Critical Reading – How to Read a Scientific Journal Article

I. **Abstract**
   a. What is the purpose of an abstract?
   b. What major question are the authors addressing? (Objective)
   c. What did the authors do? How did they attempt to answer the question?
   d. What are the major findings presented by the authors? (Intellect. Merit)
   e. What are the broader implications of this study? (Broader Impact)

II. **Introduction/Background**
   f. Describe the overarching structure of the introduction
   g. Describe the scope of the introduction: narrow, broad? Introduction will also be tailored to each journal – high impact (broadly read), letters and reports (shorter, more concise)
   h. What is the objective of the study? If possible, state the hypothesis.
   i. Is the importance of the study clearly conveyed? Does the introduction provide enough evidence to justify the proposed studies?

III. **Experimental procedures/Methods**
   j. Note tone – no interpretation; no "I"; past tense
   k. Note the level and number of specifics. The methods section should contain enough information to replicate the major findings of the study. However, universal and well-described procedures are often referenced or omitted.
   l. Non-traditional controls should be described when appropriate.

IV. **Results**
   m. What is the purpose of the results section?
   n. Describe structure of the results section
   o. Again, note tense – past tense
   p. Are the figures easy to understand? Are the figure legends clear and concise? Are they called at the appropriate time?
   q. Note how additional background is provided to put method and experimental findings in context (with respect to field)
   r. Note use of transition sentences and phrases like, "To gain insight into potential mechanisms…we…"

V. **Discussion**
   s. What is the purpose of the discussion section?
   t. Describe the structure of the discussion section.

While the focus is on content and clarity, a scientific article should read as a narrative in the sense that the transitions (including headings & titles) guide the reader through the different sections and present why the various steps were taken.

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

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The summary explains the intellectual merit of the article and its broader impact, *why* it's important

Through sequencing analysis of blood or bone marrow samples from patients with chronic myeloid leukemia, we identified *BCR-ABL* kinase domain mutations in 29 of 32 patients whose disease relapsed after an initial response to the tyrosine kinase inhibitor imatinib. Fifteen different amino acid substitutions affecting 13 residues in the kinase domain were found. Mutations fell into two groups—those that alter amino acids that directly contact imatinib and those postulated to prevent BCR-ABL from achieving the inactive conformational state required for imatinib binding. Distinct mutations conferred varying degrees of imatinib resistance. Mutations detected in a subset of patients with stable chronic phase disease correlated with subsequent disease progression. Multiple independent mutant clones were detected in a subset of relapsed cases. Our data support a clonal selection model of preexisting BCR-ABL mutations that confer imatinib resistance.

This section contains the clinical summary

Introduction

The tyrosine kinase BCR-ABL is the fusion product of a reciprocal chromosome translocation between chromosomes 9 and 22, known as the Philadelphia (Ph) chromosome, and is present in the leukemic cells of more than 95% of patients with chronic myeloid leukemia (CML) (Sawyers, 1999). The ability of *BCR-ABL* to produce a CML-like disease in mouse models (Daley et al., 1990; Elefanty et al., 1990; Kelliher et al., 1990) led to efforts to develop inhibitors of its kinase activity. Imatinib (STI571, Gleevec) was approved for CML treatment after striking efficacy was demonstrated in all stages of the disease (Druker et al., 2001a, 2001b; Kantarjian et al., 2002; Sawyers et al., 2002; Talpaz et al., 2002). The clinical success of this small molecule kinase inhibitor has led to the notion that other selective tyrosine kinase inhibitors may have significant activity against a range of human malignancies.

Ninety percent of patients with early-stage (chronic phase) CML achieve hematologic responses to imatinib, and the Ph chromosome is no longer detected in about 50% of these patients using standard cytogenetic assays (termed cytogenetic response) (Druker et al., 2001b; Kantarjian et al., 2002). Approximately 60% of patients with advanced-stage CML (blast crisis) respond to imatinib, but nearly all these patients relapse despite continued therapy (Druker et al., 2001a; Sawyers et al., 2002). We previously demonstrated that BCR-ABL kinase activity is inhibited in leukemic cells at the time of response but is reactivated at relapse (Gorre et al., 2001a). This result suggested that BCR-ABL maintains a crucial role throughout disease progression in CML. Detailed analysis of 11 such patients revealed at least two distinct mechanisms for resistance—*BCR-ABL* gene amplification or point mutations in the kinase domain that presumably interfere with drug-protein binding without compromising kinase activity (Gorre et al., 2001a). While these findings have been confirmed in additional small studies (Branford et al., 2002; Hofmann et al., 2002; von Bubnoff et al., 2002), there is

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S I G N I F I C A N C E

Chronic myelogenous leukemia represents the first human malignancy to be successfully treated with a small molecule tyrosine kinase inhibitor. Clinical resistance to this inhibitor (imatinib) has been reported in small numbers of patients due to *BCR-ABL* gene mutation or amplification. Here we present a comprehensive analysis of CML patients in varying stages of disease that documents a very high frequency of *BCR-ABL* kinase domain mutations in patients that develop imatinib resistance. These results reinforce the central importance of BCR-ABL in the pathogenesis of this disease, even at the most advanced stages, and define the challenges necessary to design second generation targeted therapies. Our findings may also have implications for other kinase inhibitors currently in development against a broad range of cancers.

The Introduction of an Honors Thesis will include more background and details that explain why the experiment is taking place to make the context and significance clear. "Significance" is usually not separated into its own section, however the scope of the article can be addressed within the intro or on its own.

disagreement about the frequency with which mutations occur (Barthe et al., 2001; Hochhaus et al., 2001). In addition, other mechanisms of imatinib resistance have been proposed, such as drug sequestration mediated by the serum protein α-1 acid glycoprotein (AGP) (Gambacorti-Passerini et al., 2000), or drug efflux by multidrug resistance pumps (Mahon et al., 2000). To date, neither of the latter mechanisms has been implicated in patients (Jorgensen et al., 2002).

