped and reprobed with an anti-abl antibody 8E9 (**second panel from top**). The other filter was probed with an anti-phospho-CrkL antibody (**third panel from the top**), then stripped and probed with an anti-CrkL antibody (**bottom panel**). **Lanes 1 and 2** were prepared from lysates from patient 1, and **lanes 3 and 4** were prepared from lysates from patient 2. **Lane 5** contains a lysate from K562 cells. Chemiluminescence was measured using a STORM 860 phosphoimager and ImageQuant software. In the **right panel**, the levels of tyrosine phosphorylation of $p210^{BCR-ABL}$ and CrkL were determined by the ratios of chemiluminescent intensities derived from the binding of antibodies to phosphotyrosine and $p210^{BCR-ABL}$, and posphorylated CrkL and CrkL, respectively. Data shown are for cells from three different patients. In each case, the ratios were normalized to the corresponding value obtained for cells from patient 1 that were not treated with IM ($P = .95$ and .88 for tyrosine phosphorylation of $p210^{BCR-ABL}$ and CrkL, respectively, by Student's t test for paired samples). The **error bars** are from the data for three separate patient samples, each studied twice. **B**) Representative karyotype showing the $17p^+$ abnormality present in all four Giemsa-banded metaphases obtained from a single colony generated from the cells present after 3 weeks in culture with IM (patient 1). The panel shows the additional material present on the short arm of one chromosome 17, which is possibly indicative of an isochromosome 17 abnormality reflecting a loss of the short arm (17p) and a duplication of the long arm (17q). **C**) BCR–ABL transcript levels in $lin^-CD34^+CD38^-$ cells isolated from three patients and in cultured cells derived from them or from individual colonies grown from the cultured cells in the presence or absence of IM. The BCR–ABL transcript levels measured in cells harvested from the suspension cultures were from three independent experiments that combined cultured cells from each patient. A total of 20 individual colonies from the three patients were used to measure the BCR–ABL transcript levels, and **error bars** represent the 95% confidence intervals derived from the 20 separate measurements under each condition. BCR–ABL transcript levels were determined by quantitative real-time polymerase chain reaction. The transcript levels in the cultured cells and in the colonies derived from them are expressed as a percent of the corresponding absolute values in the starting population. *P<.001 compared with the group from day 0, 95% confidence intervals = 0.9 to 7.2 in the absence of IM and 4 to 15 in the presence of IM; **P = .006 compared with the levels in nonmutant colonies without IM treatment from day 35 (**white bars**), 95% confidence intervals = 4 to 19 for both groups.

cells had differentiated into morphologically recognizable granulocytes and macrophages, as expected from previous studies (32,40).

To determine whether 3 weeks in culture selected for the outgrowth of an initially minor residual normal stem cell population, we used FISH and metaphase karyotyping to genotype the colonies generated in methylcellulose from harvests of the 3-week suspension cultures established from two of the CML patients (patients 1 and 2). A total of 84 metaphases were analyzed from colonies generated in the presence or absence of growth factors and imatinib, and more than 300 cells from the 3-week suspension cultures maintained under the same treatment conditions were analyzed by FISH. The results of these analyses confirmed that essentially all the cells in the 3-week cultures established from patient 1 were leukemic (i.e., 100% BCR/ABL^+/Ph^+), regardless of whether they had been grown in the absence or presence of imatinib or added growth factors. However, FISH analysis did identify some normal cells in the experiment initiated with the $lin^-CD34^+CD38^-$ cells from patient 2 that originally contained 45% normal cells (67%–77% BCR–ABL^+ cells detected in the absence of imatinib and 65%–72% BCR–ABL^+ cells in its presence). The normal (Ph^-) cells that we observed in cultures established from some suspensions of freshly isolated $lin^-CD34^+CD38^-$ cells reinforces the importance of continued genotyping whenever primitive CML cells are cultured to ensure that resurgent growth from normal stem cells is not confused with in vitro selection of imatinib-resistant CML cells.

We also compared the imatinib sensitivity of the CFCs in the freshly isolated $lin^-CD34^+CD38^-$ cells with the imatinib sensitivity of the leukemic CFCs obtained from the 3-week suspension cultures established with the same $lin^-CD34^+CD38^-$ cells by assessing the ability of the cells from both sources to form colonies in semisolid medium containing 0, 1, or 5 μM imatinib. The results of these measurements show that the CFCs harvested from the 3-week cultures (Fig. 2, C) were substantially less sensitive to imatinib than those present in the original $lin^-CD34^+CD38^-$ populations (Fig. 2, B).

