

## Mutation in the ATP-binding Pocket of the ABL Kinase Domain in an STI571-resistant BCR/ABL-positive Cell Line<sup>1</sup>

Clara Ricci,<sup>2</sup> Barbara Scappini,<sup>2</sup> Vladimir Divoky, Simona Gatto, Francesco Onida, Srdan Verstovsek, Hagop M. Kantarjian, and Miloslav Beran<sup>3</sup>

Department of Leukemia, Division of Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [C. R., B. S., S. G., F. O., S. V., H. M. K., M. B.], and Departments of Biology and Hemato-Oncology, Palacky University, 77200 Olomouc, Czech Republic [V. D.]

### Abstract

The major mechanism of action of STI571 is a competitive interference with the ATP-binding site of the Bcr/Abl tyrosine kinase. In the BCR/ABL-positive cell line KBM5, we studied cellular events associated with the *in vitro* acquisition of resistance to STI571. The emergence of the STI571-resistant phenotype was accompanied by only a marginal increase in the number of copies of the BCR/ABL gene and its level of expression. The activity of the Bcr/Abl kinase (level of autophosphorylation) in resistant cells was, however, incompletely inhibited by STI571, and the acquisition of the high degree of resistance was associated with a single-point mutation leading to a substitution of a threonine-to-isoleucine at position 315 of Abl. In the resistant KBM5-STI571<sup>R1.0</sup> cells, 20% of the BCR/ABL transcripts and 10% of BCR/ABL gene copies on the DNA level were mutated. The mutation was present in all 10 STI571-resistant clones derived from low density clonogenic assay, confirming its presence in all colony-forming cells but only in a fraction of the BCR/ABL gene copies in each cell. The contribution of this mutation to STI571-resistant phenotype remains unknown. Preliminary data showing partial reversibility of resistance in these cells suggest that resistance may be multifactorial. No other mutations were identified in the kinase domain of the BCR/ABL gene.

### Introduction

The lack of response to STI571 or the recurrence of disease after a transient response in patients with variously advanced stages of BCR/ABL-positive CML<sup>4</sup> indicates an STI571-resistant phenotype that develops either by the selection of preexisting resistant clones or the *de novo* induction of resistance. *In vitro* studies in STI571-resistant BCR/ABL-positive cell lines demonstrated an association between resistance to STI571 and quantitative changes in the Bcr/Abl protein expression (1–3). To date, no qualitative changes such as BCR/ABL gene rearrangements or mutations in the kinase domain have been identified in cell lines with STI571-induced resistance *in vitro* (1–3). Recently, however, point mutations within the DNA sequences coding for the ATP-binding pocket of the BCR/ABL gene were identified in cells from some patients with CML who had STI571-refractory disease or who had a relapse during the treatment (4–10). A point mutation resulting in a threonine-to-isoleucine change at amino acid position 315 (T315I) has been described in detail (4). This mutation,

when engineered into wild-type p210 Bcr/Abl and transiently transfected into 293T cells or to Ba/F3 cells, interfered with the inhibition of Bcr/Abl kinase activity in cells exposed to STI571 (4, 7). However, the extent to which this mutation contributes to STI571 resistance *in vivo* remains unknown. An *in vitro* model that allows study of the role of this mutation in human cells has not been available, and neither this nor any other mutation has been reported in any BCR/ABL-positive cell lines.

In this study, we describe the occurrence of the T315I mutation in a proportion of copies of the BCR/ABL gene within cells in a cell line rendered resistant to STI571 by *in vitro* exposure to the drug. The STI571-resistant cells represent a model for additional investigation of the role of this mutation in the development of STI571-resistant CML phenotype. These cells may be also useful in development of methods to overcome STI571 resistance in experimental systems.

### Materials and Methods

**Cell Lines and STI571 Treatment.** KBM5 cells were derived from a patient with myeloid blastic phase of CML; the cells contain multiple copies of the Philadelphia chromosome (11) while lacking the normal ABL gene (12). To select for the resistant phenotype, the KBM5 cells were exposed to increasing concentrations of STI571 (Novartis, Basel, Switzerland), starting with 0.05  $\mu\text{M}$  and increasing by 0.1  $\mu\text{M}$  when the cells resumed a near normal growth kinetics. Parental cells were maintained concomitantly without the drug. After 4 months, the treated cells, designated KBM5-STI571<sup>R1.0</sup>, were able to grow in the presence of 1.0  $\mu\text{M}$  STI571 and were maintained at this concentration. The reported studies were performed after the cells had grown in the presence of this drug concentration for 6–8 months. During this period, no changes in the sensitivity of parental cells to STI571 were observed.