Clinical efforts to overcome imatinib resistance must begin by defining the most common mechanisms of resistance in a larger number of patients. As a first step, we conducted comprehensive *BCR-ABL* kinase domain sequencing analysis of 45 CML patients who demonstrated imatinib resistance. Mutations were detected in over 90% of patients (29 of 32) who relapsed on initial response to imatinib, including those with chronic phase, myeloid blast crisis, and lymphoid blast crisis CML. Mutations led to 15 amino acid substitutions in 13 different residues which, based on known crystal structure data, include direct drug contact sites as well as sites that presumably impair the conformational change in the kinase domain required to accommodate imatinib binding. Mutant *BCR-ABL* alleles retain biologic activity but show varying degrees of resistance to imatinib in biochemical and biological assays. Mutations were also detected in 4 of 13 chronic phase patients with stable disease and correlated with subsequent clinical relapse. Additional observations that multiple independent imatinib-resistant clones were detected in some patients (polyclonal resistance) and that kinase domain mutations were detected prior to treatment in some patients with imatinib-refractory myeloid blast crisis provide evidence that mutations develop as a consequence of clonal selection rather than generalized hypermutability. Collectively these results establish the primary role of *BCR-ABL* kinase domain mutations as a cause of imatinib resistance in CML and provide evidence that knowledge of mutation status might influence clinical management. The spectrum of mutations identified provides new insights into kinase domain function and into novel treatment strategies to overcome resistance.

Results

Kinase domain mutations are detected in 90% of CML patients who relapse after responding to imatinib

The ability to detect *BCR-ABL* mutations in imatinib-resistant CML cells is dependent on patient selection and the sensitivity of the assay used. Because the Ph chromosome translocation occurs in a pluripotent hematopoietic stem cell, nearly all the blood and bone marrow cells in untreated CML patients express the *BCR-ABL* fusion gene. In patients with a hematologic response to imatinib (normalization of blood counts) and a cytogenetic response (reduction in the fraction of Ph chromosome-containing cells), a significant fraction of the *BCR-ABL*-expressing cells isolated at the time of relapse will represent the drug-resistant clone. However, in patients who relapse on imatinib after a hematologic response but no cytogenetic response, imatinib-resistant cells represent only a subset of the total *BCR-ABL*-expressing cells (often 20% or less) (Gorre et al., 2001b). To optimize the sensitivity of mutation detection, we performed RT-PCR amplification of *BCR-ABL* mRNA from CML patient blood or bone marrow cells, subcloned the PCR products (rather than sequence the products directly), and sequenced ten independent PCR products per patient sample. To improve the specificity of our methodology, a mutation was deemed present if it was detected on both strands of DNA in two independent isolates from the same patient (therefore representing approximately 20% of the total population of *BCR-ABL* mRNA transcripts).

Samples from 32 CML patients who relapsed after a hematologic response to imatinib were analyzed (16 with myeloid blast crisis, 5 with lymphoid blast crisis, and 11 with chronic phase). Mutations were identified at the time of relapse in 29 of the 32 patients (15 of 16 with myeloid blast crisis, 4 of 5 with lymphoid blast crisis, and 10 of 11 with chronic phase) (Table 1). Mutations mapped to 13 different amino acid residues in the *ABL* kinase domain and predicted 15 different codon changes (Figure 1). Nucleotide changes affecting two residues, Q252 and Y253, led to two alternative amino acid substitutions each—Q252H/R and Y253F/H. Mutations in seven of these residues have been reported in smaller studies (Branford et al., 2002; Gorre et al., 2001a; Hofmann et al., 2002; von Bubnoff et al., 2002); six mutations detected are novel. Mutations were detected at each of the variable (X) amino acids of the kinase consensus sequence Gly-X-Gly-X-X-Gly-X-Val located in the P loop, which provides a number of important contacts for ATP binding. One of these mutations, E255K, in addition to mutations T315I and M351T, accounted for over 60% of the cases. To address whether the high frequency and variety of kinase domain mutations represented artifact introduced during the PCR amplification process, we sequenced ten independent subclones of the *ABL* kinase domain obtained from each of two healthy blood donors. Using our criteria of at least two independent isolates out of ten clones, we found no evidence of *ABL* kinase domain mutation. We therefore conclude that the *BCR-ABL* kinase domain mutations described here are unlikely to be the result of PCR-introduced error.

To gain insight into potential mechanisms by which these mutations might confer imatinib resistance, we modeled each amino acid substitution onto the known cocrystal structure of the ABL kinase domain bound to imatinib (Figure 2A; Nagar et al., 2002; Schindler et al., 2000). The mutations fall into two groups. The first group includes mutations at sites that directly contact imatinib (T315, F317, and F359) and presumably impair drug binding without significantly affecting the binding of ATP (Figure 2A, red spheres). The second group includes mutations in residues located in distinct regions, all of which are implicated in the unique conformational change that the kinase domain must undergo to accommodate imatinib. For example, the ATP phosphate binding loop (P loop) is distorted in the imatinib/ABL structure such that it forms a hydrophobic cage for the drug. Resistance mutations observed in the P loop region (G250, Q252, Y253, and E255) are likely to destabilize the distorted shape (Figure 2A, green spheres). Y253 inserts into the ATP binding site, forming one wall of a hydrophobic pocket for the drug, and is stabilized by hydrogen bonds formed by the hydroxyl group of the side chain (Figure 2B). Y253H/F substitutions would break the hydrogen bonding interactions of the tyrosine side chain, which is also specific for the distorted conformation of the P loop. E255 forms hydrogen bonds that bridge the side chains of K247 and Y257, an interaction that appears to be specific to the distorted conformation of the P loop. Mutation of E255 presumably destabilizes the P loop conformation that is required for imatinib binding.