Using immunoblotting approaches, we determined the levels of $p210^{BCR-ABL}$, its kinase activity (as reflected in the extent of Crk-L phosphorylation), and the extent to which it was tyrosine phosphorylated in cells that had been grown from $lin^-CD34^+CD38^-$ cells in the presence of 10 μM imatinib. We then compared these values to those obtained for cells grown from the same input cells in the absence of imatinib. Blots prepared from electrophoresed extracts

Table 2. Types and frequency of BCR–ABL transcripts with kinase domain mutations detected by sequencing cloned transcripts present in extracts of individual colonies generated from CFCs harvested from 3-week cultures initiated with lin$^-$CD34$^+$CD38$^-$ CML cells*

		No. of mutant transcripts : No. of wild-type transcripts (% mutant transcripts)			
		No imatinib in the suspension culture		+ 5 μM imatinib in the suspension culture	
Patient No.	CFC No.	No imatinib in the CFC assay	+ 5 μM imatinib in the CFC assay	No imatinib in the CFC assay	+ 5 μM imatinib in the CFC assay
1	CFC-1	3 : 2	3 : 2	2 : 3	3 : 2
	CFC-2	0 : 3	2 : 3	5 : 1	1 : 4
	CFC-3	0 : 3	6 : 0	3 : 3	
	CFC-4	0 : 2		2 : 1	
	Total	3 : 10 (23)	11 : 5 (69)	12 : 8 (60)	4 : 6 (40)
2	CFC-1	1 : 3	1 : 3	0 : 3	0 : 4
	CFC-2	2 : 2			0 : 2
	CFC-3	0 : 3			4 : 0
	CFC-4				3 : 1
	Total	3 : 8 (27)	1 : 3 (25)	0 : 3 (0)	7 : 7 (50)
3	CFC-1	0 : 2	2 : 2	1 : 3	3 : 1
	CFC-2	1 : 2	2 : 2	1 : 2	3 : 0
	CFC-3	3 : 1	2 : 2	1 : 3	2 : 2
	CFC-4	2 : 2		2 : 2	2 : 2
	Total	6 : 7 (46)	6 : 6 (50)	5 : 10 (33)	10 : 5 (68)

* CFC = colony-forming cells; CML = chronic myeloid leukemia.

of the cells were first probed with an antibody to phosphotyrosine and one specific for the phosphorylated form of CrkL. The blots were then stripped and reprobed with antibodies that recognized p210$^{BCR–ABL}$ or CrkL, irrespective of their phosphorylation status. The chemiluminescence intensities of the bands corresponding to phosphorylated and total protein were then compared. The cells grown in the 3-week suspension cultures that contained 10 μM imatinib showed no detectable decrease in the extent of phosphorylation of either p210$^{BCR–ABL}$ or CrkL (P = .95 and .88, respectively) by comparison to the same cells cultured in the absence of imatinib (Fig. 3, A). This finding suggested that 3 weeks growth in vitro under these conditions had selected for cells that had acquired some genetic form of imatinib resistance. We did not detect any evidence of BCR–ABL gene amplification in these cells by FISH analysis (after analyzing more than 300 cells, data not shown). These findings prompted us to determine whether the time in culture had selected for pre-existing BCR–ABL mutations and/or whether new BCR–ABL mutations had been acquired.

Detection of New BCR–ABL Mutations in Cells Derived From Chronic Myeloid Leukemia Stem Cells Incubated In Vitro

The first indication that new mutations might be present in the progeny of cultured CML stem cells was provided by cytogenetic analysis of Giemsa-banded metaphases obtained from individually processed CFC-derived colonies produced from the 3-week suspension culture cells (a total of 84 metaphases). These studies identified one colony in which four of four metaphases analyzed contained a previously undetected 17p$^+$ abnormality (Fig. 3, B). To look for mutations in the BCR–ABL gene of the cultured cells, we sequenced 235 subcloned PCR-amplified cDNA fragments obtained both from extracts of the 3-week suspension cells (58 clones, Table 1) from three patients and the rest from 37 individual colonies generated from these cells (listed in Table 2). By sequencing, we identified 65 point mutations in the sequence encoding the BCR–ABL kinase domain that had not been detected in the original lin$^-$CD34$^+$CD38$^-$ cells from which the cultures had been established (Table 3, Fig. 4, A and B).