**Isolation of Clones and Clonally Derived Cell Lines by Cloning in Methylcellulose.** To obtain single cell-derived clonal sublines, leukemic cells were cultured at low cell density using semisolid medium in CFU colony assay. The resistant KBM5-STI571<sup>R1.0</sup> cells were plated at low density ( $0.5 \times 10^3$  cell/ml) in Iscove's methylcellulose medium (Methocult H4230; Stem Cell Technologies, Vancouver, Canada) containing 1  $\mu\text{M}$  concentration of STI571. After 8 days of culture, well-separated individual colonies were removed under sterile conditions and either analyzed directly or transferred to liquid cultures and expanded. Five single cell-derived clones, expanded in the presence, and five expanded in the absence of 1.0  $\mu\text{M}$  STI571 were used for additional studies of the cellular changes associated with the resistant phenotype, including the presence of BCR/ABL mutations.

**Stability of Resistance.** The KBM5-STI571<sup>R1.0</sup>-resistant cell line and two clonally derived cell lines were grown for 25 passages either in the presence of STI571 (1  $\mu\text{M}$ ) or in the absence of the drug. The degree of resistance was then estimated by the MTT assay.

**Cell Proliferation Assay and the Measurement of STI571 Resistance.** The cell proliferation was measured using the MTT (Sigma Chemical, St. Louis, MO) colorimetric reduction method. The exponentially growing cells were washed, plated in triplicate in 96-well plates at a cell density of  $4 \times 10^4$ /100  $\mu\text{l}$ , and STI571 was added at various concentrations. After 72 h of exposure, the level at which proliferation was inhibited was measured as a percentage of control growth (no drug in the sample). The survival curves were constructed, and the drug concentration resulting in IC<sub>50</sub> was determined. The

Received 4/5/02; accepted 9/13/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work has been supported by a grant from the Ladies Leukemia League (to F. O.), the Salners Family Foundation Fund for Leukemia Research (to M. B.), and Ministry of Health, Czech Republic Grant No. NC6652-3.

<sup>2</sup> Both authors contributed equally to this work.

<sup>3</sup> To whom requests for reprints should be addressed, at Department of Leukemia, Box 428, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-2248; Fax: (713) 794-4297; E-mail: mberan@mdanderson.org.

<sup>4</sup> The abbreviations used are: CML, chronic myelogenous leukemia; CFU, colony-forming unit; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RI, resistance index; gDNA, genomic DNA; RT PCR, reverse transcription PCR.

resistance index (RI) was calculated by dividing  $IC_{50}$  for resistant cells by  $IC_{50}$  for the parental, STI571-sensitive cells.

**Western Blot Analysis.** Cells were incubated in the presence or absence of graded concentrations of STI571. After 2 h, cells were washed twice with cold PBS containing protease inhibitors [protease inhibitor mixture (Complete, Mini Roche Molecular Biochemical, Indianapolis, IN), 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin], and phosphatase inhibitors (AEBSF 1 mM, sodium fluoride 10 mM, ortovanadate 1 mM), and  $1 \times 10^7$  cells were lysed in 1 ml of lysis buffer [0.125 M Tris-HCL (pH 6.8), 1% SDS, 0.01% bromphenol blue, 5% glycerol, 2% 2-mercaptoethanol, protease and phosphatase inhibitors]. Cell lysates corresponding to  $5 \times 10^5$  were boiled for 10 min, resolved on 7.5% SDS-PAGE gels, and transferred onto a polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). This procedure proved optimal for the detection of Bcr/Abl and minimized its degradation which, although absent in the control K562 cell line, was consistently observed in KBM5 parental and resistant cells. Anti-c-Abl (PharMingen, San Diego, CA) and antiphosphotyrosine (4G10 monoclonal; Upstate Biotechnology, Lake Placid, NY) were used for Western blot analyses. After overnight incubation at 4°C with primary antibody, the membranes were washed and incubated with secondary antibody (antimouse, Bio-Rad) horseradish peroxidase-conjugated antibodies at room temperature for 1 h. The membranes were washed, and bound antibodies were detected with enhanced luminol and oxidizing reagent by chemiluminescence as specified by manufacturer (Amersham, Arlington Heights, IL). After stripping the membranes in the stripping buffer [0.5 mM Tris-HCL (pH 6.7), 2% SDS, 100 mM 2-mercaptoethanol] for 30 min at 56°C, they were reprobed with anti  $\beta$ -actin antibodies (Sigma Chemical) to assess the comparability of the protein loading. Intensity of Bcr/Abl bands was evaluated using BioMax 1D software and compared with intensity of the corresponding  $\beta$ -actin bands on the same membranes.