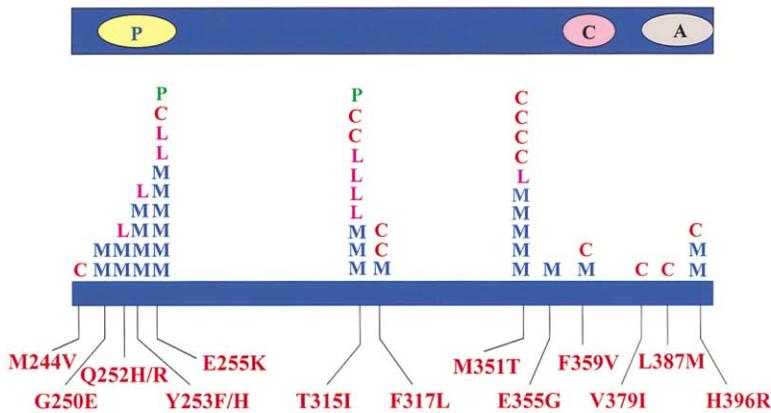A second structural change required for imatinib binding is

Table 1. Detailed summary of *BCR-ABL* kinase domain mutations by disease category prior to imatinib therapy

Patient no.	Duration of treatment with imatinib at time of analysis	*BCR-ABL* kinase domain mutation(s)	Number of independent clones containing mutation
1 MBC	4.5 months	G250E	7/10
		H396R	2/10
2 MBC	13 months	T315I	10/10
3 MBC	8 months	none	n/a
4 MBC	3.5 months	M351T	5/10
5 MBC	2 months	Q252H	5/10
6 MBC	13 months	M351T	8/10
7 MBC	13 months	M351T	6/10
8 MBC	5 months	E255K	4/10
		Y253H	2/10
9 MBC	7 months	E355G	5/10
		F317L	2/10
10 MBC	6 months	G250E	8/10
11 MBC	3 months	Y253F	3/10
		E255K	2/10
		M351T	2/10
		H396R	2/10
12 MBC	2 months	M351T	3/10
		T315I	2/10
		Y253H	2/10
		E255K	2/10
13 MBC	3 months	Y253F	2/10
		E255K	2/10
		T315I	2/10
14 MBC	3 months	E255K	2/10
15 MBC	2 months	E255K	2/10
		Q252H	2/10
16 MBC	4 months	F359V	8/10
17 LBC	2 months	M351T	3/10
		E255K	2/10
		T315I	2/10
		Y253F	2/10
18 LBC	3 months	T315I	5/10
		E255K	2/10
		Q252R	2/10
19 LBC	2 months	T315I	5/10
20 LBC	9 months	T315I	6/10
21 LBC	3 months	none	n/a
22 CP	17 months	T315I	8/10
23 CP	10 months	F317L	10/10
24 CP	9 months	E255K	7/10
25 CP	38 months	F359V	10/10
26 CP	25 months	L387M	7/10
27 CP	25 months	M244V	6/10
		M351T	2/10
28 CP	20 months	F317L	4/10
		M351T	4/10
29 CP	17 months	M351T	7/10
30 CP	17 months	T315I	8/10
31 CP	33 months	none	n/a
32 CP	25 months	M351T	4/10
		H396R	4/10
33 P-MBC	n/a	T315I	2/10
		M343T/F382L	2/10
34 P-MBC	n/a	E255K	2/10
35 P-MBC	n/a	none	n/a
36 P-MBC	n/a	none	n/a
37 NCR	14 months	V379I	7/10

Duration of imatinib therapy at the time of analysis is shown. The fraction of independent clones for each mutation is given. Patients 2, 6, 8, 18, and 20 had major cytogenetic responses and patients 17 and 29 had minor cytogenetic responses prior to relapse, using criteria previously described (Kantarjian et al., 2002). Patients 10, 13, and 31 had evidence of *BCR-ABL* gene amplification at relapse by FISH analysis. Patient 37 (NCR) harbors a kinase domain mutation but has not suffered progressive disease (see also Figure 4). Eight additional NCR patients, as depicted in Figure 4, had no evidence of kinase domain mutation, and are not represented here.
Abbreviations: MBC, relapsed myeloid blast crisis; LBC, relapsed lymphoid blast crisis; CP, relapsed chronic phase; P-MBC, preimatinib sample from myeloid blast crisis patients whose disease was subsequently refractory; and NCR, chronic phase with hematologic response but no cytogenetic response.

BCR-ABL kinase domain

M244V Q252H/R E255K M351T F359V L387M
G250E Y253F/H T315I F317L E355G V379I H396R

Top: Schematic representation of the BCR-ABL kinase domain. P loop (P), catalytic domain (C), and activation loop (A) are depicted. Bottom: Each letter represents a patient in whom the corresponding mutation was detected. Relapsed chronic phase patients are represented by the letter C (for exception, see below). Relapsed myeloid blast crisis patients are indicated by the letter M. Patients with relapsed lymphoid blast crisis are represented by the letter L. P indicates mutations detected prior to imatinib treatment in patients with myeloid blast crisis who exhibited primary resistance to treatment. V379I was detected in a single chronic phase patient with persistence of the Philadelphia chromosome in the setting of sustained hematologic response. Note that kinase domain is not drawn to scale.