To determine whether the BCR–ABL kinase domain is particularly prone to mutation in CML cells, we used the same cDNA-based sequencing approach to search for mutations at two additional locations in the BCR–ABL gene in cells from six colonies generated from the same 3-week cultures that had already been analyzed for kinase domain mutations. Both locations were 5′ of the kinase domain; one extended into the BCR sequence. From sequencing 23 PCR-amplified cDNA fragments from the three patient samples analyzed, 21 mutations were identified (Table 4). These results show that these additional regions were also highly susceptible to mutations. In contrast, using specific primers to amplify ABL cDNAs from similarly cultured lin$^-$CD34$^+$CD38$^-$ cells derived from two normal adult human bone marrow samples and sequencing a total of 17 cDNA fragments, we detected only four mutations (23%, Table 4).

Because the studies of transcripts from the leukemic CFC–derived colonies were performed on single colonies, it was possible to determine whether the mutations were already present in the individual CFCs from which the colonies arose (preexisting mutations would be expected to be present in all transcripts from a single colony, whereas mutations acquired de novo during colony growth would lead to a mixed pattern of wild-type and mutant transcripts). Colonies that had been grown from single cells that were harvested from the 3-week suspension cultures (containing either 0 or 5 μM imatinib) and then plated in methylcellulose assays were picked, RNA extracts were prepared, and cDNA fragments containing the BCR–ABL kinase domain were then amplified by PCR. After purification, these cDNAs were cloned into a

Table 3. Comparison of the sets of BCR–ABL kinase domain mutations identified in this and previous studies*

BCR–ABL mutations in primitive cells		Mutations in imatinib-resistant patients (15,16,18,38,44–46)	Mutations in CD34+ cells in cytogenetic remission (48)	Mutations by a comprehensive mutational screen (49)
Nucleotide change†	**Amino acid change‡**			
GTG-GCG	V228A			
AAC-TAC	N231Y	N231D		
TAC-CAC	Y232H			
AAG-GAG	K234E			
TGG-GGG/CGG/TAG	W235G/R/stop			
GAA-GAT	E238D			
ACG-GCG	T240A			
GAC-GCC	D241A	D241G		
AAG-GAG/ATG/AGG	K245E/M/R			
AAG-GAG/AGG/TAG	K247E/R/stop			
GGG-CGG/AGG	G250R	G250A/E/R		G250E/R
GGG-GAG/AGG	G254E/R			
GTG/GCG/ATG	V256A/M			
GTG-GCG	V260A			
AAG-AGG	K262R			
TAC-TGC	Y264C			
AGC-TGC/GGC	S265C/G			S265I/T
CTG-CCG	L266P			L266M/V
ACG-ATG	T267M			
GTG-GCG/ATG	V268A/M			V268/270A
AAG-AGG	K271R			
ACC-GCC	T272A			
AAG-GAG	K274E			
GAG-GGG	E275G			E275K
GAC-GGC	D276G	D276G	D276G	D276V
ACC-GCC	T277A	T277A		
GAA-GGA/TAA	E281/G/stop			E281K
GAG-GGG	E282G			E282D
TTG-TCG/GTG	L284S/V			L284F
AAA-AGA	K285R			
GAA-GGA/AAA	E286G/K			
GTC-GCC	V289A	V289A		V289S
AAA-AGA	K291R			K291E/R
ATC-ACC	I293T			
AAA-GAA	K294E			K294R
CAC-CGC	H295R			
CCT-TCT/ACT	P296S/T			
GTG-GCG/ATG	V299A/M			
CAG-CGG	Q300R			Q300H
CTC-CCC	L301P			L301F
GTC-GCC	V304A		V304G	
CGG-CAG/TGG	R307Q/W			
ATC-ACC	I313T			
ATC-ACC	I314T			
ACT-GCT/ATT	T315A/I	T315I		T315G/I/S
GAG-GGG	E316G			E316D
ATG-GTG	M318V			
AAC-GAC	N322D			
CTC-TTC/CCC	L323F/P			
TAC-TTC	Y326F			
CTG-CCG	L327P			
AAC-AGC	N331S			N331S
CAG-CTG	Q333L			
GAG-GGG	E334G			
GCC-TCC	A337S			
GTG-GAG	V338E			V338G
TAC-CAC	Y342H			
ATG-GTG	M343V	M343T		M343T
ATC-GTC	I347V			
GCC-GTC	A350V			
GAG-AAG	E352K		E352G	E352K

(Table continues)

Table 3 (continued).

