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Clinical outcome of chronic myeloid leukemia imatinib-resistant patients: do BCR–ABL kinase domain mutations affect patient survival? First multicenter Argentinean study

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Abstract

In imatinib-treated patients with chronic myeloid leukemia (CML), BCR–ABL mutations are the most common mechanism of resistance. Here we report the first multicenter Argentinean study investigating mutations in those patients with CML who fail or lose response to imatinib, with or without previous interferon treatment. Point mutations were detected in 36 of 154 patients by direct sequencing. In our series, the single most common mutations were G250E, E255K/V, and M351T. The presence of mutations correlated significantly with accelerated phase, lack of molecular response, and lower cytogenetic and hematological responses. While overall survival did not differ between patients with or without mutations, the probability of progression was higher in patients with mutations. Cases with non-P-loop mutations showed a significantly better overall survival from diagnosis. Multivariate analysis showed that the most significant variables related to the development of mutations were accelerated phase, duration of imatinib treatment, and time delay to starting imatinib. Our results demonstrated that mutation frequency increased with the progression of disease, and suggest that imatinib treatment should be started early.

Keywords: *CML, imatinib, mutations, BCR–ABL, P-loop, resistance*

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by a reciprocal chromosomal translocation between chromosomes 9 and 22, t(9;22)(q34;q11), known as the Philadelphia chromosome (Ph). The resulting chimeric *BCR–ABL* oncogene encodes a constitutively activated oncoprotein ($p210^{BCR–ABL}$ tyrosine kinase) that induces CML [1,2].

Imatinib mesylate (IM) is a novel targeted agent, available orally, that has drastically changed the therapy paradigm of the disease and which currently represents the gold standard front-line treatment in patients in chronic phase, also being used in more advanced phases of CML and Ph+ acute lymphoblastic leukemia [3–7]. In CML chronic phase, the vast majority of patients attain a complete cytogenetic response when treated with IM, and molecular

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responses improve over time. Nevertheless, relapses or lack of response are observed in a minority of patients [6,8,9].

The two most common mechanisms of resistance are point mutation in the ABL kinase domain (KD) and overexpression of the BCR–ABL protein due to amplification of the *BCR–ABL* gene. Other BCR–ABL independent mechanisms have been extensively described [8,10–13]. More than 100 different BCR–ABL mutations have been identified [14,15]. These mutations cluster within four regions of the ABL-KD: (1) adenosine triphosphate (ATP) binding site (P-loop), (2) IM binding site, (3) catalytic domain, and (4) activation site (A-loop) [6,16].

The aim of this study was to identify ABL point mutations and/or amplification in patients who failed or lost response to imatinib in order to determine the association with clinical characteristics and the influence of mutations on disease outcome.

Materials and methods

Patient characteristics

This was a multicenter Argentinean study carried out in the Institute of Hematological Research in Buenos Aires, to detect mutations in the peripheral blood (PB) of IM-resistant patients with CML. From October 2005 to August 2008 a total of 154 samples from resistant patients were analyzed.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was reviewed by the ethics committee of the National Academy of Medicine in Buenos Aires. All patients provided written informed consent according to institutional regulations.

The patients included in this study were on IM therapy at 400 to 600 mg/day with or without previous IFN-α, failing to achieve hematological response at 3 months or patients failed to achieve a major cytogenetic response at 12 months, patients who lost cytogenetic response in chronic phase on IM, and patients in accelerated phase or blast crisis progression on IM.

Criteria for resistance were defined according to those reported by Hochhaus and colleagues [14], and disease phases according to the World Health Organization (WHO) classification [17].

The clinical characteristics of patients (age, Sokal score, phase of disease, prior interferon therapy) are described in Table I.

Table I. Clinical characteristics of patients at time of assessment for mutations.

