

Clinical Resistance to STI-571 Cancer Therapy Caused by BCR-ABL Gene Mutation or Amplification

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Clinical studies with the Abl tyrosine kinase inhibitor STI-571 in chronic myeloid leukemia demonstrate that many patients with advanced stage disease respond initially but then relapse. Through biochemical and molecular analysis of clinical material, we find that drug resistance is associated with the reactivation of BCR-ABL signal transduction in all cases examined. In six of nine patients, resistance was associated with a single amino acid substitution in a threonine residue of the Abl kinase domain known to form a critical hydrogen bond with the drug. This substitution of threonine with isoleucine was sufficient to confer STI-571 resistance in a reconstitution experiment. In three patients, resistance was associated with progressive *BCR-ABL* gene amplification. These studies provide evidence that genetically complex cancers retain dependence on an initial oncogenic event and suggest a strategy for identifying inhibitors of STI-571 resistance.

Cancers are characterized by multiple oncogenic events that collectively contribute to the phenotype of advanced stage disease. With the advent of new drugs that target specific molecular abnormalities, it is important to know whether the initial oncogenic event continues to play a functional role at later stages of tumor progression and at relapse with the development of chemotherapy resistance. This question has been addressed in transgenic mice through regulated expression of the initial oncogene. In three models testing different oncogenes in different tissues, the primary oncogene was required to maintain the tumor phenotype, despite the presence of numerous additional oncogene and tumor suppressor mutations (1–3). Recent clinical trials of the Abelson tyrosine kinase (Abl) inhibitor STI-571 in chronic myeloid leukemia (CML) allow this question to be addressed directly in human cancer (4, 5).

CML is a pluripotent hematopoietic stem cell disorder characterized by the Philadelphia (Ph) chromosome translocation (6, 7). The resulting *BCR-ABL* fusion gene encodes a cytoplasmic protein with constitutive tyrosine kinase activity (8). Numerous experimental models have established that *BCR-ABL* is an oncogene and is sufficient to produce CML-like disease in mice (9, 10). CML progresses through distinct clinical stages. The earliest stage, termed the

chronic phase, is characterized by the expansion of terminally differentiated neutrophils. Over several years the disease progresses to an acute phase termed blast crisis, characterized by maturation arrest with excessive numbers of undifferentiated myeloid or lymphoid progenitor cells. The *BCR-ABL* oncogene is expressed at all stages, but blast crisis is characterized by multiple additional genetic and molecular changes. STI-571 is a 2-phenylamino pyrimidine that targets the adenosine triphosphate (ATP) binding site of the kinase domain of ABL (11). In phase I clinical trials, STI-571 induced remissions in patients in chronic phase as well as blast crisis (4, 5). Whereas responses in chronic phase have been durable, remissions observed in blast crisis patients have usually lasted only 2 to 6 months, despite continued drug treatment (4).

To characterize the mechanism of relapse in STI-571-treated patients, we first assessed the status of BCR-ABL signaling in primary leukemia cells (12). Our goal was to distinguish between BCR-ABL-dependent and BCR-ABL-independent mechanisms of relapse. If BCR-ABL remains critical for the proliferation of the leukemia clone, then the BCR-ABL signaling pathway should be reactivated. Alternatively, if the expansion of the leukemia clone is independent of BCR-ABL, then signaling through the BCR-ABL pathway should remain impaired by STI-571. The most direct measure of signaling through the BCR-ABL pathway is through the enzymatic activity of BCR-ABL protein itself (8, 13, 14). Although readily measured in cell lines, this assay is difficult to perform in a

reproducible, quantitative fashion with clinical material because BCR-ABL is subject to rapid degradation and dephosphorylation upon cell lysis (15). In a search for alternative measures of BCR-ABL kinase activity, we found that the phosphotyrosine content of Crkl, an adaptor protein that is specifically and constitutively phosphorylated by BCR-ABL in CML cells (16–18), could be measured reproducibly and quantitatively in clinical specimens. Crkl binds BCR-ABL directly and plays a functional role in BCR-ABL transformation by linking the kinase signal to downstream effector pathways (19). When phosphorylated, Crkl migrates with altered mobility in SDS-polyacrylamide gel electrophoresis (PAGE) gels and can be quantified by means of densitometry. As expected, Crkl phosphorylation in primary CML patient cells was inhibited in a dose-dependent manner when exposed to STI-571 and correlated with dephosphorylation of BCR-ABL (Fig. 1A) (20).

