Original article

Frequency of ABL gene mutations in chronic myeloid leukemia patients resistant to imatinib and results of treatment switch to second-generation tyrosine kinase inhibitors

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\textbf{ABSTRACT}

\textit{Background and objectives:} Tyrosine kinase inhibitors (TKI) have improved the management of patients with chronic myeloid leukemia (CML). However, a significant proportion of patients do not achieve the optimal response or are resistant to TKI. ABL kinase domain mutations have been extensively implicated in the pathogenesis of TKI resistance. Treatment with second-generation TKI has produced high rates of hematologic and cytogenetic responses in mutated ABL patients. The aim of this study was to determine the type and frequency of ABL mutations in patients who were resistant to imatinib or had lost the response, and to analyze the effect of second-generation TKI on their outcome.

\textit{Patients and methods:} The presence of ABL mutations in 45 CML patients resistant to imatinib was evaluated by direct sequencing and was correlated with the results of the cytogenetic study (performed in 39 cases). The outcome of these patients after therapy with nilotinib or dasatinib was analyzed.

\textit{Results:} ABL mutations were detected in 14 out of 45 resistant patients. Patients with clonal cytogenetic evolution tended to develop mutations more frequently than those without clonal evolution. Nine out of the 15 patients with ABL mutation responded to a treatment switch to nilotinib (n = 4), dasatinib (n = 2), interferon (n = 1) or hematopoietic stem cell transplantation (n = 2).

\textit{Conclusion:} The frequency of ABL mutations in CML patients resistant to imatinib is high and is more frequent among those with clonal cytogenetic evolution. The change to second-generation TKI can overcome imatinib resistance in most of the mutated patients.

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Frecuencia de mutaciones del gen ABL en pacientes con leucemia mieloide crónica resistentes a imatinib y resultado del cambio de tratamiento a inhibidores de tirosín-cinasa de segunda generación

\textbf{RESUMEN}

\textit{Fundamento y objetivos:} La mayoría de los pacientes con leucemia mieloide crónica (LMC) obtienen respuesta clínica bajo tratamiento con imatinib. Sin embargo, una proporción significativa de ellos no alcanza dicha respuesta o son resistentes al tratamiento, implicándose en ello mutaciones del gen ABL. El...
Introduction

Chronic myeloid leukemia (CML) is a clonal malignant disease of the hematopoietic stem cell characterized by the presence of the Philadelphia chromosome (Ph) caused by a reciprocal translocation between the long arms of chromosomes 9 and 22, resulting in the BCR-ABL1 fusion gene. This gene encodes a chimeric protein with constitutive tyrosine kinase activity that enhances cellular proliferation, resistance to apoptosis and oncogenesis, and contributes to unregulated expansion of mature myeloid cells. Imatinib (Novartis Pharmaceuticals, Basel, Switzerland), the first tyrosine kinase inhibitor (TKI) approved for the treatment of CML, targets the tyrosine kinase activity of BCR-ABL1, and binds to this fusion gene in the inactive conformation.

In order to best determine the individual response to therapy, an operational set of goals defined within specific periods of time have been established for all patients according to the European Leukemia Net recommendations. According to the international scale, patients who achieve major molecular response (MMR), equivalent to a reduction in BCR-ABL1 ratio less than 0.1% before 18 months, are predicted to have a remarkably low risk of disease progression. Patients failing to achieve defined responses in this chronological time point are described as primarily resistant to therapy. A number of patients still do not succeed in obtaining complete hematologic response (CHR), 20–25% of patients do not achieve a complete cytogenetic response (CCyR, defined as <1% of Ph-positive metaphase cells at 12 months) and less than 10% of patients achieve complete molecular response at 18 months. Loss of previous responses to imatinib is termed secondary resistance and occurs in 20–25% of patients that reach CHR and/or CCyR. The lack of MMR leads to a loss of CCyR in a proportion of patients. It is important to keep these data in mind in order to choose the best therapeutic choice for each patient. Mutations in the ABL domain are the most important mechanism of imatinib resistance. Numerous mutations have been characterized throughout the ABL sequence. The frequency of mutations seems to increase as patients progress from the chronic phase through to the accelerated or blast phase disease. Mutations in the ABL domain of the BCR-ABL1 gene represent 40% of secondary resistances to first and second-generation TKI.