	$n = 154$ (%)
Sex	
Female	68 (44.1)
Male	86 (55.8)
Sokal score	
Low	77 (50.0)
Intermediate	38 (24.7)
High	39 (25.3)
Phase of disease	
Chronic	97 (63.0)
Accelerated	37 (24.0)
Blastic	20 (13.0)
Pevious interferon therapy	
Yes	76 (49.3)
No	78 (50.7)

Phases of disease

Chronic phase (CP) was defined as less than 10% blasts in the peripheral blood (PB) or bone marrow (BM), less than 20% blasts plus promyelocytes in the PB or BM, less than 20% basophils, and no extramedullary involvement apart from the liver and spleen. Accelerated phase (AP) was defined by the presence of 11–29% blasts in the PB or BM, the presence of at least 20% blasts plus promyelocytes in the PB or BM, or the presence of at least 20% basophils in the PB. Blast phase (BP) was defined by the presence of at least 30% blasts in the PB or BM or the presence of extramedullary blastic disease.

BCR–ABL *gene amplification*

The number of copies of the *BCR–ABL* rearrangement was assessed using dual-color fluorescence *in situ* hybridization (FISH) using commercial probes (ES; Vysis). Analysis of interphase nuclei allowed confirmation of the Ph-positive cytogenetic status, duplication or amplification of the *BCR–ABL* gene.

Level of BCR–ABL

Fusion transcripts were quantified by real-time polymerase chain reaction (PCR) using a Light Cycler Roche 2.0 instrument and Syber Green I dye assay; ABL endogenous expression was used to normalize all data. The results were expressed according to the International Scale (IS): a complete molecular response (CMR) was defined as either BCR–ABL/ABL undetectable or below 0.01%, while a major molecular response (MMR) was defined as a transcript level between 0.1 and 0.01% [18].

Mutation analysis

RNA was isolated from total cells from the peripheral blood by Trizol solubilization (Invitrogen). One microgram of RNA was reverse transcribed to

cDNA using random hexamer primers and M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega). The ABL-KD, exons 4–7 of rearranged *BCR–ABL*, was amplified using semi-nested PCR [19]. Briefly, the *BCR–ABL* allele was first amplified; the product of 1327 bp was used as a template for the second PCR of 579 bp that covered amino acids 220–412 of the KD.

PCR products were purified through GFX columns (GE Healthcare). The purified products underwent direct sequencing in an ABI 3130xl (Applied Biosystems) set-up using the Big Dye Terminator Cycle Sequencing Kit. Sequences were compared with the wild-type sequence (Gen Bank #NM_005157).

Statistical analysis

Univariate and multivariate analyses were used to assess correlation between patients' clinical characteristics and mutation status. Descriptive statistics and univariate analysis using the χ^2 test, Fisher exact test, and Student *t*-test were performed on categorical and continuous data. To estimate overall survival (OS) and progression-free survival (PFS) curves, the Kaplan–Meier product limit method was used, whereas between-group comparisons were performed using the log-rank test [20]. OS and PFS were defined based on two starting points: from the time of receiving IM and the time of mutation detection to the date of death or last follow-up.

Clinical characteristics were analyzed for their association with mutation development using standard Cox proportional hazards models [21]. Predictive variables with *p*-values of less than 0.1 for the univariate analysis were included in the multivariate model.

Results

Assessment of mutations was performed in 154 patients, but only 136 had adequate follow-up for a complete evaluation. Ninety-seven patients (63%) were in the chronic phase of the disease, 37 (24%) in the accelerated phase, and 20 (13%) in blast phase (Table I). At the time of mutation analysis, the overall median time from diagnosis was 44.4 months (range 2.8–208); the median age was 49 years (range 20–84); there were 68 female and 86 male patients (gender ratio M/F: 1.26:1). The median time on IM treatment to mutation study was 26.8 months (range 3–80). Seventy-six out of 154 patients (49%) were formerly treated with IFN before IM introduction during their chronic phase.

Point mutations in the KD of BCR–ABL were detected in 36 patients, at 13 different amino acid positions (Table II). These mutations were located within a sequence of 579 nucleotides, involving amino acids 220–412. Simultaneous double mutation was observed in three patients, two in accelerated phase and one patient in blast phase. One of the 36 mutations listed in Table II, V289F, has not been reported in previous international publications. Codon 289 has already been described and published [22], but encoded a different nucleotide substitution.