the closed conformation of the ABL activation loop, which is part of a network of interactions that help stabilize the drug binding site. In the closed conformation, Y393 in the activation loop is positioned such that it mimics the tyrosine residue of a peptide substrate. The H396R substitution is located close to Y393, such that mutations in this region might disturb the ener-

getic balance required to stabilize the closed conformation of the activation loop (Figure 2C). Another cluster of mutations involves amino acids located in the C-terminal lobe of the kinase (E355, M351, and V379) in a region that provides a platform for the base of the activation loop. While it is difficult to predict the precise energetic consequences of mutations at these residues,
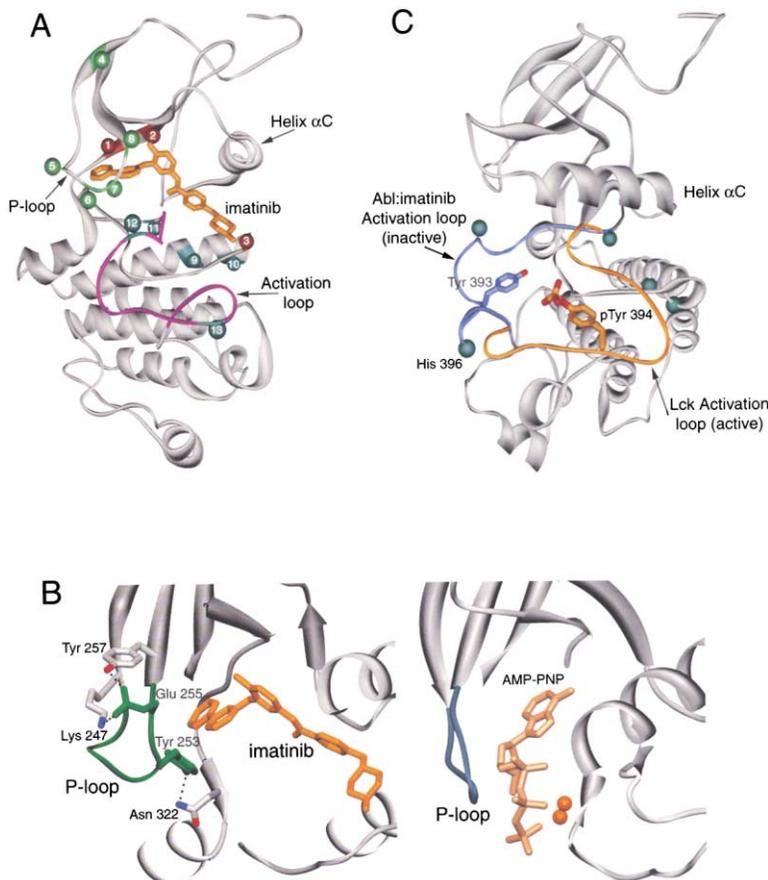

Figure 2. Proposed mechanisms of action of imatinib-resistant mutations based upon the crystal structure of ABL complexed with imatinib

A: Ribbon representation of the kinase domain of ABL complexed to imatinib (Nagar et al., 2002), depicting resistant mutations. Imatinib is shown in gold. Positions 1–3 (red) are mutations that directly affect imatinib binding. All other positions are those that likely affect the ability of the kinase to achieve the conformation required to bind imatinib, including those in the P loop (4–8; green) and those in the vicinity of the activation loop (9–13; cyan). The activation loop is colored purple. The positions of amino acids found mutated are depicted by spheres: 1, F317; 2, T315; 3, F359; 4, M244; 5, G250; 6, Q252; 7, Y253; 8, E255; 9, M351; 10, E355; 11, V379; 12, L387; 13, H396. **B:** Comparison of the P loop in the ABL:imatinib structure (left) with that of active insulin receptor kinase (IRK) (right) (Hubbard, 1997). Glu 255 in the P loop (green) of ABL makes strong interactions with Tyr 257 and Lys 247. The P loop is distorted, with Tyr 253 swung into the ATP binding site, forming a hydrophobic face against which imatinib packs. In IRK, the P loop (blue) is folded out over the nucleotide. Orange spheres indicate Mg^{2+} ions associated with nucleotide. Representation constructed using a previously described program (Evans, 1993). **C:** Mutations located at sites that could influence conformational changes undergone by the activation loop. For comparison, the activation loop of Lck (an active tyrosine kinase) is shown in gold, including the phosphorylated Tyr residue (pTyr 394) (Yamaguchi and Hendrickson, 1996). Tyr 393, the site of activating phosphorylation in ABL, is shown in blue. Positions of mutations M351T, E355G, V379I, L387M, and H396R are depicted as spheres (cyan).

The transitions present *why* these steps were taken before specifying the results

all three are located adjacent to a region where large torsional rotations are required to accommodate imatinib. Comparative crystallographic studies of mutant *ABL* alleles are required to precisely define the structural consequences of these mutations.