BCR–ABL mutations in primitive cells		Mutations in imatinib-resistant patients (15,16,18,38,44–46)	Mutations in CD34+ cells in cytogenetic remission (48)	Mutations by a comprehensive mutational screen (49)
Nucleotide change†	**Amino acid change‡**			
TAC-CAC	Y353H		Y353G/H	
AAA-GAA/CAA	K357E/Q			
TTC-CTC/TCC	F359L/S	F359A/I/V		F359C
ATC-ACC	I360T			I360F
CAC-CGC	H361R			
AAC-AGC	N368S			
CAC-CGC	H375R			
GTG-GCG	V377A			
TTT-TCT	F382S	F382L		
ATG-ACG/GTG	M388T/V			M388I
ACA-GCA	T389A		T389A	
GAC-GGC	D391G			
ACC-GCC	T392A			
TAC-TGC	Y393C			
AAG-GAG/AGG	K400E/R			
AA A/ACA	K404T			

* References for previous studies are shown in parentheses.

† Nucleotide changes are according to GenBank accession number M14752. Each mutant with a nucleotide change is underlined.

‡ Amino acid positions are those of ABL type 1a, which has the following subdomains: P-loop 244–255, C-helix 276–290, SH3 contact 294–304, SH2 contact 331–353, activation loop 381–402.

bacterial vector to generate transformants. Several transformants were isolated for each extract investigated and DNA extracts obtained from each for sequencing (Table 2). Most of the 37 colonies examined were found to contain both wild-type and mutant transcripts, and 18 contained more than a single type of mutant transcript. A lower frequency of BCR–ABL kinase domain mutations was observed in the colonies grown in the absence of imatinib than its presence in all three patient samples studied (patient 1: 23% versus 40%, patient 2: 27% versus 50%, and patient 3: 46% versus 68%; Table 2). These results demonstrate that BCR–ABL kinase domain mutations arose at a high rate during the 2 weeks of in vitro colony growth. Thus, the imatinib-resistant phenotype displayed in these CFC assays was not due solely to the acquisition during the preceding 3 weeks of culture of BCR–ABL kinase domain mutations.

To determine whether imatinib resistance of the CFCs obtained from the 3-week cultures was due in part to increased BCR–ABL expression, we used real-time RT–PCR to measure the levels of BCR–ABL transcripts in the cells of individual colonies produced in the methylcellulose media. The results showed that the cells from colonies containing some or exclusively mutant BCR–ABL transcripts expressed BCR–ABL at 30-fold higher levels than cells