**Fluorescence *in Situ* Hybridization Analysis.** Interphase nuclei of parental and STI571-resistant cells were hybridized with fluorescently labeled ABL and BCR probes (Vysis, Downers Grove, IL) according to recommendation of the manufacturer. Two hundred interphase nuclei were analyzed, and the number of fusion signals/cell was enumerated.

**Sequence Coding the Abl-Kinase Domain.** Two  $\mu$ g of total RNA were converted to cDNA using SuperScriptII reverse transcriptase (Life Technologies, Inc., Carlsbad, CA) and oligo(dT) primers. A seminested PCR for BCR/ABL fusion transcript was carried out using a mixture of TaqGold polymerase (Perkin-Elmer, Foster, CA) with Pfu polymerase (Stratagene, La Jolla, CA) and the following primers and conditions: forward primer B2A (5'-tcagaagcttcctcctgacat-3', exon 13 of the BCR gene) and the reverse primer NTPE- (5'-CTTCGTCTGAGATACTGGATTCCT-3', exon 9 of the ABL gene) for the first PCR round [40 cycles, annealing temperature ( $T_{ann}$ ) = 65°C]; and forward primer NTPB<sub>+</sub> (5'-aagcgcaacaagccactgtctat-3', exon 4 of the ABL gene) and NTPE- as a reverse primer for the second PCR round (28 cycles,  $T_{ann}$  = 63°C). The first round of PCR generated a 1615-bp product, which was diluted 1:10, and 1  $\mu$ l was then subjected to the second round of amplification. An 862-bp product was obtained, which included a coding sequence for the whole ATP-binding pocket and activation loop of the Bcr/Abl kinase domain.

The 862-bp PCR products from both the parental and resistant KBM5 cells were used for direct sequencing. In addition, the amplified fragments were subcloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA). Twenty KBM5 parental and 35 KBM5STI<sup>R1.0</sup> plasmid clones were then screened by PCR and endonuclease digestion of the PCR product. A 177-bp region of the Abl kinase domain was amplified (ABL-F1, 5'-gtgcccgtgaagaccttgaag-3', exon 4; ABL-R1, 5'-GTAGTCCAGGAGGTTCCCGT-3', exon 7; 32 cycles,  $T_{ann}$  62°C) and digested with the restriction endonuclease DdeI (New England Biolabs, Beverly, MA). The presence of the C-T mutation abolishes the recognition site for this enzyme. Finally, selected plasmid DNA segments were sequenced in forward and reverse directions using the ABI Prism 377 automatic sequencer. To verify the mutation in clonally derived sublines of KBM5-STI<sup>R1.0</sup>, 10 sublines were evaluated by direct analysis of the 177-bp RT-PCR product after digestion with DdeI endonuclease.

Similar strategies were used to analyze gDNA extracted from 10 clonal-resistant sublines using DNAzol (Molecular Research Center, Cincinnati, OH). Total gDNA (600 ng) was subjected to PCR analysis using primers and conditions as described previously (4). A 344-bp gDNA fragment, including the whole exon 3 of the ABL, was amplified (primers ABL-F2, 5'-GCA-

GAGTCAGAATCCTTCAG-3' and ABL-R2 5'-TTTGTAAGGCTGCCCGGC-3') and sequenced in forward and reverse directions. In addition, a 169-bp gDNA PCR product (primers ABL F2 and ABL R1) obtained from gDNA of two resistant KBM5-STI571<sup>R1.0</sup>-derived clonal sublines was subcloned into pZErO-2. Thirty plasmid clones each were screened for the presence of C-T (ABL nucleotide 944) mutation by PCR amplification followed by DdeI digestion.