To establish the dynamic range of this assay in patient material, we measured Crkl phosphorylation in cells from BCR-ABL-negative individuals ($n = 4$), untreated CML patients ($n = 4$), and patients who responded to STI-571 therapy but whose bone marrow cells remained 100% Ph chromosome-positive ($n = 8$). The mean level of Crkl phosphorylation in cells from CML patients before STI-571 treatment was $73 \pm 13.3\%$ (Fig. 1B). At the time of response the mean was $22 \pm 9.9\%$ (Fig. 1B), similar to the mean level of Crkl phosphorylation in cells from BCR-ABL-negative individuals ($22 \pm 6.0\%$) (21). We next measured levels of Crkl phosphorylation in primary leukemia cells from 11 patients who responded to STI-571 but subsequently relapsed on treatment. In these cases, which included one patient with lymphoid blast crisis, three with Ph+ acute lymphoid leukemia, and seven with myeloid blast crisis, the mean level of Crkl phosphorylation at relapse was $59 \pm 12.5\%$ (Fig. 1C). Antibodies to phosphotyrosine (anti-phosphotyrosine) immunoblot analysis of a subset of these samples confirmed that BCR-ABL was phosphorylated on tyrosine at relapse (Fig. 1C). Longitudinal analysis of blood or bone marrow samples obtained from a subset of these patients before and throughout the course of STI-571 treatment confirmed that Crkl phosphorylation fell during the response to treatment but increased at the time of relapse (Fig. 1D). Therefore, disease progression in patients who initially respond to STI-571 is associated with the failure to maintain effective inhibition of BCR-ABL kinase activity.

A recent preclinical study of STI-571 resistance in mice engrafted with a human blast crisis CML cell line demonstrated that $\alpha 1$ acid glycoprotein, an acute-phase reactant synthesized by the liver, can bind STI-571 in serum and block its activity against BCR-ABL (22). This observation raises the possibility that STI-571 resistance in patients is due to a host-mediated response against the drug. Alternatively, resis-

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tance might be mediated by a cell-autonomous event in a leukemia subclone that allows escape from kinase inhibition by STI-571. To distinguish between these two possibilities, we determined the sensitivity of patient cells taken before treatment and at the time of relapse to STI-571 by measuring inhibition of Crkl phosphorylation (23). If STI-571 resistance is a consequence of a host response, pretreatment and relapse leukemia cells should be equally sensitive to ex vivo STI-571 treatment. However, if STI-571 is cell-intrinsic, leukemia cells obtained at relapse should be less sensitive to STI-571 than pretreatment cells. In those patients for whom we had sufficient matched clinical material, a 10-fold or greater shift in sensitivity to STI-571 was observed at relapse (Fig. 2A). Aggregate analysis of 11 samples confirmed that higher concentrations of STI-571 are required to inhibit Crkl phosphorylation in patients' cells obtained at relapse compared with pretreatment (Fig. 2B).

Because these ex vivo studies provide evidence that STI-571 resistance is cell-intrinsic, we considered several possible mechanisms. Some CML cell lines that develop resistance to

STI-571 after months of in vitro growth in subtherapeutic doses of the drug show amplification of the *BCR-ABL* gene (24–26). We performed dual-color fluorescence in situ hybridization (FISH) experiments to determine if *BCR-ABL* gene amplification could be similarly implicated in STI-571 resistance in human clinical samples (27). Multiple copies of the *BCR-ABL* gene were detected in interphase nuclei in three (two myeloid blast crisis, one lymphoid blast crisis) of the 11 patients who relapsed after initially responding to STI-571 (Fig. 3). Further cytogenetic and FISH characterization of metaphase spreads from these patients showed a unique inverted duplicate Ph chromosome with interstitial amplification of the *BCR-ABL* fusion gene (Fig. 3C). In one patient, the inverted duplicate Ph chromosome could be detected before the initiation of STI-571. In all three cases, additional copies of the aberrant Ph chromosome were observed as STI-571 treatment continued, as well as ring chromosomes harboring multiple copies of the *BCR-ABL*. Patient MB14 was reevaluated by FISH 1 month after receiving alternative treatment for her leukemia. *BCR-ABL* amplification was no longer detectable 4

weeks after discontinuation of STI-571, raising the possibility that persistent STI-571 administration might select for increased copies of the *BCR-ABL* gene in some patients. Quantitative polymerase chain reaction (PCR) analysis of genomic DNA (gDNA) obtained from these three patients confirmed increased *ABL* gene copy number at relapse when compared to a patient without *BCR-ABL* gene amplification (Fig. 3D) (28).