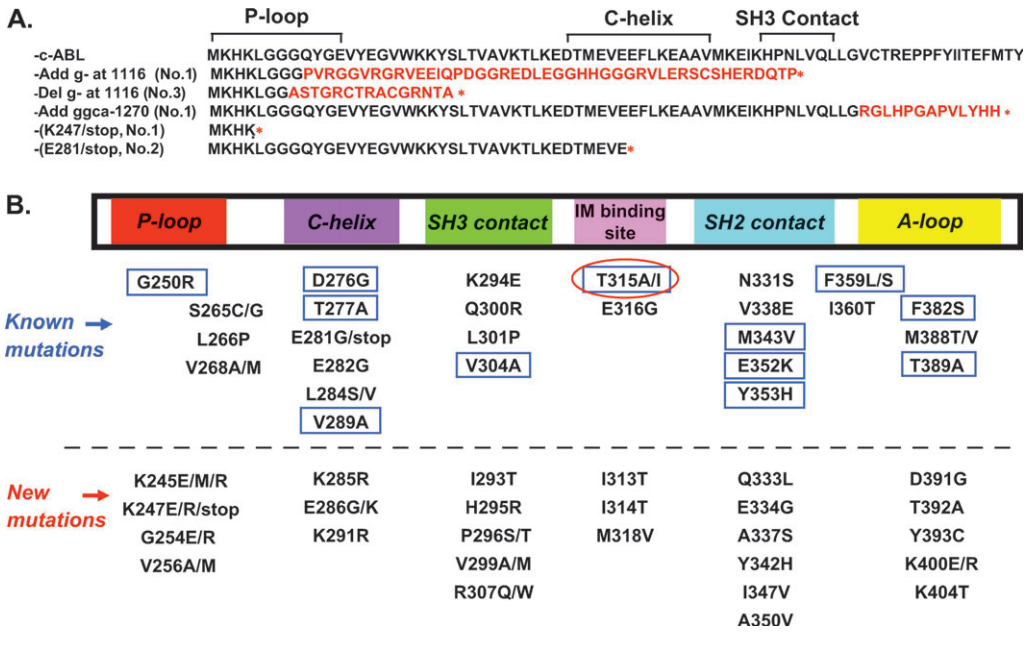


Fig. 4. BCR–ABL kinase domain mutations identified in freshly isolated and cultured chronic myeloid leukemia cells. **A**) Three frameshift mutations (in **red**) that generate new amino acid sequences and two premature stop codons (denoted by **asterisks**) within the BCR–ABL kinase domain identified in this study. **B**) Locations of the single amino acid substitutions identified in previous studies and in this one in relation to different subdomains within the BCR–ABL kinase domain. The amino acid changes that have been associated with imatinib resistance in patients are indicated by the **boxes**. The T315I mutation that has been reported to confer resistance to dasatinib and/or nilotinib is indicated by the **red oval**. P-loop = phosphate-binding loop; C-helix = catalytic domain; SH3 contact = Src homology 3 domain contact; IM binding site = imatinib binding site; SH2 contact = Src homology 2 domain; A-loop = activation loop; No. = patient number.

Table 4. Types and frequency of BCR–ABL transcripts with mutations in the tyrosine kinase domain or in a BCR–ABL sequence immediately upstream of the kinase domain detected in colonies generated from CFCs harvested from 3-week cultures initiated with $lin^-CD34^+CD38^-$ cells*

Origin of mutations	Patient No. 1	Patient No. 2	Patient No. 3	Normal adult No. 1	Normal adult No. 2
3′ BCR region	L882P, N900P	T883A L879P Q890R	T898A,N900P	N/A	N/A
5′ ABL region	N78S, W110R E86G, G109S V186F	H202R N193S N94D,V199A S187P G169E	F72S T224A K183E	N/A	N/A
ABL kinase domain	K271R, K357Q V228A,K245E, K247R, K274E,T315A, Y393C A337S, K404T E286G, nucleotide 1116 add g W235R, L327P, nucleotide 1270 add ggca W235R, L327P, nucleotide 1270 add ggca I347V V228A K247Stop	K245M,Y342H K234E,V377A,D391G K234E,H361R R307W	N231Y, T267M, M388T, nucleotide 1116 del g K274E, I313T L301P E281Stop	A269T A288T	M278V R307W

* Results are shown for a subset of the colonies analyzed for kinase domain mutations in Table 2. Each result shown indicates the one or more mutations detected by sequencing subcloned PCR-amplified cDNA fragments from six CFC-derived colonies (two per patient) in the screen for kinase domain mutations (26 cDNA clones) plus PCR-amplified cDNA fragments for a region upstream of the kinase domain (23 cDNA clones). CFCs = colony-forming cells; N/A = not applicable.

from colonies in which only wild-type transcripts were present (Fig. 3, C). Colony growth in the presence of imatinib did not affect the level of BCR–ABL transcripts because mutant CFCs obtained from all three patient samples studied in either the presence or absence of imatinib contained similar levels of BCR–ABL transcripts. This observation suggests that the acquisition of BCR–ABL kinase domain mutations may be associated with a diminished ability to reduce BCR–ABL expression. The overall levels of BCR–ABL transcripts in the cultured cells as compared with the starting population were also reduced (Fig. 3, C), as occurs when CML stem cells differentiate in vivo (7,39).