A DNA fragment containing T315I mutation, derived from a patient with blastic phase CML in Dr. Sawyer's laboratory (and obtained from Dr. Arlinghaus) was amplified and used as a positive control throughout the studies.

## Results

### Generation and Characterization of STI571-resistant Cell Line.

After 4 months of exposure of KBM5 cells to increasing concentrations of STI571, the cells were able to grow in the presence of 1  $\mu$ M concentration of the drug (KBM5-STI571<sup>R1.0</sup>). There were no apparent differences in the growth kinetics of the parental cells growing in the absence and the resistant cells growing in the presence of 1  $\mu$ M STI571.

The  $IC_{50}$  of parental and resistant cell lines was 0.55 and 15.5  $\mu$ M, respectively, as determined by the MTT assay. The calculated RI was 28.2. The  $IC_{50}$  of the 10 single CFU-derived, clonal sublines, assayed by the same methodology, varied between 7.0 and 30.3  $\mu$ M and the corresponding RIs varied between 12.7 and 30.3.

The growth of KBM5-STI571<sup>R1.0</sup>-resistant cell line and of the two resistant clonal derivatives was comparable in the presence and absence of 1  $\mu$ M STI571. Removal of the drug from the growth media for 25 passages resulted in a partial reversal of the resistance in all of the lines studied (Fig. 1).

The fluorescence *in situ* hybridization analysis of KBM5 and KBM5-STI571<sup>R1.0</sup> cells documented only a marginal increase in the number of BCR/ABL fusion signals. The proportion of parental cells containing 2, 3, and 4–8 fusion signals was 5, 20, and 75%; the corresponding numbers in KBM5-STI571<sup>R1.0</sup> cells were 3, 11, and 86%.

**Expression and Phosphorylation of the p210<sup>Bcr/Abl</sup> Protein.** The acquisition of resistance to STI571 was characterized by a slight overexpression of Bcr/Abl as assessed by immunoblotting (Fig. 2). Using densitometry and correction against  $\beta$ -actin expression, the expression of Bcr/Abl in KBM5-STI571<sup>R1.0</sup> was found to be increased 3.8-fold (Fig. 2).

**Inhibition of p210<sup>Bcr/Abl</sup> Phosphorylation by STI571.** Exponentially growing sensitive and resistant cells were washed and incubated for 2 h in 1, 5, and 10  $\mu$ M concentrations of STI571. The Western blot analysis with antiphosphotyrosine antibody demonstrated a dose-

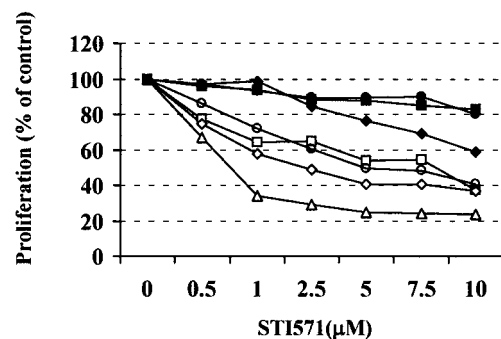


Fig. 1. The degree and stability of STI571 resistance. KBM5-STI571<sup>R1.0</sup> cells (●, ○) and two clonal derivatives, KBM5-STI571<sup>R1.0</sup> C1 (■, □) and KBM5-STI571<sup>R1.0</sup> C6 (◆, ◇) were grown in the presence (closed symbols) or absence (open symbols) of 1.0  $\mu$ M STI571 for 25 passages. The level of resistance was assessed by MTT assay. The sensitivity of the parental KBM5 cells (△) is shown for comparison. The mutational status of clonal cell lines C1 and C6 at the time of the removal from the STI571-containing media is shown in Fig. 3 on cDNA level (D) and on gDNA level (E).