Four patients harbored the T315I mutation, making its frequency 11% of all mutations found in this series. Three of these patients progressed to blast phase; one died and two remained alive. One patient relapsed after stem cell transplant and remained under control in accelerated phase.

In 21 out of 36 (58%) patients, mutations were detected within the P-loop at amino acids 244, 248, 250, 252, 253, or 255, and in 15 patients (42%), mutations were outside the P-loop (non-P-loop) at residues 289, 298, 311, 315, 351, 355, or 359.

Patients with mutations were switched to second-generation tyrosine kinase inhibitors (TKIs): 14 to dasatinib, four to nilotinib, five to both inhibitors subsequently; seven increased the imatinib dose and six had a BM transplant, four of these followed by dasatinib.

Analysis by FISH of interphase nuclei showed duplication of BCR–ABL in nine cases, indicating clonal evolution, and only one case with 4–6 copies of BCR–ABL, indicating gene amplification.

Clinical characteristics of patients with mutations versus cases without mutations were analyzed. Univariate analysis showed that accelerated phase, lack of molecular response, and lower cytogenetic

Table II. Listed ABL kinase domain mutations (36 patients).

AA substitution	Mutation	Mutation location	No. of patients*
Met244Arg	M244V	P-loop	2
Leu248Val	L248Val	P-loop	1
Gly250Glu	G250E	P-loop	6
Gln252His	Q252H	P-loop	2
Tyr253His	Y253H	P-loop	4
Glu255Val	E255V	P-loop	3
Glu255Lys	E255K	P-loop	3
Val289Phe	V289F	IB	1
Leu298Val	L298V	IB	2
Phe311Ile	F311I	IB	1
Thr315Ile	T315I	IB	4
Met351Thr	M351T	C	5
Glu355Gly	E355G	C	1
Phe359Cys	F359C	C	2
Phe359Ile	F359I	C	1
Phe359Val	F359V	C	1

*Three patients had two different mutations.
AA, amino acid; P-loop, phosphate binding loop; IB, imatinib binding site; C, catalytic domain.

and hematological responses significantly correlated with patients harboring mutations (Table III). Moreover, we observed that patients starting IM after 3 months or more from diagnosis had a higher incidence of mutations (8/25, 32%) when compared with those treated earlier during the first 3 months (7/53, 13%) ($p < 0.05$) (Figure 1). For this evaluation, we excluded patients previously treated with IFN to avoid bias, assuming that this group probably included patients in late chronic phase most likely associated with mutations. Namely, the results suggest that the presence of mutations is significantly associated with the delay from diagnosis to IM onset. No significant differences were observed regarding sex, age, prior IFN treatment, Sokal score, or median duration of disease (Table III). OS from the time of mutation and from the start of IM, comparing the two groups of patients (with or without mutations), showed no significant differences.

Table III. Univariate analysis of clinical characteristics of 136 patients according to mutation status.

	With mutation, $n = 36$ (%)	Without mutation, $n = 100$ (%)	p-Value
Sex			
Female	15 (41.6)	45 (45)	0.826
Male	21 (58.3)	55 (55)	
Age (median)	46 years	50 years	0.221
Prior interferon therapy	20 (57)	45 (45)	0.216
Sokal score			
Low	16 (44.4)	52 (52)	0.275
Intermediate/high	20 (55.5)	48 (48)	
Duration of disease (median)	54.9 months	39 months	0.435
Duration of imatinib therapy (median)	31.3 months	26 months	0.467
Phase of disease			
Chronic phase	11 (30.5)	70 (70)	
AP + BC	25 (69.4)	30 (30)	0.0001
Molecular response			
Complete/major	0 (0)	15 (15)	0.01
Minor/null	36 (100)	85 (85)	
Cytogenetic response			
Major	1 (2.8)	26 (26)	
Minor	14 (38.9)	32 (32)	0.01
Null	21 (58.3)	42 (42)	
Hematologic response			
Complete	7 (19.4)	40 (40)	
Partial	10 (27.8)	33 (33)	0.01
No response	19 (52.8)	27 (27)	
Resistance			
Primary	16 (44.4)	61 (61)	
Secondary	20 (55.6)	39 (39)	0.11
Treatment delay*			
>3 months	8 (53)	17 (27)	0.05
<3 months	7 (47)	46 (73)	

*Patients with previous interferon therapy were excluded.
AP, accelerated phase; BC, blast crisis.