Discussion

In this study, we found that the BCR–ABL fusion gene in CML stem cells is highly unstable as indicated by an unusual rate of accumulation of mutations in this compartment in vivo and in their progeny generated in vitro, even in the absence of imatinib exposure. This finding is consistent with the clinical observation that many chronic phase CML patients can achieve a complete cytogenetic remission (as evidenced by the absence of the BCR–ABL translocation [t 9;22] in 20 bone marrow metaphases), but complete molecular remissions (as evidenced by a reduction of BCR–ABL transcripts to levels that are undetectable by real-time RT–PCR) are rare (18,41,42), and resurgence of imatinib-resistant leukemic cells remains a clinical concern. The high mutation rate in the BCR–ABL fusion gene is also consistent with recent laboratory studies that have suggested that the most primitive and/or quiescent fraction of chronic phase CML cells are relatively insensitive to either imatinib or more potent BCR–ABL–targeted agents like dasatinib at doses that kill the more mature leukemic cells (23,43). These studies have focused attention on the importance of these unresponsive primitive/quiescent cells in the inability of some CML patients to achieve durable responses to new therapies.

It has been reported that the most common mechanism of acquired resistance to imatinib in CML patients involves either an amplification of the BCR–ABL gene or the appearance of point mutations in the kinase domain (15,38). However, another clinical study has concluded that BCR–ABL gene amplification is probably a less frequent mechanism of clinical resistance to imatinib than acquisition of mutations that affect imatinib binding (16). None of the patients studied here exhibited evidence of BCR–ABL gene amplification, as indicated by FISH analysis of their primitive cells, nor was amplification of the BCR–ABL gene detected in cells cultured with or without imatinib. BCR–ABL mutations that affect imatinib binding have occasionally been detected in patients before the initiation of imatinib therapy (16,17) and have been hypothesized to result from an inherent genetic instability in BCR–ABL–transformed cells (38,44). However, their presence in the $lin^-CD34^+CD38^-$ cells from imatinib-naive chronic phase CML patients has not been documented previously. Here, we have shown that numerous mutations in the sequence encoding the kinase domain of BCR–ABL were present at detectable frequencies in the most primitive leukemic cells of all three chronic phase CML samples that were examined, even though the samples were from patients who had never been treated with imatinib. More recent studies with samples from an additional 12 chronic phase CML patients have corroborated these results (Jiang X, Forrest D,

Smith C, Eaves A, Eaves C: unpublished data). These data clearly support the idea that kinase domain mutants arise spontaneously and at a clinically relevant frequency in chronic phase CML stem cells expanding in vivo.

Another important contribution of this study was the observation of the high incidence and variety of mutations identified in cultured CML cells (either with or without imatinib). Overall, point mutations affecting 77 different nucleotides within the kinase domain of BCR–ABL were identified by sequencing transcripts obtained from either fresh CML cells or their progeny. Most were point mutations, and up to three different substitutions were identified at some sites in different samples. The majority were A → G (46%), T → C (27%), and C → T (5%) transitions previously associated with imatinib resistance (28,42). In some instances, more than a single mutation could be found in the same cloned transcript (Fig. 4 and Tables 3 and 4). Of the mutations identified, 30 have been previously reported. Ten of the substitutions (N231Y, D241A, G250R, D276G, T277A, V289A, T315I/A, M343V, F359L/S, and F382S) have been associated with imatinib resistance in patients and two (G250R and T315I) with imatinib resistance in vitro as well (15,18,38,45–49). T315I has recently been reported to confer resistance to dasatinib and/or nilotinib, two more recently developed inhibitors of the BCR–ABL kinase (9–11). Another five substitutions (D276G, V304A, E352K, Y353H, and T389A) have been identified in $CD34^+$ cells from imatinib-treated CML patients in complete cytogenetic remission (48). To the best of our knowledge, the other 47 point mutations have not been previously described. In addition, we discovered three novel frameshift mutations and three mutations that result in premature termination codons, leading to truncated proteins (Fig. 4, A and Table 3). Mutations were also found outside the kinase domain of BCR–ABL, consistent with the concept that CML cells have heightened genetic instability in vitro (28). Taken together, these findings strongly implicate BCR–ABL instability in CML stem cells in the development of imatinib resistance.