Mutant *BCR-ABL* alleles show varying degree of imatinib resistance in vitro

To address the causal role of these mutations in imatinib resistance, we generated mutant alleles by site-directed mutagenesis of wild-type *BCR-ABL* and expressed the mutants in the interleukin-3 (IL-3)-dependent cell line Ba/F3. BCR-ABL activity has been shown to render Ba/F3 cells IL-3 independent (Daley and Baltimore, 1988). The T315I and E255K mutations are known to confer IL-3 independence as well as biochemical and biological resistance to imatinib in this system (Gorre et al., 2001a, 2002; von Bubnoff et al., 2002) and were included as controls. Six previously uncharacterized mutants were chosen for this initial analysis based on their frequency of isolation and location within the kinase domain. IL-3-independent Ba/F3 populations were derived for all six mutants studied, demonstrating that each of these mutants retains biologic activity comparable to wild-type BCR-ABL in this system. The effect of imatinib concentrations ranging from 0.5 to 10 μM on BCR-ABL kinase activity and cellular viability was determined using standard assays (Figure 3). As expected, the T315I and E255K mutants were highly resistant to imatinib with IC_{50} values greater than 10-fold higher than wild-type BCR-ABL (Table 2). G250E-expressing cells were also markedly resistant. Y253F also resulted in considerable resistance, but clearly less than T315I, consistent with another report that characterizes the effects of this same mutation on *ABL* kinase function (Roumiantsev et al., 2002). Four mutations (Q252H, F317L, M351T, and E355G) conferred only moderate resistance to imatinib, retaining biochemical sensitivity at drug concentrations of 1–4 μM. Since drug levels approaching this concentration can be clinically achieved in some patients, these results raise the possibility that resistance caused by certain mutations might respond to high-dose imatinib treatment. The fact that all *BCR-ABL* mutants tested show imatinib resistance in vitro provides strong evidence that each of these mutations plays a causal role in clinical imatinib resistance. Biochemical characterization of the five remaining mutations found, consisting of the novel mutations M244V, F359V, V379I, and L387M, in addition to the previously described H396R, will require additional studies.

Potential predictive value of mutation detection in patients with stable disease

The overwhelming majority of CML patients on imatinib therapy have chronic phase disease. Although 90% of patients with chronic phase CML achieve excellent hematologic control of their disease with imatinib, about half of these patients continue to have a significant number of Ph chromosome-containing cells in the bone marrow ("cytogenetic nonresponders"). Recent data suggests that these patients progress to blast crisis more quickly that those who obtain cytogenetic remissions (Kantarjian et al., 2002). The reason for failure to achieve a cytogenetic response is unknown, but may involve incomplete inhibition of BCR-ABL kinase activity in progenitor cells or activation of BCR-ABL-independent survival pathways. Since *BCR-ABL* kinase domain mutations are prevalent in imatinib-treated patients with

hematologic relapse, we asked if similar mutations might be found in cytogenetic nonresponders with sustained hematologic remissions. Four of 13 such chronic phase patients had mutations. Three of these mutations (E255K, F317L, and F359V) are identical to those observed in cases of relapsed myeloid blast crisis described above, whereas the fourth mutation (V379I) has not been previously documented. We next asked if detection of a mutation during this time of stable hematologic response correlated with clinical outcome. Three of the four patients with kinase domain mutations suffered disease progression within 18 months of mutation detection, whereas only one of nine patients without a detectable mutation progressed (Figure 4). Of note, the patient without evidence for a kinase domain mutation had *BCR-ABL* gene amplification at relapse based on fluorescence in situ hybridization analysis (data not shown). The patient harboring the novel V379I mutation continues to have a complete hematologic remission but no cytogenetic response at 18 months. Although the number of patients is small, these findings suggest that periodic assessment of the mutational status of the *BCR-ABL* kinase domain may have prognostic utility.

Evidence for clonal selection of preexisting *BCR-ABL* kinase domain mutations

The most frequent explanation for single-nucleotide substitutions in the human genome are methylation-deamination reactions that give rise to C/T or G/A transitions in the context of the CpG dinucleotide sequence. This mechanism is estimated to account for about 25% of all reported nucleotide changes based on comprehensive analysis of human genetic disorders (Vogelstein and Kinzler, 1997). Remarkably, only 1 of the 15 mutations we observed (E255K) could be explained on that basis. Alternatively, the high frequency of kinase domain mutations observed in our study could reflect a global increase in mutation frequency (hypermutation) as a consequence of genetic instability. To address this possibility, we looked for mutations in other regions of the genome by sequencing a 700 bp region of *BCR-ABL* immediately 5′ to the kinase domain in five patients in whom kinase domain mutations were detected in a large fraction of the original clones sequenced. No evidence of additional mutation was found in these samples. We also assessed the kinase domain of the related tyrosine kinase *KIT*, which resides on chromosome 4 and exhibits sensitivity to imatinib at concentrations equivalent to *BCR-ABL*, in the same group of five patients. No evidence of *KIT* kinase domain mutation was detected, arguing against a model of widespread genomic instability.