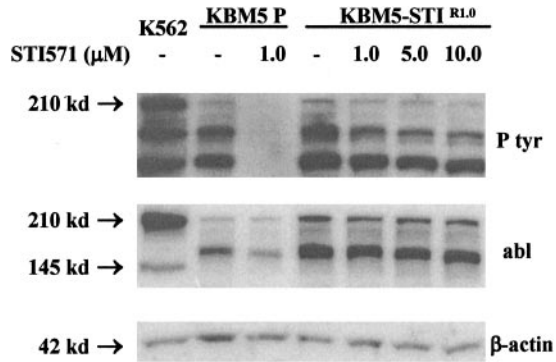


Fig. 2. Bcr/Abl expression and phosphorylation in KBM5 and KBM5-STI571<sup>R1.0</sup> cell lines. Western blots were probed with antiphosphotyrosine (*P-tyr*), anti-abl (*abl*), and  $\beta$ -actin antibodies. The top panel shows the level of Bcr/Abl phosphorylation in parental KBM5 P cell line and its STI571-resistant derivative and the inhibition of Bcr/Abl phosphorylation by STI571. KBM5 and KBM5-STI571<sup>R1.0</sup> cells were incubated with graded concentrations of STI571 for 2 h, and the extent of p210 phosphorylation was visualized with antiphosphotyrosine antibodies on immunoblots. The middle panel shows the level of expression of Bcr/Abl (210 kDa) after probing of the stripped membranes with anti-Abl antibodies. Note the absence of Abl expression (145-kDa band) in both KBM5 P and KBM5-STI571<sup>R1.0</sup> cell lines. The K562 leukemic cell line, expressing both Bcr/Abl and Abl, are shown as control (Lane 1). Expression of  $\beta$ -actin was used for loading control and quantitation of the Bcr/Abl levels.

dependent inhibition of Bcr/Abl phosphorylation in both sensitive and resistant cells. Although the autophosphorylation in KBM5 cells was completely abolished by 1.0  $\mu$ M STI571, concentrations up to 10.0  $\mu$ M inhibited phosphorylation of the kinase in KBM5-STI571<sup>R1.0</sup> cells only partially (Fig. 2).

**Mutational Analysis.** To assess the possibility that *BCR/ABL* mutation confers resistance to STI571, we performed molecular analysis of the KBM5-STI<sup>R1.0</sup> cells growing in the presence of 1.0  $\mu$ M STI571. In addition, we extended such analyses on single cell (CFU)-derived clonal sublines, using both cDNA and gDNA.

The analysis of cDNA with direct sequencing of the 862-bp PCR products derived from both the parental and resistant KBM5 cell lines suggested the presence of a C-T point mutation at *ABL* nucleotide 944, leading to T315I substitution of Abl either in a portion of KBM5-STI<sup>R1.0</sup> cells or in some of the multiple copies of the *BCR/ABL* gene (Fig. 3B). The same mutation was suggested by *DdeI* restriction analysis of the 177-bp PCR product; the mutation abolishes the recognition site for this enzyme (Fig. 3A). No changes in the cDNA sequence were identified in the parental cells. These findings provided the impetus for additional studies, which involved subcloning of the 862-bp PCR product, restriction digests, and sequencing of selected plasmid clones. Restriction analysis of the PCR-derived plasmid clones revealed that 0 of 20 parental KBM5 cells and 7 of 35 (20%) KBM5-STI<sup>R1.0</sup> cDNA clones were positive for the mutation (Fig. 3C). The presence of the mutation was also confirmed by sequencing of the plasmid DNA.

The proportion of mutant *BCR/ABL* transcripts based on the PCR cloning experiments was 20%. To determine whether the mutation affects only a portion of the KBM5-STI<sup>R1.0</sup> cells, 10 CFU-derived sublines were tested for the presence of the mutation using *DdeI* analysis. All these clonally derived sublines displayed a degree of STI571 resistance comparable with that of the KBM5-STI<sup>R1.0</sup> cell line from which they were derived. The 177-bp RT-PCR *BCR/ABL* products from these subclones were partially digested, documenting a mixture of wild-type and mutant cDNA transcripts in all 10 clones studied. (Fig. 3D). This finding suggested the presence of T315I mutation in all KBM5-STI<sup>R1.0</sup> cells and provided evidence that the mutation affected only a portion of the Bcr/Abl molecules in every resistant cell.

Using gDNA as a template, a 344-bp PCR product consisting of the whole exon 3 of the *ABL* was obtained from 10 clonal derivatives of KBM5-STI571<sup>R1.0</sup> cell line. Direct sequencing in both forward and reverse directions showed presence of both wild-type and mutated sequences in the STI571-resistant cell line and all 10 resistant clones.