We also considered the possibility that mutations in *BCR-ABL* might confer resistance to STI-571. A 579-base pair (bp) region corresponding to the ATP binding pocket and the activation loop of the kinase domain of *BCR-ABL* was sequenced in the nine patients for whom RNA was available at the time of relapse (Fig. 4A) (29). A single, identical C → T nucleotide (nt) change was detected at *ABL* nt 944 in six of nine cases examined (Fig. 4A). In all six patients a mixture of wild-type and mutant cDNA clones was found, with the frequency of mutant clones ranging from 17 to 70%. The mutation was found in three of three patients with lymphoid disease [two Ph⁺ acute lymphoid leukemia (ALL), one lymphoid blast crisis] and

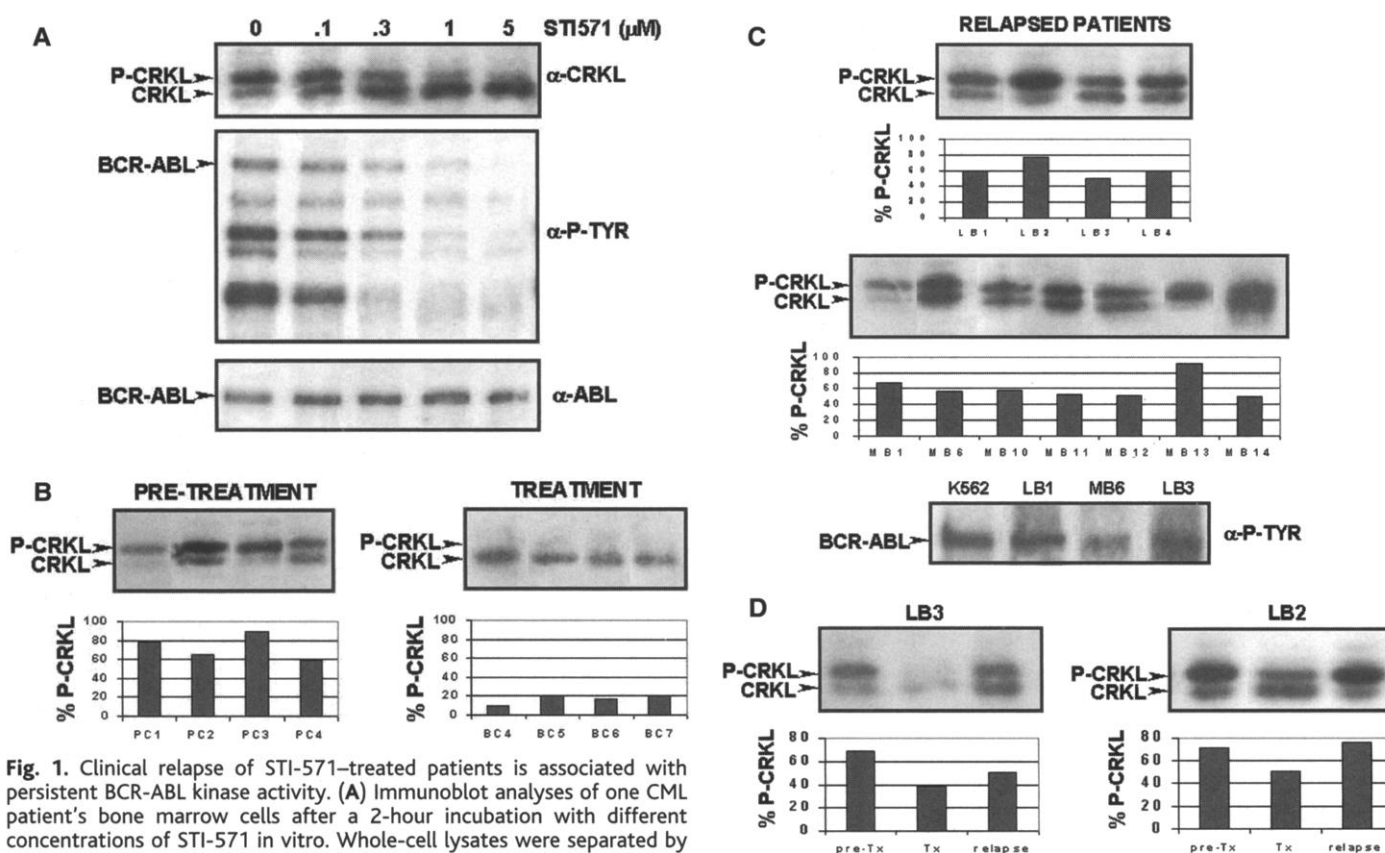
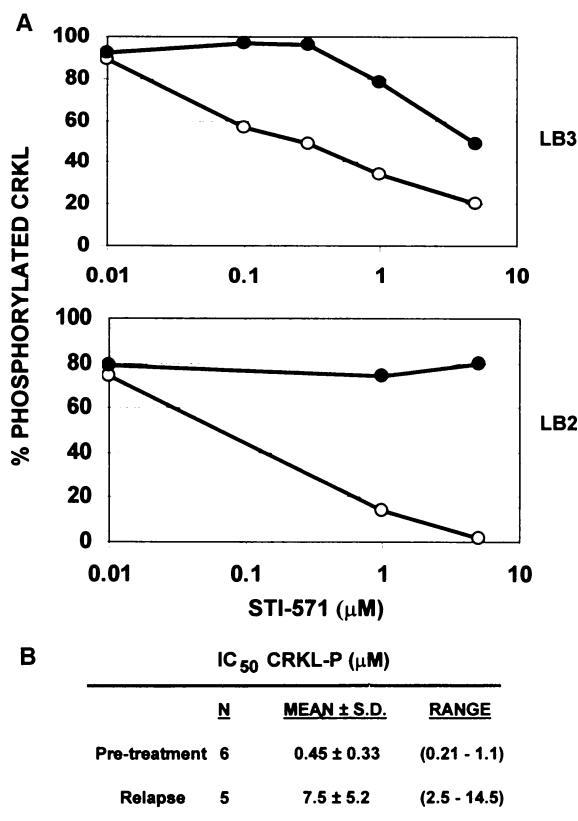