The second-generation TKI, dasatinib (Bristol-Myers Squibb, New York, USA) and nilotinib (Novartis Pharmaceuticals, Basel, Switzerland), are both effective in a proportion of patients with imatinib failure. Dasatinib, a dual SRC-family kinases/ABL kinase inhibitor, and nilotinib, a more specific inhibitor of ABL kinase, also inhibit Kit and PDGFR receptors and have greater inhibitory potency than imatinib.

The aims of this study were: (1) to determine the type and frequency of ABL mutations in 45 consecutive patients who were sent to our institution for study of ABL mutations because of resistance or loss of response to imatinib, and (2) to analyze their response to the second-generation TKI, dasatinib and nilotinib.

Patients and methods

Patient samples

The study population consisted of 320 patients diagnosed with CML according to the World Health Organization classification in six Spanish centers since 2004. Forty-five out of these patients (38 in chronic phase, 2 in accelerated phase and 5 in blast crisis) were resistant or had lost their response to imatinib and were sent to our laboratory for study of ABL mutations. All samples were collected after obtaining informed consent in accordance with approved Institutional Research Boards protocols. Peripheral blood (PB) was obtained when patients were on imatinib treatment, but 8 were switched to second-generation TKI before the study of mutations. Analysis of ABL mutations was performed in 23 patients with suboptimal response, 14 with treatment failure, 4 who lost molecular response and 4 patients who progressed to blast phase.

Cytogenetic study

This study was performed in 39 out of 45 patients. Bone marrow cells from each patient were cultured in RPMI1640 medium supplemented with fetal calf serum, antibiotic and glutamine for 24 h. Conventional chromosome G-banding preparations were performed using standard techniques and karyotypes were formulated according to the International System for Cytogenetic Nomenclature.

Study of mRNA expression of BCR-ABL1

Whole PB samples were collected in 10 mL EDTA tubes. White blood cells were isolated by lysing the red cell component with a buffer containing ammonium chloride (157 mM), EDTA (94 μM) and HEPES (10 mM) in sterile water. RNA was extracted from approximately 1 × 10^6 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until use. Complementary DNA was synthesized using Random Primers and MMLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). BCR-ABL1 was amplified using PCR primers as previously described. Real-time quantitative PCR was carried out on ABI7900 PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). Messenger RNA (mRNA) expression of BCR-ABL1 was calculated as a percentage relative to ABL expression to determine if the allelic burden was sufficient to succeed with the ABL mutational study.
The normal ABL allele was excluded from mutational study by amplifying a BCR-ABL1 amplicon from exon 13 of the BCR gene to exon 10 of the ABL gene using primers B2A: 5’-TCCA-GAAGCTCTCCACATCAT-3’ and JAMR: 5’-GTACTCACGCCAC-CAGGA-3’.15

Direct sequencing

The BCR-ABL1 amplicon from each subject in the study was carried out by nested PCR using primers NTPB+: 5’-AACCGCCAA-CAACCTTCTGATGAC-3’ and NTPE–: 5’-CTTGCGTCTGATGAT-3’ to generate an 863-bp fragment containing the entire BCR-ABL1 kinase domain. An aliquot of the PCR product was electrophoresed through 2.0% agarose gel and the PCR fragment containing the ABL kinase domain was isolated using a commercially available gel purification kit (QiAquick, QiAgen, IZASA, Germany). The purified amplicons were then subjected to Sanger’s dideoxy chain termination reaction using Big-Dye V3 in an ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA). The reaction was primed with oligonucleotides NTPB+, NTPE–, ABLK: 5’-AACCGCCAGGCCTCTGAC-3’; 41R: 5’-CTTGGCTGACGCT-3’ and CR: 5’-GTATCTCAAGGCCTGTTGCA-3’.16 In each case the sequence obtained was compared with the published ABL1 sequence, GenBank U07563, using BLAST 2 software.