Analyses of BCR–ABL transcripts present in individual colonies generated in CFC assays of the 3-week suspension cultures revealed cases in which an individual colony of cells sometimes contained a mixture of wild-type transcripts and one, or two, or even three different mutant transcripts. These data indicate that new mutants arose during the process of colony formation; i.e., within 8–10 cell divisions. This rapid generation of BCR–ABL mutants in primary CML cells in vitro is the same type of genome instability that was recently demonstrated in murine BaF3 cells (50) and adult bone marrow cells (51) transduced with a BCR–ABL vector. Future experiments will be required to determine whether the genetic instability manifested by cultured CML cells is due to a heightened sensitivity to reactive oxygen species produced in cultures maintained in 20% oxygen, as has been recently suggested (28).

Our study clearly demonstrates that CML stem cells are an important target population for the development of the BCR–ABL mutations associated with the development of imatinib resistance in vivo and in vitro. Nevertheless, some potential limitations of this study should be considered. First, the studies of CML stem cell properties presented here were largely confined to a small number of chronic phase CML patients' samples whose stem cell compartment contained predominantly leukemic cells. Caution needs to be exercised in extrapolating from these findings to more typical chronic phase CML patients in whom the leukemic stem cells are much less numerous. A second limitation of this study is the lack of mechanistic data to explain the altered genomic stability seen due to the very small numbers of leukemic stem cells that can be accessed, even from patients' in whom the majority of stem cells are leukemic.

Taken together, our findings support the concept that chronic phase CML stem cells exhibit heightened genomic instability that allows these cells to diversify genetically. As a result, with slow but continued expansion of the chronic phase stem cell compartment over time in vivo, subclones of mutant stem cells with a variety of altered features would be expected to appear. The subclones could have mutations that confer a growth disadvantage leading to their extinction as well as mutations that would be growth neutral but confer resistance to BCR–ABL–targeted therapies and thus provide a selective growth advantage upon the administration of such therapies. Although this study was not designed to identify mutations that would contribute to disease progression, our results suggest that these would also be found. These considerations highlight the importance of gaining further understanding of the control of DNA replication and repair in the leukemic stem cells from patients with chronic phase CML in future efforts to devise therapies with curative potential.

References

(1) Goldman JM, Melo JV. Chronic myeloid leukemia—advances in biology and new approaches to treatment. N Engl J Med 2003;349:1451–64.

(2) Jiang X, Smith C, Eaves A, Eaves C. The challenges of targeting chronic myeloid leukemia stem cells. Clin Lymphoma Myeloma 2007;7(Suppl 2):S71–81.

(3) Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of *bcr-abl* oncogene products. Science 1990;247:1079–82.

(4) Sattler M, Griffin JD. Molecular mechanisms of transformation by the BCR-ABL oncogene. Semin Hematol 2003;40:4–10.

(5) ten Hoeve J, Arlinghaus RB, Guo JQ, Heisterkamp N, Groffen J. Tyrosine phosphorylation of CRKL in Philadelphia$^+$ leukemia. Blood 1994;84:1731–6.

(6) Hamilton A, Elrick L, Myssina S, Copland M, Jorgensen H, Melo JV, et al. BCR-ABL activity and its response to drugs can be determined in CD34+ CML stem cells by CrkL phosphorylation status using flow cytometry. Leukemia 2006;20:1035–9.

(7) Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, Eaves A, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia 2007. [Epub ahead of print].

(8) Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996;2:561–6.

(9) Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399–401.

(10) Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, et al. Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. Proc Natl Acad Sci U S A 2005;102:11011–6.

(11) Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 2005;7:129–41.

(12) Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001;344:1031–7.