Fig. 1. Clinical relapse of STI-571-treated patients is associated with persistent BCR-ABL kinase activity. (A) Immunoblot analyses of one CML patient's bone marrow cells after a 2-hour incubation with different concentrations of STI-571 in vitro. Whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiserum to Crkl (top), anti-phosphotyrosine (middle), and antibodies to Abl (bottom). (B) Crkl immunoblot of whole cell lysates from CML patients before STI-571 therapy (left) and from Ph-positive blast crisis patients who achieved hematological remission (<5% blasts) on STI-571 but remained 100% BCR-ABL-positive (right). (C) Crkl immunoblots of whole-cell lysates from lymphoid blast crisis or Ph-positive acute lymphoid leukemia patients (top) and myeloid blast crisis patients (middle) who relapsed after initially responding to

STI-571 therapy. Phosphotyrosine immunoblot of patient cell lysates at time of relapse (bottom). A Ph-positive cell line, K562, was used as a positive control for autophosphorylated BCR-ABL. (D) Crkl immunoblots of cell lysates from relapse patients (LB3 and LB2) taken before (pre-Tx) and during the course of (Tx and relapse) STI-571 therapy. Densitometric analyses of Crkl immunoblots (expressed as a percentage of phosphorylated Crkl over total Crkl protein) are presented in bar graphs.

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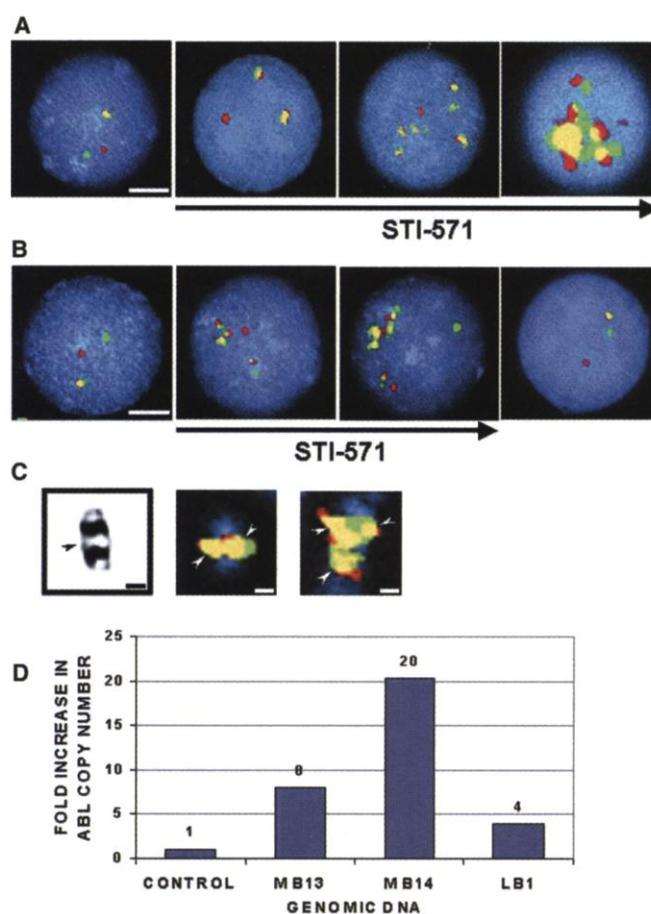
Fig. 2. Altered sensitivity of relapsed patient cells to STI-571. **(A)** STI-571 dose-response curves of Crkl phosphorylation in cells taken from blast crisis patients (LB3 and LB2) before STI-571 therapy (open circles) and at the time of relapse (solid circles). Cells from both time points were exposed to increasing concentrations of STI-571, harvested, and analyzed by Crkl immunoblot and densitometry. **(B)** The median inhibitory concentration (IC_{50}) values for STI-571 inhibition of Crkl phosphorylation determined by exposure of cells isolated from untreated versus relapsed CML patients to increasing concentrations of STI-571 and subsequent Crkl immunoblot and densitometric analyses. Crkl phosphorylation in one relapsed patient sample (LB2) could not be inhibited with high concentrations of STI-571.