Results

Cytogenetic results

Cytogenetic studies were available in 39 out of 45 patients (28 unmuted and 11 mutated). Seventeen showed a normal karyotype at the time of the mutational study. Fourteen patients (3 mutated) presented the same karyotype as at the diagnosis (12 cases had the t(9;22) as the only chromosomal alteration and two patients had the t(9;22) in the context of a complex karyotype) while 8 cases (5 mutated) developed additional chromosomal abnormalities (Table 1). A significant correlation was established between the clonal cytogenetic evolution and the presence of ABL gene mutations (p = 0.028) (Table 2a).

Results of mutational study

Mutations of the ABL gene were detected in 14 out of 45 studied patients (31%), with more than one mutation in 3 (Table 1). The incidence of kinase domain mutations was 26% for chronic phase and 67% for accelerated and blast crisis phases. In 3 out of 15 patients, mutations were detected in the P-loop at amino acids 244, 250 and 253, and in 11 out of 14 patients, mutations were outside the P-loop and affected residues 276, 277, 311, 315, 317, 340, 351, 359, 379 and 417. We could not establish a recurrence of the type of mutations detected among the different patients or a higher frequency of P-loop mutations in cases of accelerated phases of the disease.

Cases with suboptimal response at the time of the mutational study presented less susceptibility to develop mutations, although a significant correlation could not be established (p = 0.062). Only 6 out of 20 patients with suboptimal response developed mutations and 2 of these 6 mutated patients had a clonal cytogenetic evolution. On the contrary, patients who progressed to accelerated phases of the disease showed a tendency to develop mutations and additional chromosomal abnormalities (3 out of 4 patients) (Table 2b).

In our study, two patients with the F317L mutation had a blast crisis CML at the time of the mutational study. These two patients received dasatinib prior to the study of ABL mutations and died before a change of treatment could be implemented. We identified

### Table 1

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<th>Characteristics of the 14 patients harboring ABL mutations</th>
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**AP:** accelerated phase; **CP:** chronic phase; **BC:** blast crisis; **IM:** imatinib; **DA:** dasatinib; **NI:** nilotinib; **del:** deletion; **HR:** hematologic response; **CCyR:** complete cytogenetic response; **PCyR:** partial cytogenetic response; **MCyR:** minor cytogenetic response; **MR:** molecular response.
three patients with T315I mutations, two in chronic phase and one in blast crisis.

Treatment and outcome of patients

After the mutational study, 6 patients were treated with second-generation TKI (4 with nilotinib and 2 with dasatinib) as a single agent. Two achieved a MMR, one patient is in CCyR and the remaining two are in MCyR and PCyR, respectively. One patient treated with nilotinib was submitted to hematopoietic stem cell transplantation (HSCT) and is in MMR. Two patients underwent HSCT on detection of mutation and achieved MMR. In summary, 3 out of 7 patients treated with second-generation TKI have reached CCyR (n = 1) or MMR (n = 3) independently of the presence of t(9;22) either isolated or with additional chromosomal abnormalities. Only one patient treated with nilotinib as the second option did not reach a cytogenetic response one year after detection of the mutation.

The two chronic phase patients with the T315I mutation were treated with IFN and nilotinib achieving PCyR and MCyR, respectively, and are alive. The other T315I patient, who was treated with imatinib 400 mg/24 h and with dasatinib 70 mg/12 h previously to the study of mutations, died before a change of treatment could be performed.

Eight out of 31 patients without ABL mutations achieved CCyR, 2 were in PCyR and the remaining 10 reached MMR after treatment with second-generation TKIs. Only one unmutated patient died after HSCT due to infection by Fusarium.

Discussion

On the basis of the IRIS study data, 30–35% of patients would need to change therapy at some point in the treatment due to imatinib resistance. Approximately 30–40% of patients showing failure to imatinib may be successfully rescued by second-generation TKIs. In our study, 31% of the cases analyzed presented mutations in the ABL gene, according to the literature.