PFS from the time of mutation in the two groups of patients displayed a borderline statistically significant shorter time to progression for cases with ABL mutations ($p = 0.053$) (Figure 2). However, no significant differences were observed when calculating from the start of IM.

Stratifying patients according to point mutation loci (i.e. P-loop vs. non-P-loop or P-loop + T315I vs. non-P-loop without T315I), we found a significantly better OS calculated at the time of diagnosis only for those patients with non-P-loop mutations (97.70 months vs. 154.2 months, $p < 0.01$) (Figure 3). OS and PFS from the time of mutation detection for patients with or without P-loop mutations showed no significant differences.

Finally, in an attempt to detect those factors that could better predict the development of mutations, considering mutations as a time-dependent variable, we performed a multivariate analysis. Patient age,

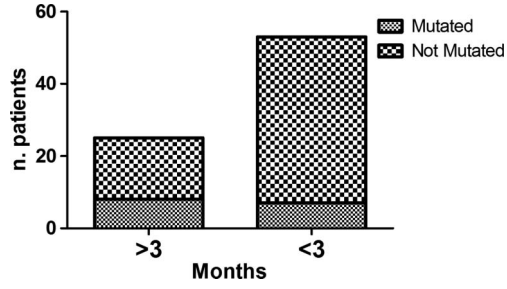

Figure 1. Start of IM therapy more than 3 months from diagnosis had a higher incidence of mutations (8/25, 32%) vs. patients treated with IM within 3 months from diagnosis (7/53, 13%) ($p < 0.05$).

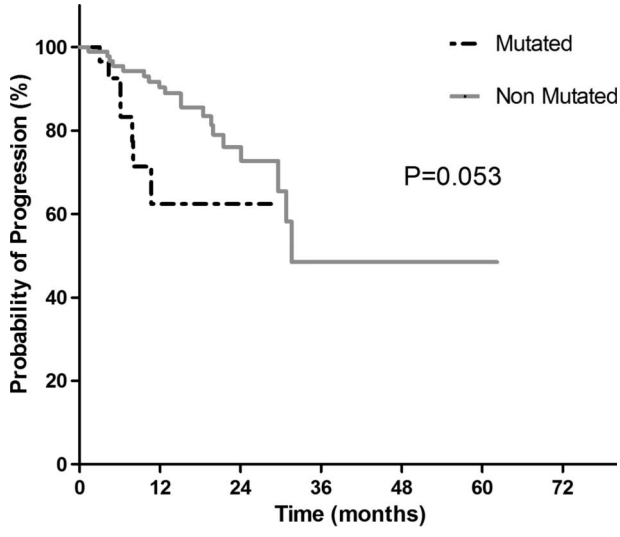

Figure 2. Progression-free survival (PFS) of patients with and without mutations from time of assessment for mutation. We observed a borderline statistically significant ($p = 0.053$) shorter PFS in mutated cases.