in three of six patients with myeloid blast crisis. The presence of the mutation was confirmed by analysis of gDNA (Fig. 4A) (30). Analysis of RNA or gDNA from pretreatment samples failed to provide evidence of the mutation before STI-571 therapy; however, we cannot rule out the possibility that rare cells bearing the mutation exist before treatment.

This single nucleotide C \rightarrow T change results in a threonine to isoleucine substitution at position 315 (Thr³¹⁵ \rightarrow Ile; T315I) of c-Abl. The recently solved crystal structure of the catalytic domain of Abl complexed with a variant of STI-571 identified both the amino acid residues within the ATP binding site and the activation loop of c-Abl that are required for STI-571 binding and, thus, the inhibition of Abl kinase activity (31). Thr³¹⁵ is among those that form critical hydrogen bonds with STI-571. The potential consequence of the T315I substitution on the STI-571 binding pocket was modeled based on the crystal structure of the wild-type Abl kinase domain in complex with STI-571 (Fig. 4B). The absence of the oxygen atom normally provided by the side chain of Thr³¹⁵ would preclude formation of a hydrogen bond with the secondary amino group of STI-571. In addition, isoleucine contains an extra hydrocarbon group in the side chain, which would result in a steric clash with STI-571 and presumably inhibit binding. Notably, the model predicts that the

Fig. 3. BCR-ABL amplification in patients who relapsed after an initial response to STI-571. **(A)** BCR-ABL FISH analyses of interphase nuclei from blast crisis patient M13 before and during STI-571 therapy. Nuclei are visualized with 4',6'-diamidino-2-phenylindole stain (blue), ABL probe is labeled with Spectrum Orange (red signal), and BCR probe is labeled with Spectrum Green (green signal). BCR-ABL gene fusions, indicated by yellow signals, show an increase in BCR-ABL gene amplification during STI-571-resistant disease progression. (Bar, 10 μ m.) **(B)** BCR-ABL FISH analyses of interphase nuclei from blast crisis patient M14 before, during, and after removal from STI-571 therapy showing BCR-ABL-amplified phenotype and the reversion to nonamplified phenotype on removal from STI-571 therapy. (Bar, 10 μ m.) **(C)** Giemsa-stained image (left; bar, 5 μ m) and dual color FISH images (middle and right; bars, 3 μ m) of sample from patient LB1 showing duplicated inverted Ph-chromosome. Arrows indicate BCR-ABL gene fusions. **(D)** Quantitative PCR analysis of gDNA from BCR-ABL-amplified patients (MB13, MB14, and LB1) and one nonamplified patient LB3 (control) confirming increased ABL gene copy number in all three patients.



T315I mutation should not interfere with ATP binding (32).

To confirm that this amino acid substitution interferes with STI-571 activity, we engineered the T315I mutation into wild-type p210 BCR-ABL (33). Cells were transfected with wild-type or T315I p210 BCR-ABL and cultured in the presence of increasing concentrations of STI-571 (34). As shown by Abl immunoblot analysis, the expression of wild-type and T315I mutant BCR-ABL proteins was similar and was not changed by STI-571 (Fig. 4C, bottom panels). On the basis of anti-phosphotyrosine immunoblot analysis, the kinase activities of wild-type BCR-ABL and the T315I mutant appear com-

parable in the absence of STI-571. Whereas wild-type BCR-ABL kinase activity was inhibited by STI-571, the T315I mutant retained high levels of phosphotyrosine at all concentrations of the inhibitor tested (Fig. 4C, top panels).