*DdeI* digestion of 169-bp gDNA PCR product obtained from KBM5-STI571<sup>R1.0</sup>-derived clones verified the presence of both wild-type and mutant *BCR/ABL* gene copies in all 10 clones (Fig. 3E). To determine proportion of wild-type and mutated gDNA copies, the 169-bp PCR products obtained from gDNA of two CFU-derived KBM5-STI571<sup>R1.0</sup> clonal sublines (nos. 4 and 10) were subcloned and analyzed with *DdeI* restrictase. Restriction analysis of 30 plasmid clones each showed 15 and 6% frequency of mutant amplicon copies derived from clones 4 and 10, respectively.

## Discussion

The cellular and molecular mechanisms involved in the development of STI571-resistance *in vitro* and *in vivo* involves both quantitative and qualitative changes of the Bcr/Abl tyrosine kinase. Here, we report development of STI571 resistance in p210<sup>Bcr/Abl</sup>-positive myeloid cell line KBM5-STI571<sup>R1.0</sup> growing in the continuous presence of STI571; the acquisition of the resistant phenotype was characterized by only a marginal increase in the number of *BCR/ABL* gene copies and the level of expression of Bcr/Abl protein. The resistant cells display, however, a Bcr/Abl tyrosine kinase that is resistant to

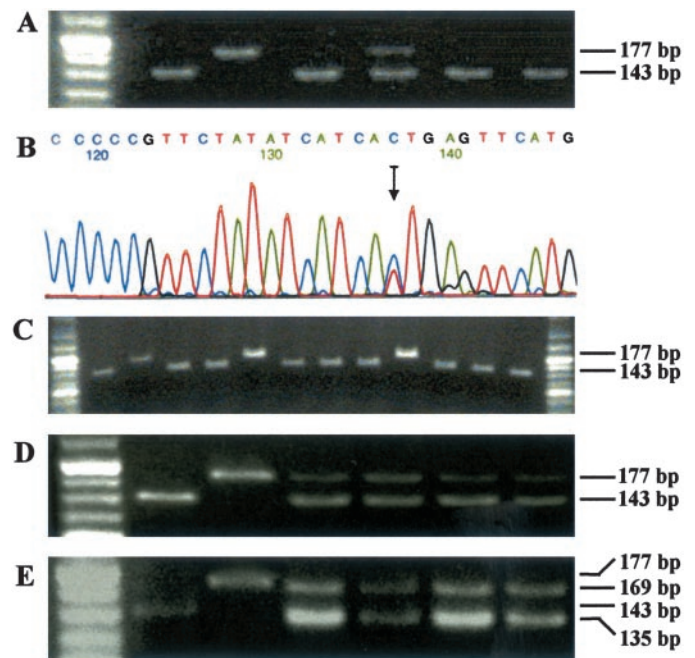


Fig. 3. Detection of the point mutation. A, incomplete digest of the 177-bp PCR product. The wild-type *ABL* sequence (C/TGAG) is digested by the *DdeI* restrictase, and two fragments of 143-bp and 34-bp can be visualized on a 3% agarose gel (the 34-bp fragment is out of the gel area shown). The mutation abolishes the recognition site (T/TGAG) leaving the 177-bp product uncut. Lanes 1, 5, and 6, negative controls; Lane 2, positive control for the mutation; Lane 3, KBM5; Lane 4, KBM5-STI<sup>R1.0</sup>. B, direct sequencing of the KBM5-STI571<sup>R1.0</sup>-derived, amplified cDNA fragment containing the ATP-binding pocket of Abl kinase. A minor peak (arrow) representing C-T substitution first suggested a presence of the T315I mutation along with the wild-type product. C, *DdeI* restriction analysis of plasmids, containing cDNA-derived PCR product (for details see "Materials and Methods"). Lane 1, negative control; Lane 2, positive control for the mutation; Lanes 5 and 9 represent undigested (mutated) and lanes 3, 4, 6, 7, 8, and 10–12 wild-type transcripts derived from the KBM5-STI<sup>R1.0</sup>-resistant cell line. D, incomplete digestion of the PCR product amplified from the cDNA of 4 of 10 CFU-derived clones. Lane 1, negative control; Lane 2, positive control for the mutation; Lanes 3–7, clones 1, 4, 6, and 10. E, incomplete digestion of PCR product amplified from the genomic DNA of the same clones as in D. Lane 1, negative control; Lane 2, positive control for the mutation; Lanes 3–6, clones 1, 4, 6, and 10 derived from KBM5-STI<sup>R1.0</sup>.