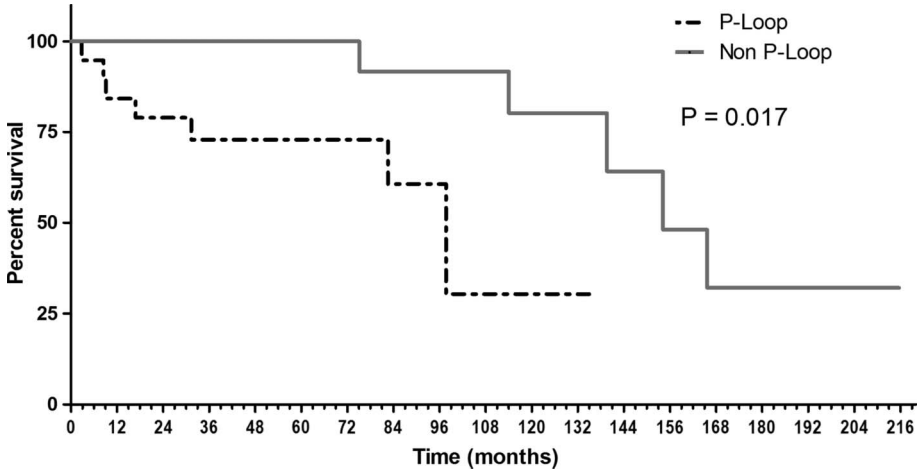

Figure 3. Overall survival (OS) of patients with mutations in P-loop vs. non-P-loop mutations from time of diagnosis of CML. Patients with P-loop mutations had a significantly shorter time of survival ($p = 0.017$).

gender, Sokal score, duration of imatinib therapy, delay time to imatinib start, disease phase, and previous interferon treatment were evaluated. Multivariate analysis showed that accelerated phase ($p < 0.001$), duration of IM treatment ($p < 0.01$), and delay time from diagnosis to IM onset ($p < 0.05$) were the variables independently associated with an increased risk of mutation occurrence.

Discussion

This is the first multicenter Argentinean study for mutations in patients with CML who fail or lose response to IM, with or without previous IFN treatment. The purpose of the present study was to evaluate the mutational status and clinical outcome in a group of IM-resistant patients. Mutations were detected in 36 of 154 patients by conventional direct nucleotide sequencing. As previously reported by others, mutations in the P-loop were more frequent than in IM binding or catalytic domains [10,22–24].

In our series, the single most frequent mutations were G250E, E255K/V, and M351T. These three mutations were reported by Branford *et al.* [25] as the commonest mutations clustered within the ATP binding region of BCR–ABL in resistant patients. The frequency of the T315I mutation in our cohort was 2.6% (4/154); this value varies widely in different published series [10,14,23,26].

In this study, the presence of point mutations in the KD of BCR–ABL showed a significant correlation with the accelerated phase of the disease and with lack of molecular response and lower cytogenetic and hematological responses.

We evaluated the impact of ABL-KD mutations on the clinical outcome of IM-resistant patients. The OS calculated according to the time of mutation study and from IM start did not differ between patients with and without mutations. However, the probability of PFS was higher (borderline significance) in patients with mutations, indicating that nucleotide substitutions are associated with the progression of disease, in concordance with other publications [10,27]. This observation would be relevant to predict disease progression.

Several studies have pointed out that mutations in the P-loop confer a particularly poor outcome when evaluating OS or PFS from both IM onset or mutation detection [10,23], although controversial results have been published [28]. Our data indicate that P-loop mutations were not associated with poor OS or PFS evaluated from the time of mutation. Similar results have been published by Jabbour *et al.* [28]. However, evaluating OS from the time of diagnosis, P-loop mutations were associated with shorter survival. These data may be explained by the presence of small mutated clones from diagnosis, becoming more aggressive malignant clones over time [29–31]. Low levels of specific mutant clones could have a different clinical significance, suggesting the use of more sensitive mutation detection methods as routine analysis for BCR–ABL mutations.

In our series, gene amplification of BCR–ABL, assessed by FISH, was detected in 10/154 (6.5%) patients. This frequency is within the range reported in clinical samples from patients who were resistant to imatinib [19,25,32]. Nine cases showed duplication of BCR–ABL, indicating double Ph chromosome, and one case showed multiple copies. Fifty percent of these cases died in blast crisis, indicating that the mechanism of amplification produces BCR–ABL overexpression and progression of the disease.

A further issue that emerges from our study is the higher probability of harboring mutations for those

patients with delay to starting IM from diagnosis. In the multivariate analysis, therapy onset after 3 months from diagnosis was associated with a higher incidence of mutations, suggesting that IM treatment should be started earlier. Whether this finding could become a predictive factor for mutations or not is a matter for discussion, and further analysis will be necessary to confirm this not previously reported correlation.

In conclusion, our results indicate that mutation characterization could be important to anticipate disease progression, and that early and sensitive detection could provide clinical benefit for patients with CML by leading to early reconsideration of therapeutic strategies.

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