Our analysis of 11 patients with advanced stage CML who underwent disease progression after an initial response to STI-571 shows that reactivation of BCR-ABL signaling occurred in all patients, despite continued STI-571 treatment. Therefore, the primary explanation for disease progression in these patients appears to be BCR-ABL-dependent proliferation rather than secondary oncogenic signals that permit BCR-ABL-independent growth. It is possible

that studies of a larger number of patients may identify exceptions to this theme, as has been reported in transgenic mice expressing conditional oncogenes where an occasional tumor can escape dependence on the initiating oncogene (1–3). In 8 of the 11 patients we studied, the mechanism of resistance was a consequence of mutation (six patients) or amplification (three patients) of the target oncogene *BCR-ABL* (one patient had both events). These results provide evidence in a genetically complex human cancer that a single molecular target remains relevant in late stage, relapsed disease.

The identity of the Abl kinase domain mutation found in these patients bears remarkable

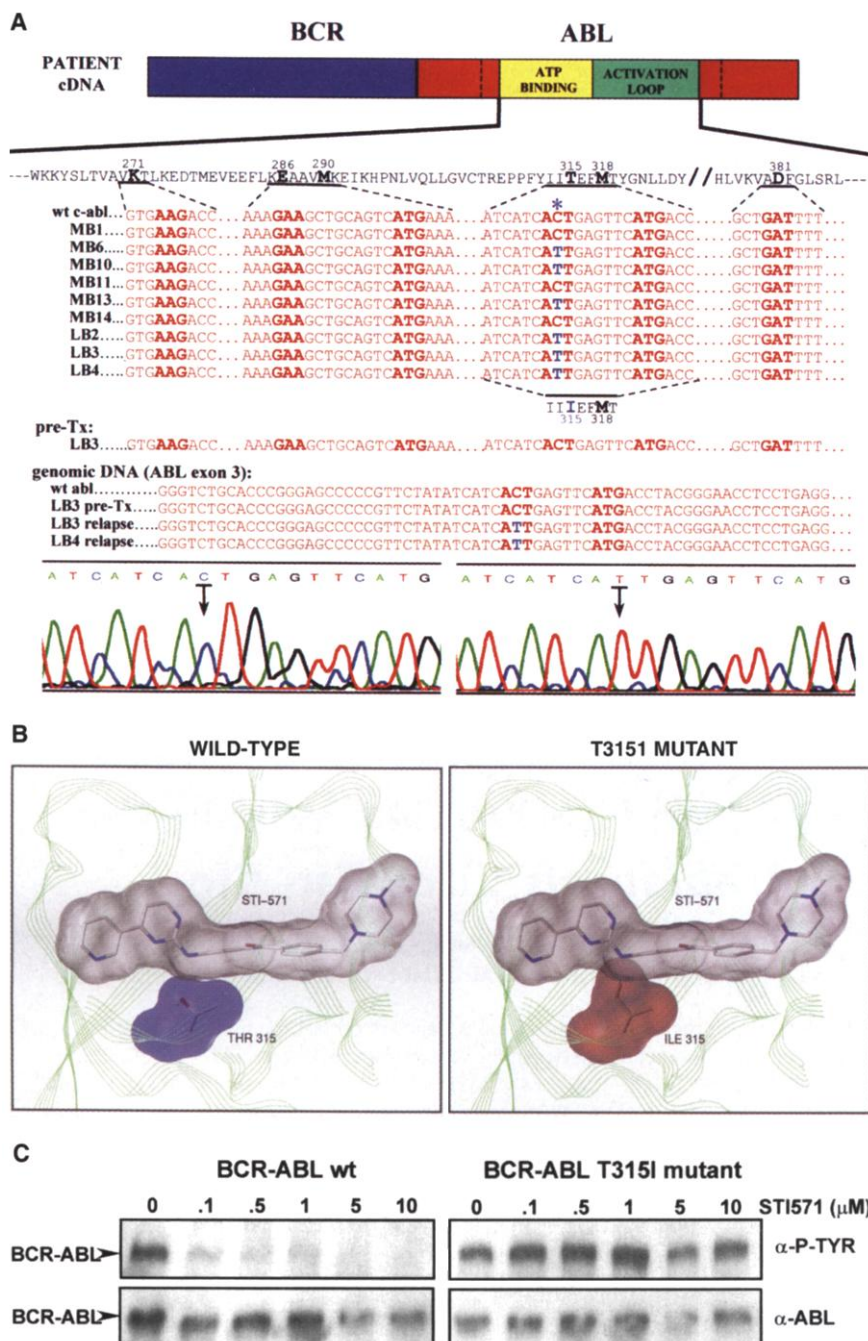


Fig. 4. Point mutation in the ATP binding pocket of the Abl kinase domain confers STI-571 resistance in relapsed patients. **(A)** Schematic of PCR strategy to determine the sequence of a 579-bp region of BCR-ABL that corresponds to the ATP binding pocket and activation loop of the kinase domain in patient samples. Amino acid sequence of the region of Abl analyzed is shown in black (40). Residues predicted to form hydrogen bonds with STI-571, on the basis of crystal structure data, are in boldface and are numbered from the first amino acid of c-Abl (GenBank accession number M14752). Corresponding nucleotide sequence (shown in red) was aligned with sequences obtained from nine patient cDNAs. The C → T mutation at ABL nt 944 (detected in six patients at relapse and in no pretreatment samples) is shown in blue. The sequence of wild-type ABL exon 3 (GenBank accession number NT008338.2) was aligned with sequences obtained from patient gDNA before