(13) Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med 2002;346:645–52.
(14) O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl Med 2003;348:994–1004.
(15) Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001;293:876–80.
(16) Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, Lai JL, Philippe N, Facon T, et al. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. Blood 2002;100:1014–8.
(17) Roche-Lestienne C, Lai JL, Darre S, Facon T, Preudhomme C. A mutation conferring resistance to imatinib at the time of diagnosis of chronic myelogenous leukemia. N Engl J Med 2003;348:2265–6.
(18) Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood 2005;105:2640–53.
(19) O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res 2005;65:4500–5.
(20) Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–41.
(21) Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006;354:2542–51.
(22) Holyoake TL, Jiang X, Drummond MW, Eaves AC, Eaves CJ. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia. Leukemia 2002;16:549–58.
(23) Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 2002;99:319–25.
(24) Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood 2002;99:3792–800.
(25) Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood 2003;101:4701–7.
(26) Calabretta B, Perrotti D. The biology of CML blast crisis. Blood 2004; 103:4010–22.
(27) Penserga ET, Skorski T. Fusion tyrosine kinases: a result and cause of genomic instability. Oncogene 2007;26:11–20.
(28) Koptyra M, Falinski R, Nowicki MO, Stoklosa T, Majsterek I, Nieborowska-Skorska M, et al. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. Blood 2006; 108:319–27.
(29) Petzer AL, Eaves CJ, Barnett MJ, Eaves AC. Selective expansion of primitive normal hematopoietic cells in cytokine-supplemented cultures of purified cells from patients with chronic myeloid leukemia. Blood 1997;90:64–9.
(30) Eisterer W, Jiang X, Christ O, Glimm H, Lee K, Pang E, et al. Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. Leukemia 2005;19:435–41.
(31) Holyoake TL, Jiang X, Jorgensen HG, Graham S, Alcorn MJ, Laird C, et al. Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth in vitro in association with up-regulation of expression of interleukin-3. Blood 2001;97:720–8.
(32) Jiang X, Lopez A, Holyoake T, Eaves A, Eaves C. Autocrine production and action of IL-3 and granulocyte colony-stimulating factor in chronic myeloid leukemia. Proc Natl Acad Sci U S A 1999;96:12804–9.
(33) Hogge DE, Lansdorp PM, Reid D, Gerhard B, Eaves CJ. Enhanced detection, maintenance and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human Steel factor, interleukin-3 and granulocyte colony-stimulating factor. Blood 1996;88:3765–73.
(34) Fraser C, Eaves CJ, Kalousek DK. Fluorodeoxyuridine synchronization of hemopoietic colonies. Cancer Genet Cytogenet 1987;24:1–6.
(35) Jiang X, Ng E, Yip C, Eisterer W, Chalandon Y, Stuible M, et al. Primitive interleukin 3 null hematopoietic cells transduced with BCR-ABL show accelerated loss after culture of factor-independence in vitro and leukemogenic activity in vivo. Blood 2002;100:3731–40.
(36) Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25: 169–93.
(37) Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29: 23–39.
(38) Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2002;2:117–25.
(39) Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 2004;351:657–67.
(40) Jiang X, Fujisaki T, Nicolini F, Berger M, Holyoake T, Eisterer W, et al. Autonomous multi-lineage differentiation in vitro of primitive $CD34^+$ cells from patients with chronic myeloid leukemia. Leukemia 2000;14: 1112–21.
(41) Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 2003;349:1423–32.
(42) Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, et al. Detection of *BCR-ABL* mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 2003;102:276–83.
(43) Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML, but does not eliminate the quiescent fraction. Blood 2006;107:4532–9.
(44) Sorel N, Bonnet ML, Guillier M, Guilhot F, Brizard A, Turhan AG. Evidence of ABL-kinase domain mutations in highly purified primitive stem cell populations of patients with chronic myelogenous leukemia. Biochem Biophys Res Commun 2004;323:728–30.
(45) Shah NP, Sawyers CL. Mechanisms of resistance to STI571 in Philadelphia chromosome-associated leukemias. Oncogene 2003;22:7389–95.
(46) Corbin AS, La Rosee P, Stoffregen EP, Druker BJ, Deininger MW. Several Bcr-Abl kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. Blood 2003;101:4611–4.
(47) Burgess MR, Skaggs BJ, Shah NP, Lee FY, Sawyers CL. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. Proc Natl Acad Sci U S A 2005;102:3395–400.
(48) Chu S, Xu H, Shah NP, Snyder DS, Forman SJ, Sawyers CL, et al. Detection of BCR-ABL kinase mutations in $CD34^+$ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. Blood 2005;105:2093–8.
(49) Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. Cell 2003;112:831–43.
(50) von Bubnoff N, Veach DR, van der KH, Aulitzky WE, Sanger J, Seipel P, et al. A cell-based screen for resistance of Bcr-Abl-positive leukemia identifies the mutation pattern for PD166326, an alternative Abl kinase inhibitor. Blood 2005;105:1652–9.
(51) Flamant S, Turhan AG. Occurrence of de novo ABL kinase domain mutations in primary bone marrow cells after BCR-ABL gene transfer and Imatinib mesylate selection. Leukemia 2005;19:1265–7.

Notes

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