Clonal evolution is present at the time of imatinib failure in 24% of patients and BCR-ABL kinase mutations occur more frequently in patients with clonal evolution than in those without it. Our results are in accordance with these observations. We detected additional cytogenetic alterations in 5 out of 11 mutated cases while we could only observe additional abnormalities in 3 out of 28 unmutated cases (Table 2). All patients with clonal cytogenetic evolution achieved a response to the switch to the TKI.

The detection of additional mutations is an important issue as mutation frequency also appears to increase as patients progress from a chronic to a blast phase. It has been postulated that certain mutations seem to occur more often in different disease phases. For example, substitutions at M244, L248, F317, H396 and S417 are more likely to occur in patients in chronic phase disease, whereas those at Q252, Y253, E255, T315, E459 and F486 are associated with advanced phase disease. However, we found a F317 mutation in two patients in blast crisis who were treated with dasatinib prior to the mutational study. This is in concordance with what has been described in previous studies in which dasatinib treatment induced the appearance of new mutations such as F317. It has also been postulated that mutations in the P-loop tend to have a worse prognosis and more rapid progression to advanced stage disease. In our case, we could not establish an association between P-loop mutations and prognosis.

Although the T315I mutation has been described in advanced phases, we identified this mutation in the chronic phase, probably due to the early study of ABL mutational status in patients with suboptimal response or failure to treatment.

According to the literature, approximately 50% of cases resistant to imatinib achieve CCyR with a second-generation TKI, and the 2-year PFS rate after therapy with these agents is 64–81%. Once the indication for treatment change is established, the challenge lays in selecting the best agent. The presence of a mutation can provide guidance, since some mutants have a higher sensitivity to one agent than to others. For example, in patients with a F317L mutation, nilotinib is a better choice than dasatinib, whereas in those with a F359V, dasatinib is preferable. All of our patients were treated according to this criterion after mutational study and almost 50% achieved MMR. Similar results were obtained in the unmutated cases on switching the treatment to second-generation TKIs.

One of the possible limitations of our current analysis is that some of the patients were treated with more than one TKI before the study of the ABL gene mutations. This feature makes it difficult to determine the significance of the appearance of one mutation or another. Patients were referred to our center from different hospitals and the treatment choice and mutational analysis request were not uniform in all centers. This makes it difficult to establish a correlation between the duration of the initial treatment, the moment of appearance of the mutation and the type of mutation due to the number of patients included in this study. On the other hand, our series of patients is too small to establish correlations between the type of mutations detected and the prognosis and between the initial response and the presence of mutations and additional chromosomal alterations.

Changing therapy as soon as failure or suboptimal response is recognized is important, since the outcome seems to improve in patients treated as soon as criteria for cytogenetic failure are met rather than waiting until hematologic response is also lost. In our series 9 out of the 14 patients who carried an ABL mutation benefited from a switch in treatment to second-generation TKIs independently of the cytogenetic status, even in cases with additional chromosomal abnormalities. Further studies in larger sample sizes are required to support these results.

Funding

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Table 2
Correlation between ABL gene mutations and clonal cytogenetic evolution (a) and response to initial treatment (b).

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<tr>
<th>(a) Clonal evolution (n=8) No clonal evolution (n=31)</th>
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<th>(b) Mutation (n=11) No mutation (n=28)</th>
<th>Suboptimal response (n=20) Failure (n=11) Progression (n=4) Loss of molecular response (n=4)</th>
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No Mutation Clonal Evolution

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Conflict of interest

The authors declare no conflict of interest.

Appendix A

Grupo ICO de estudio de mutaciones de ABL en pacientes afectos de LMC: Silvia Marcé, Lurdes Zamora, Marta Cabezon, Blanca Xicoy, Concha Boqué, Olga García, Diana Dominguez, Jordi Ribera, Cristina Fernández, Lluis Rodriguez, Imma Portal, Ester Plensa, Patricia Velez, Jorge Medina, Marisol Xandri, José-Tomàs Navarro, Alberto Fernández de Sevilla, Josep-Maria Ribera, Evarist Feliu, Fuensanta Millà.

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