Another possible explanation for the high frequency of mutations is that exposure to imatinib might induce genetic instability, particularly since the wild-type ABL kinase (which is also inhibited by imatinib) has been implicated in DNA repair (Kharbanda et al., 1997; Shafman et al., 1997; Yoshida et al., 2002). Several lines of evidence argue against this hypothesis. First, patients with blast crisis CML often relapse within 1–3 months of initiating imatinib therapy, providing a short window of time for the acquisition of mutations and expansion of a drug-resistant clone. Second, we found multiple independent *BCR-ABL* mutations (2–4 clones per patient) in 12 of the 32 patients with imatinib resistance, including 5 patients who relapsed within 3 months of starting therapy (Table 1). Unless we postulate an extremely high mutation rate, these results are most consistent with a
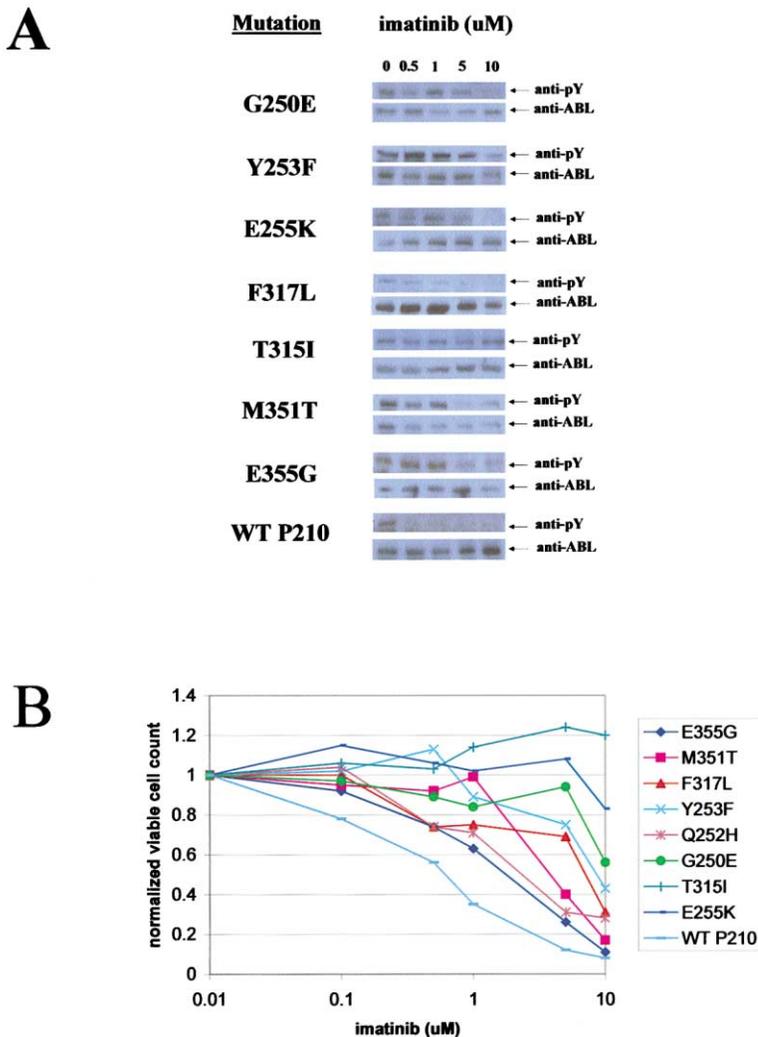
A

B

Figure 3. BCR-ABL kinase domain mutants exhibit varying degrees of biochemical and biological resistance to imatinib

A: Western blot analysis using an anti-phospho-tyrosine antibody (4G10) or anti-ABL antibody of lysates prepared from IL-3-independent Ba/F3 populations infected with retroviruses expressing the *BCR-ABL* isoforms indicated. Cells were exposed to the concentrations of imatinib indicated for 2 hr. **B:** Ba/F3 populations stably expressing mutant *BCR-ABL* isoforms were plated in the presence of varying concentrations of imatinib in triplicate, and viable cell counts were performed after 48 hr of exposure. Cell counts were normalized to the number of viable cells from control cultures grown in the absence of imatinib.

model whereby mutant clones preexist in some patients at levels below our threshold for detection, then expand under the selective pressure of imatinib treatment. Definitive evidence for this hypothesis was obtained by analysis of pretreatment samples from four blast crisis patients who failed to achieve even a transient response to imatinib (primary or de novo hematologic resistance). One patient exhibited T315I prior to initiation of therapy, as well as a separate *BCR-ABL* clone that contained two mutations, M343T and F382L, on the same DNA sequence. A second patient had the E255K mutation (Table 1). Collectively, these data indicate that *BCR-ABL* kinase domain mutations can occur during the natural evolution of CML and that those capable of conferring imatinib resistance expand under the selective pressure of imatinib treatment. The fact that certain mutations can be detected in a significant fraction of cells prior to imatinib therapy suggest that some mutations may confer gain of function, analogous to a kinase domain mutation present in v-Abl, which has been shown to contribute to its oncogenic potential (Jackson et al., 1993). Members of the Src family of kinases, which have increased kinase activity relative to ABL, all have a phenylalanine at amino acid position 5 in the P loop, whereas ABL has a tyrosine at this position. Furthermore, the Y253F

mutation, when engineered into ABL, has demonstrated oncogenic activity (Allen and Wiedemann, 1996). The mechanism for the underlying increase in genetic instability that presumably leads to these mutations in CML cells remains to be defined.

Discussion

In a comprehensive analysis of CML patients treated with imatinib in all phases of the disease, we find that mutations in the *BCR-ABL* kinase domain occur in over 90% of patients who relapse after an initial response. The high frequency of mutation detection in our study relative to smaller studies reported by other investigators is most likely explained by differences in assay sensitivity. By subcloning and sequencing ten independent clones from each patient, we can reliably and confidently detect mutant clones representing approximately 20% of the total population of *BCR-ABL*-expressing cells. This strategy also allows detection of polyclonal resistance, which would be extremely difficult to find using direct sequencing methods (without subcloning) because the mixture of wild-type and mutant clones would most likely be read as consensus wild-type sequence. A second issue likely to explain differences in mutation

Table 2. Summary of *BCR-ABL* kinase domain mutations, associated disease state(s) prior to imatinib therapy, biochemical and biologic properties, and proposed mechanism of resistance