treatment and at relapse. Examples of primary sequence data (represented as chromatographs) from wild-type BCR-ABL (left) and BCR-ABL with the C → T point mutation (right). **(B)** Model of STI-571-binding pocket of wild-type Abl in complex with STI-571 (left) and predicted structure of STI-571-binding pocket of T315I mutant Abl in complex with STI-571 (right). In the molecular structures representing STI-571 and Abl residue 315, nitrogen atoms are shown in blue and oxygen atoms are shown in red. Van der Waals interactions are depicted in gray for STI-571 (both panels), in blue for wild-type Abl residue Thr³¹⁵ (left), and in red for mutant Abl residue Ile³¹⁵ (right). The polypeptide backbone of the Abl kinase domain is represented in green. **(C)** Immunoblots of whole-cell lysates isolated from transfected 293T cells (wild-type p210 BCR-ABL shown in left panels and T315I mutant shown in right panels) after a 2-hour incubation with different concentrations of STI-571. Blots were probed with phosphotyrosine (top) and antibodies to Abl (bottom).

similarity to a Thr → Ile change in v-Src versus c-Src at position 338, which corresponds to Thr³¹⁵ in c-Abl. Despite the fact that v-Src and c-Src have almost identical kinase domain sequences (98% identity), v-Src is about 50-fold more resistant than c-Src to kinase inhibition by the Src inhibitor PP1 (35).

Although the development of STI-571 resistance presents new therapeutic challenges, the fact that BCR-ABL remains active in STI-571-resistant cells suggests that the chimeric oncoprotein remains a rational drug target. Because several patients examined to date share an identical mutation associated with drug resistance, it may be possible to identify an inhibitor of the mutant BCR-ABL allele that would have broad utility. In addition, knowledge of this mutation should permit the development of assays to detect drug-resistant clones before clinical relapse. It should be noted that this study has focused on a small, selected group of patients. Analyses of larger sample sizes are required to determine the true frequency of this mutation among resistant patients.