STI571 inhibition. The uniqueness of the resistant cells lies in the presence of a specific mutation within the ATP-binding domain of Bcr/Abl. Whereas such mutation has recently been found by Gore *et al.* (4) in patients with a STI571-resistant blastic phase of CML, this is the first report of mutation in the ATP-binding pocket of the Abl kinase domain, which has been found in *BCR/ABL*-positive cell line rendered resistant to STI571 exposure *in vitro*. When studied in patients, the mutation was detected in 17–80% of RT-PCR-derived *BCR/ABL* plasmid clones (4). The presence of both mutated and wild-type *BCR/ABL* transcripts was postulated to reflect mutant and wild-type *BCR/ABL* patients' cells (4, 7). Possible different proportions of mutant and wild-type *BCR/ABL* DNA copies within individual cells have not been evaluated.

Our study revealed a mutation only in KBM5-STI571<sup>R1.0</sup> cells and not in parental KBM5, suggesting that the mutation arose during the drug exposure. These data favor a *de novo* acquisition of the mutation and the preferential survival of mutant cells under selective drug pressure albeit not completely ruling out the possibility of a preexistent *BCR/ABL* mutation in a parental cell. Of interest is the question of how only a minor fraction of mutant Bcr/Abl molecules can account for the observed level of STI571 resistance. Is this minor fraction of mutant Bcr/Abl copies involved in more critical pathways required for survival/growth of these cells, or is it acting in a dominant negative fashion? A relatively modest increase of p210 expression in cells containing high number of *BCR/ABL* copies of which only a minority is mutated suggests a possibility of a dominant negative effect. A slightly lower proportion of mutant copies on gDNA than cDNA level suggests such an effect on the transcriptional levels. A partial suppression of the kinase phosphorylation in KBM5-STI<sup>R1.0</sup> cells by STI571 suggests that the wild-type molecules might still be blocked by the drug and that only the mutant fraction of Bcr/Abl molecules is sufficient to trigger the kinase-dependent activation of proliferation/survival. Additional studies are required to answer these questions. There is still a remote possibility that there are other undetected mutations within the pseudo wild-type transcripts.

We tested the possibility that the point mutation may have resulted from a PCR error. Besides including a DNA polymerase with proof-reading activity to our PCR, we tested the reproducibility of our findings in repeated experiments involving a single PCR round and different primer combinations. In all instances, we confirmed the presence of the C-T point mutation of the KBM5-STI<sup>R1.0</sup> cells and absence of any nucleotide change involving the ATP-binding pocket coding sequence in the parental cells. No other mutations, recently reported in patients clinically resistant to STI571 (5–10), were identified in KBM5 parental or any STI571-resistant KBM5 sublines. Interestingly, growth of KBM5-STI<sup>R1.0</sup> cells and two clonally derived sublines in the absence of STI571 for 25 passages resulted in a partial reversal of resistance (Fig. 1). Additional studies will be required to explain such partial reversal of the STI571 resistance and to assess biological significance of this mutation, occurring in a fraction of

amplified *BCR/ABL* genes in resistant cells, and the role of this mutation in the STI571-resistant phenotype.

In summary, systematic analysis of the DNA sequence in the region corresponding to the ATP-binding pocket of the kinase domain of the *BCR/ABL* gene identified a single nucleotide substitution C-T at *ABL* position 944 that results in the T315I mutation reported in a proportion of STI571-resistant CML patients (4). The analysis of gDNA and cDNA yielded results documenting that each clonogenic cell contains both wild-type- and mutated *BCR/ABL* copies and transcripts.

## Acknowledgments

We thank Dr. Hui Lin and Dr. Ralph Arlinghaus (M. D. Anderson Cancer Center, TX) for initial advice and for providing us with the positive control for *DdeI* digests of mutant *BCR/ABL* and some of the primers. We also thank Dr. Arlinghaus for advice and critical review of this manuscript. We thank Li Dong for technical assistance with the initial parts of the study.