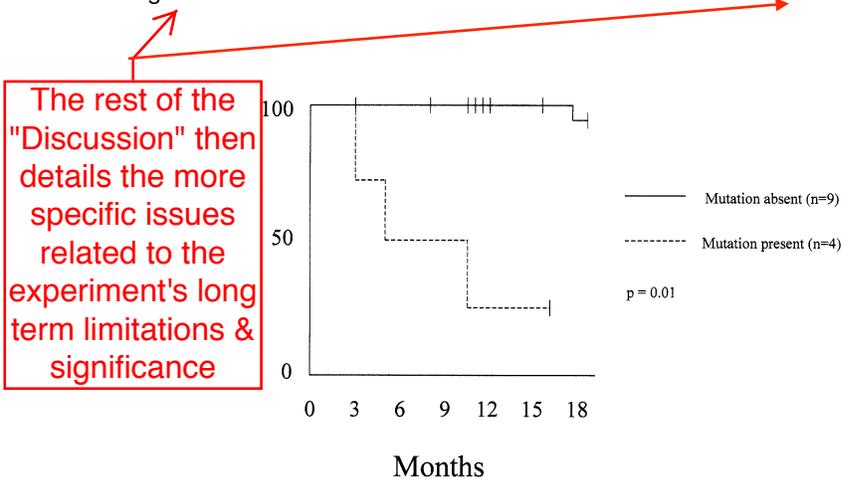| Mutation	Phase of disease	Biochemical IC_{50} (μM)	Biological IC_{50} (μM)	Proposed mechanism of resistance
M244V	CP	not assessed	not assessed	impairs conformational change (P loop)
G250E	MBC	7.40	>10	impairs conformational change (P loop)
Q252H/R	MBC, LBC	2.93	3.12	impairs conformational change (P loop)
Y253F/H	MBC, LBC	5.96	8.94	impairs conformational change (P loop)
E255K	MBC, LBC, CP, P-MBC	7.81	>10	impairs conformational change (P loop)
T315I	MBC, LBC, CP, P-MBC	>10	>10	directly affects imatinib binding
F317L	MBC, CP	3.30	7.50	directly affects imatinib binding
M351T	MBC, LBC, CP	1.07	4.38	impairs conformational change (adjacent to activation loop)
E355G	MBC	2.82	2.38	impairs conformational change (adjacent to activation loop)
F359V	MBC, CP	not assessed	not assessed	directly affects imatinib binding
V379I	CP-NCR	not assessed	not assessed	impairs conformational change (activation loop)
L387M	CP	not assessed	not assessed	impairs conformational change (activation loop)
H396R	MBC, CP	not assessed	not assessed	impairs conformational change (activation loop)
wild-type	-	0.28	0.60	not applicable

Abbreviations: MBC, myeloid blast crisis; LBC, lymphoid blast crisis; CP, chronic phase; CP-NCR, chronic phase with hematologic response in the absence of cytogenetic response (cytogenetic nonresponder).

frequency is patient selection, particularly if analysis is performed on patients who fail to have a hematologic response to imatinib. Having defined a large number of amino acid substitutions, we obtained definitive evidence from in vitro reconstitution studies that at least eight mutants (all those studied to date) play a causal role in imatinib resistance. The role of the remaining seven (less common) mutations will need to be examined using similar assays before definitive conclusions about these mutants can be made. Our results reinforce the central role of BCR-ABL kinase activity in CML at all disease stages and clearly delineate the challenge that future targeted therapeutic strategies for CML must address.

Are there any obvious strategies that should be considered? The complex array of different mutations observed in these patients makes it difficult to envision a single second generation ABL kinase inhibitor that would have activity against all mutants. However, recent insights gained from structural analysis of ABL bound to imatinib versus a dual ABL/SRC inhibitor indicate that two different inhibitors can bind to the same kinase in different conformational states (Nagar et al., 2002; Schindler et al., 2000). If a significant number of the mutations that cause imatinib resistance do so by preventing the kinase domain from achieving the closed conformation necessary for imatinib binding (as proposed in Figure 2), one can envision that inhibitors that bind ABL in the open configuration might retain activity against many of these mutants. Indeed, we have evidence that the E255K mutant remains sensitive to such a dual ABL/SRC inhibitor (M. Moasser, N. Rosen, M.E.G., and C.L.S., unpublished data). Alternatively, it may be necessary to inhibit BCR-ABL signaling using completely different strategies that do not require binding to the kinase domain. Possibilities include the induction of BCR-ABL protein degradation using inhibitors of the molecular chaperone Hsp90 or blockade of critical signal transduction events that function downstream of BCR-ABL such as the RAF/MEK/ERK and PI3K/AKT pathways. We have preclinical evidence in support of the former strategy based on the fact that two Hsp90 inhibitors, geldanamycin and 17-AAG, have potent in vitro activity against the T315I and E255K BCR-ABL mutants (Gorre et al., 2002).

As second generation inhibitors undergo development, it may be useful now to measure the mutation status of the *BCR-ABL* kinase domain in selected populations of CML patients

Figure 4. Kaplan-Meier analysis of time to progression in chronic phase CML patients with hematologic response despite lack of cytogenetic response

Thirteen chronic phase patients treated at UCLA with imatinib who had sustained hematologic response in the absence of cytogenetic response were studied. Patients were categorized with respect to the presence of *BCR-ABL* kinase domain mutations at the time of analysis. Progression is defined as loss of previously achieved hematologic response to imatinib. The four patients who progressed were reevaluated for the presence of mutation at the time of progression. Data obtained at the time of progression is included in Table 1 (patients 23–25, 31). The one patient with a kinase domain mutation who did not have disease progression is also listed in Table 1 (patient 37). P value obtained by exact logrank test.

treated with imatinib. Our analysis of a small number of chronic phase patients with stable hematologic control but no cytogenetic response suggests that detection of such mutants portends a poor prognosis. If this result is confirmed in a larger prospective trial, one can envision altering the clinical management of such patients by imatinib dose escalation (if the mutation is one that confers partial imatinib resistance), addition of a second agent such as interferon, early consideration of allogeneic stem cell transplantation, or enrollment in an experimental drug trial. Widespread testing for such mutations would clearly require a less cumbersome method for mutation detection than that described here, perhaps using mutation-specific assays. The fact that only three mutations (T315I, E255K, and M351T) account for more that 60% of the cases in our study provides a starting point for the development of such clinical tests.