References and Notes

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12. Peripheral blood and/or bone marrow samples were obtained with appropriate informed consent from CML and Ph-positive ALL patients at the University of California Los Angeles who were enrolled in multicenter clinical trials of STI-571 (sponsored by Novartis Pharmaceuticals, Basel, Switzerland). All patients had >30% blasts in the marrow before treatment. Responding patients had reduction in the percentage of bone marrow blasts to <15% (partial) or <5% (complete), as described (5). Progressive disease was defined as an increase in percentage of blasts after an initial response, despite continued STI-571 treatment. Mononuclear cells were isolated by centrifugation through Ficoll-Hypaque, washed twice in phosphate-buffered saline, counted, and used immediately or cryopreserved.
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20. Cells were lysed in 1% Triton X-100 buffer with protease and phosphatase inhibitors (36). Equal amounts of protein, as determined by the BioRad DC protein assay (Bio-Rad, Hercules, CA), were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with phosphotyrosine antibody (4G10, Upstate Biotechnologies, Lake Placid, NY), Abl antibody [pex5, (36)], β -actin antibody (Sigma, St. Louis, MO), or Crkl antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Several exposures were obtained to ensure linear range of signal intensity. Optimal exposures were quantified by densitometry with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
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27. Interphase and metaphase cells were prepared (37) and examined with Locus Specific Identifier BCR-ABL dual color translocation probe (Vysis, Downers Grove, IL).
28. gDNA was extracted from purified bone marrow or peripheral blood cells with the QiaAmp Blood Mini Kit (Qiagen, Valencia, CA). We subjected 10 ng of total gDNA to real-time PCR analysis with the iCycler iQ system (Bio-Rad, Hercules, CA). A 361-bp gDNA fragment including ABL exon 3 was amplified with two primers (5'-CAGAGTCAGATCCTTCAG-3' and 5'-TTTGTAAAGGCTGCCCGGC-3'), which are specific for intron sequences 5' and 3' of ABL exon 3, respectively. A 472-bp gDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with two primers (5'-TTCCACCACATGGAGAAGGC-3' and 5'-CAGGAATGAGCTTGACAAA-3'), which are specific for sequences in exon 5 and exon 8 of GAPDH, respectively. Fold increase in ABL copy number was determined by calculating the difference between threshold cycle numbers of ABL and GAPDH for each sample (Δ Ct). With control LB3 as reference sample, Δ Ct from each sample was subtracted from Δ Ct of control to determine $\Delta(\Delta$ Ct). Fold increase was calculated as $2^{-\Delta(\Delta$ Ct)}.
29. RNA was extracted from purified peripheral blood or bone marrow cells with Triagent-LS (Molecular Research Center, Cincinnati, OH). Two milligrams of total RNA was subjected to reverse transcriptase (RT)-PCR with Oligo dT primers. A 1327-bp cDNA fragment was amplified by PCR with a 5' BCR-specific primer (5'-GAAGCTTCTCCTGGCATCCGT-3') and a 3' ABL-specific primer (5'-CCAGGCTCTCGGTG-CAGTCC-3'). In two patients, the BCR-ABL fragment could not be amplified; therefore, a 579-bp fragment was amplified with an alternative 5' ABL-specific primer (5'-GCGCAACAAGCCCACTGTCTATGG-3') and the same 3' ABL primer. PCR products were cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA). Both strands of a 579-bp region were sequenced with the 5' ABL primer and M13 forward primer or M13 forward and reverse primer set for the 1327-bp and the 579-bp fragments, respectively, on an ABI prism 377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA). Sequence analysis was performed with the ClustalW alignment algorithm (38).
30. gDNA was extracted from purified bone marrow or peripheral blood cells with the QiaAmp Blood Mini Kit (Qiagen). A 361-bp DNA fragment was amplified by PCR with two primers (5'-GCAGAGTCAGATCCTTCAG-3' and 5'-TTTGTAAAGGCTGCCCGGC-3'), which are specific for intron sequences 5' and 3' of ABL exon 3, respectively. PCR products were cloned and sequenced.
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32. The structure of the kinase domain of Hck in complex with an ATP analog (AMP-PNP) was superimposed onto the model of the Ile315 Abl kinase domain.
33. Full-length p210 BCR-ABL was subcloned into the pSRaMSVtkNeo retrovirus vector (39). A fragment containing the C → T mutation at ABL nt 944 was made by PCR and swapped with the corresponding sequence in pSRaMSVtkNeo p210 BCR-ABL wild-type to create the pSRaMSVtkNeo p210 BCR-ABL T315I mutant. The resulting construct was confirmed by sequencing.
34. Transient transfection of 293T cells was performed with CaCl₂ (39). After a 24-hour transfection, cells were incubated with varying concentrations of STI-571 (Novartis) for 2 hours. Proteins were extracted and subjected to immunoblot analysis.
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40. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
41. We thank J. Kuriyan and B. Nagar for providing structural modeling data; E. Buchdunger for providing STI-571; K. Shannon, K. Shuai, and O. Witte for helpful comments; K. Banks for technical assistance; and L. Rose for manuscript preparation. Supported by grants from the Leukemia and Lymphoma Society, the National Cancer Institute (C.L.S.) and a United States Public Health Service National Research Service Award GM07185 (M.E.G.).

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Reciprocal Regulation Between TOC1 and LHY/CCA1 Within the Arabidopsis Circadian Clock

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The interactive regulation between clock genes is central for oscillator function. Here, we show interactions between the *Arabidopsis* clock genes *LATE ELONGATED HYPOCOTYL* (*LHY*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and *TIMING OF CAB EXPRESSION 1* (*TOC1*). The MYB transcription factors *LHY* and *CCA1* negatively regulate *TOC1* expression. We show that both proteins bind to a region in the *TOC1* promoter that is critical for its clock regulation. Conversely, *TOC1* appears to participate in the positive regulation of *LHY* and *CCA1* expression. Our results indicate that these interactions form a loop critical for clock function in *Arabidopsis*.

Circadian clocks represent a widespread endogenous mechanism that allows organisms to time different processes appropriately throughout the day-night cycle. Among the

activities controlled by the circadian clock are the regulation of transcription in cyanobacteria, the rhythmic movement of leaves in plants, and more complex activities such as