## References

1. Le Coutre, P., Tassi, E., Varella-Garcia, M., Barni, R., Mologni, L., Cabrita, G., Marchesi, E., Supino, R., and Gambacorti-Passerini, C. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood*, 95: 1758–1766, 2000.
2. Weisberg, E., and Griffin, J. D. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in *BCR/ABL*-transformed hematopoietic cell lines. *Blood*, 95: 3498–3505, 2000.
3. Mahon, F. X., Deininger, M. W. N., Schultheis, B., Chabrol, J., Reiffers, J., Goldman, J. M., and Melo, J. V. Selection and characterization of *BCR-ABL* positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanism of resistance. *Blood*, 96: 1070–1079, 2000.
4. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. Clinical resistance to STI571 cancer therapy caused by *BCR-ABL* gene mutation or amplification. *Science* (Wash. DC), 293: 876–880, 2001.
5. Hochhaus, A., Kreil, S., Corbin, A., La Rosee, P., Lahaye, T., Berger, U., Cross, N. C., Linkesch, W., Druker, B. J., Hehlmann, R., Gambacorti-Passerini, C., Corneo, G., and D'Incalci, M. Roots of clinical resistance to STI-571 cancer therapy (Letter). *Science* (Wash. DC), 293: 2163, 2001.
6. Barthe, C., Cony-Makhoul, P., Melo, J. V., and Mahon, J. R. Roots of clinical resistance to STI-571 cancer therapy (Letter). *Science* (Wash. DC), 293: 2163, 2001.
7. Von Bubnoff, N., Schneller, F., Peschel, C., and Duyster, J. *BCR-ABL* gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. *Lancet*, 356: 487–491, 2002.
8. Kreil, S., Muller, M. C., Lahaye, T., La Rosée, P., Corbin, A. S., Schoch, C., Cross, N., Berger, U., Rieder, H., Druker, B. J., Gschaidmeier, H., Rüdiger, H., and Hochhaus, A. Molecular and chromosomal mechanisms of resistance in CML patients after STI571 (Gleevec) therapy (Abstract). *Blood*, 98 (Suppl. 1): 1823, 2001.
9. Shah, N. P., Nicoll, J. M., Gorre, M. E., Paquette, R. L., Ford, J., and Sawyers, C. L. Resistance to Gleevec: sequence analysis reveals a spectrum of *BCR/ABL* kinase domain mutations in both acquired- and *de novo*-resistant cases of chronic myelogenous leukemia (CML) in myeloid blast crisis (Abstract). *Blood*, 98 (Suppl. 1): 3205, 2001.
10. Hoffman, W. K., Jones, L. C., Lemp, N. A., de Vos, S., Gschaidmeier, H., Hoelzer, G. H., Ottmann, O. G., and Koefler, H. P. Ph(+) acute lymphoblastic leukemia resistant to the tyrosine kinase inhibitor STI571 has a unique *BCR-ABL* gene mutation. *Blood*, 99: 1860, 2002.
11. Beran, M., Pisa, P., O'Brien, S., Kurzrock, R., Siciliano, M., Cork, A., Andersson, B. S., Kohli, V., and Kantarjian, H. Biological properties and growth in SCID mice of a new myelogenous leukemia cells in the blastic phase. *Cancer Res.*, 53: 3603–3610, 1993.
12. Wetzler, M., Talpaz, M., Van Etten, R. A., Hirsch-Ginsberg, C., Beran, M., and Kurzrock, R. Subcellular localization of *BCR*, *ABL*, and *Bcr-Abl* proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J. Clin. Invest.*, 92: 1925–1939, 1993.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Mutation in the ATP-binding Pocket of the ABL Kinase Domain in an STI571-resistant BCR/ABL-positive Cell Line

Clara Ricci, Barbara Scappini, Vladimir Divoky, et al.

*Cancer Res* 2002;62:5995-5998.

**Updated version** Access the most recent version of this article at:  
<http://cancerres.aacrjournals.org/content/62/21/5995>

**Cited articles** This article cites 9 articles, 7 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/62/21/5995.full#ref-list-1>

**Citing articles** This article has been cited by 15 HighWire-hosted articles. Access the articles at:  
<http://cancerres.aacrjournals.org/content/62/21/5995.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cancerres.aacrjournals.org/content/62/21/5995>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.