The long-range outlook for managing imatinib resistance should focus on defining the mechanism by which kinase domain mutations occur so that the emergence of resistant clones might be prevented. Our sequencing analysis provides very little evidence for methylation-deamination or hypermutation as factors that lead to the high frequency of mutations. By default, we assume that CML cells have increased genomic instability, as is clearly evident in blast crisis where a range of secondary chromosomal changes are frequently observed. The fact that mutations are also found in chronic phase patients (who have normal karyotypes except for the Ph chromosome) argues for additional genetic instability early in the disease. Indeed, there is some experimental evidence that expression of *BCR-ABL* can induce genetic instability in mouse models (Salloukh and Laneuville, 2000). Until the mechanism responsible for the development of these mutations is defined, it seems likely that future targeted therapy of CML will follow the model currently in practice for the treatment of HIV—combination therapy with multiple targeted agents.

Experimental procedures

After obtaining informed consent to participate in a research protocol approved by the UCLA institutional review board, blood or bone marrow samples were obtained from patients enrolled in clinical trials assessing the efficacy of imatinib in the treatment of CML. Criteria for determination of response and relapse were as defined in the phase I studies of imatinib in CML (Druker et al., 2001a, 2001b). RNA was extracted using TriReagent or TriAzol. CDNA synthesis was performed using MMLV reverse transcriptase. Polymerase chain reaction (PCR) was performed using the following primers: CM10 (5′-GAAGCTTCTCCCTGACATCCGT-3′) and 3′ ABL KD (5′-GCCAG GCTCTCGGGTGCAGTCC-3′). The resultant 1.3 kb fragment was excised from a low melting point agarose gel following electrophoresis. A second PCR was performed on the gel-purified 1.3 kb fragment to isolate the kinase domain using the primers 5′ ABL KD (5′-GCGCAACAAGCCCACTGTCTA TGG-3′) and 3′ ABL KD. The resultant 0.6 kb fragment was subcloned into pBluescript II KS+ digested with Eco RV. Bacterial transformants were plated on media containing ampicillin and X-gal. Ten white colonies per cDNA were inoculated into media and miniprep DNA was isolated. Sequencing of each clone was performed using M13 universal forward and reverse primers. Because two rounds of amplification were employed, a mutation was considered present if it was detected on both strands of at least two independent clones per patient (see Figure 1). Analysis of the *ABL* kinase domain from two healthy blood donors was performed using PCR of the *ABL* kinase domain, followed by subsequent reamplification to control for the number of amplification cycles to which patient samples were subjected. Sequence analysis of a 0.7 kb portion of *BCR-ABL* immediately 5′ to the kinase domain was performed by amplification of the previously described 1.3 kb fragment using CM10 and 5′ ABL KD reverse complement (5′-CCATAGACAGTGG GCTTGTTGCGC-3′) followed by ligation into pBluescript II KS+ as above.

The kinase domain of c-*KIT* was amplified using the following primers: 5′-TGAGGAGATAAATGGAAACAA-3′ and 5′-AACTCGTCATCCTCCATGAT-3′. To control for the number of cycles used for the *BCR-ABL* kinase domain, a second amplification was performed; the resultant 0.6 kb fragment was subcloned into pBluescript II KS+ and ten independent clones were sequenced.

Expression vectors of mutant P210 isoforms were created as follows. Oligonucleotides representing various point mutations were synthesized by GIBCO-BRL. pSRαP210*BCR-ABL* was used as the template DNA for site-directed mutagenesis reactions utilizing the mutant oligonucleotides and the QuikChange mutagenesis kit (Stratagene). Successful mutagenesis was confirmed by sequence analysis of the kinase domain.

293T cells maintained in DMEM with 10% fetal calf serum were cotransfected by the calcium phosphate method with mutant P210 expression vectors and a packaging plasmid (Ecopack, kindly provided by R. van Etten). Media containing virus was filtered and used to infect Ba/F3 cells grown in RPMI supplemented with 10% fetal calf serum and IL-3. Stable lines were selected in the presence of G418 and IL-3. After 48 hr, cells were washed thrice in PBS and subsequently grown in the absence of IL-3. Expression of BCR-ABL was documented by Western blot analysis. To determine the biochemical sensitivity of mutant BCR-ABL P^{210} isoforms to imatinib, cells were incubated in the presence of imatinib (kindly provided by Novartis Pharmaceuticals) at 0, 0.5, 1, 5, and 10 micromoles per liter. After 2 hr of incubation, cell lysates were prepared in 12.5 mM EDTA (pH 8.0), 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 μg/ml aprotinin, 100 μg/ml PMSF, and 2 μg/ml leupeptin. Seventy-five micrograms of total protein were analyzed by Western blot using anti-ABL antibody AB-3 (Oncogene Research Products) or anti-phosphotyrosine antibody 4G10 (Upstate Biochemicals). Quantification of the 210 kDa signal was achieved using ImageQuant 5.0 (Microsoft) densitometry. Tyrosine-phosphorylated BCR-ABL was normalized to the amount of P210 signal detected with anti-ABL antibody. Normalized phospho-BCR-ABL values were plotted for each mutant, and biochemical IC$_{50}$ was determined following creation of a best-fit curve. Mutant Q252H was not adequately detected by AB-3 and phospho-BCR-ABL was therefore not normalized. To determine the biological sensitivity to imatinib, 1×10^5 exponentially growing Ba/F3 cells/ml expressing various isoforms of BCR-ABL P^{210} were grown in RPMI supplemented with 10% fetal calf serum and incubated in the presence of imatinib at 0, 0.1, 0.5, 1, 5, and 10 micromoles per liter. Exposure to imatinib was performed in triplicate. After 24, 48, and 72 hr of incubation, live cells were quantitated by trypan blue stain exclusion. The mean number of viable cells at varying concentrations was normalized to mean value of viable cells in the 0 mM imatinib sample. Biological IC$_{50}$ was determined from semilogarithmic graphing of the 48 hr time point for each mutant.

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