Exon 7 Deletion in the bcr-abl Gene Is Frequent in Chronic Myeloid Leukemia Patients and Is Not Correlated with Resistance against Imatinib

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Abstract

Chronic myeloid leukemia (CML) patients treated with imatinib develop frequent resistance generally due to a point mutation. Recently, large rearrangements of abl sequence have also been described. In this study, we focused on the complete deletion of exon 7. We screened for bcr-abldelexon7 in 63 resistant patients by high-resolution melting (HRM) analysis and direct sequencing. Moreover, we analyzed expression of abldelexon7 and bcr-abldelexon7 in 17 CML patients at diagnosis, 32 patients at resistance, and 20 negative controls by quantitative PCR or fragment length analysis. bcr-abldelexon7 was detected on 34 (54%) among 63 resistant patients by HRM, showing an increase in the sensitivity of screening, because only 3.2% could be detected by direct sequencing. This deletion was not associated with a point mutation (P = 0.3362). In addition, abldelexon7 was found in all tested samples with the same pattern of expression, suggesting an alternative splicing mechanism. In the bcr-abl component, there was no statistical difference between CML patients at diagnosis and resistant patients (P = 0.2815) as regarding bcr-abldelexon7 proportion, thus arguing against involvement of deletion in resistance. Moreover, among two patients harboring bcr-abldelexon7 at diagnosis, one experienced a complete disappearance of this transcript, and the other decreased >75% at resistance. In conclusion, bcr-abldelexon7 is frequently observed in CML patients when using sensitive techniques. It seems to be the result of an alternative splicing mechanism and to be independent from the occurrence of resistance. Mol Cancer Ther; 9(11); 3083–9. ©2010 AACR.

Introduction

Chronic myeloid leukemia (CML) is a hematologic malignancy characterized by a chromosomal abnormality, the Philadelphia chromosome, due to a reciprocal translocation involving the long arms of chromosomes 9 and 22, t(9;22)(q34;q11). This rearrangement leads to a fusion gene, bcr-abl, which encodes a chimeric protein, BCR-ABL. This neoformed oncoprotein carries a kinase domain in a constitutively active form, which is essential for cell transformation, proliferation, and survival through interaction and dysregulation of intracellular processes involving RAS, phosphatidylinositol 3-kinase/AKT, and signal transducer and activator of transcription 5 pathways (1).

Before the imatinib era, IFN-α was considered as the gold standard therapy, because a major cytogenetic response rate (i.e., at least 35% of cells without Philadelphia chromosome) has been observed in 28% of patients at 24 months and an overall survival rate at 5 years in 68% (2). The IRIS phase II trial highlighted the ability of imatinib to induce complete cytogenetic response and improve overall survival (3). Namely, a 6-year follow-up of patients included in the IRIS study showed a rate of complete cytogenetic response of 82% and an estimated overall survival at 5 years of 95% (4). Imatinib mesylate is a competitive inhibitor of ATP binding to the BCR-ABL tyrosine kinase, inhibiting its phosphorylation capacity (5, 6). Despite this targeted therapy, it is not infrequent, ranging from 15% to 40% (7, 8), that patients develop resistance (5, 6). Despite this targeted therapy, it is not infrequent, ranging from 15% to 40% (7, 8), that patients develop resistance. Several mechanisms have been described, some of them being independent from BCR-ABL—like inappropriate drug level concentrations due to efflux/influx abnormalities and others being BCR-ABL dependent, like genetic amplification and acquisition of mutations in ABL tyrosine kinase domain (8). For these latter cases, resistance depends on the amino acid involved. To date, up to 100 point mutations have been described, 17 of them accounting for roughly 80% to 90% of cases (9, 10). Recently, new kinds of mutations have been described, which do not correspond to a point mutation but to a large rearrangement-like in-frame deletions in exon 4 (11), insertion of 35 bp between exon 8 and exon 9 (12), insertion of 12 nucleotides...
in exon 5 (13), and complete deletion of exon 7 (14, 15). It is to date generally assumed that these “new mutations” are related to a high-degree resistance level to imatinib.

In the present article, we studied a series of cases of resistance to imatinib with exon 7 deletion to better evaluate its frequency and its involvement in the resistance process by comparison with newly diagnosed CML cases and normal controls.

Materials and Methods

**Patients and samples**

The data set included 17 CML patients at diagnosis and 63 resistant patients defined according to ELN 2009 criteria (16) as an absence of complete cytogenetic response at 12 months, or an absence of major molecular response at 18 months (i.e., bcr-abl/abl ratio lower than 0.1%), or at any moment a loss of cytogenetic/molecular response, seen in our laboratory between 2001 and 2010. All patients received between 400 and 800 mg/d imatinib from diagnosis (or from the time this drug became available) until resistance.

Twenty patients negative on bcr-abl screening were included as negative control.

Total RNA was isolated from peripheral blood cells using the Trizol/phenol/chloroform method, followed by a linearization step at 70°C during 10 minutes. RNA reverse transcription was done using 1 μg of total RNA, random hexamer primers at 25 μmol/L of each deoxynucleoside triphosphate (dNTP), 10 mmol/L DTT, and 20 units of RNase inhibitor with 100 units of the wild-type transcript, bcr-ablwt, was sized to 351 bp (exon 7 size, 185 bp). Sequencing analysis of fragment analysis products was done in forward 5′ fluorescence-labeled one (Eurofins MWG Operon). Amplified cDNA (1 μL) was mixed with 2.5 μL of 10× Buffer, 5 μL of SolutionQ, 1.5 μL of 25 mmol/L MgCl₂, 1 μL of 5 mmol/L dNTP mix, 10 pmol of 10 μmol/L forward primer and 15 pmol of reverse primer; 0.5 μL of HotStarTaq (5 U/μL; Qiagen), and distilled water in a final volume of 25 μL. Thermal cycling conditions were as follows: 94°C for 7 minutes for denaturation, then 95°C for 60 seconds, 59°C for 40 seconds, 72°C for 90 seconds, repeated for 20 cycles, followed by 72°C for 5 minutes. After amplification, 2 μL of PCR product were mixed with 12 μL of deionized formamide and 0.4 μL of GeneScan 500 ROX Size Standard (Applied Biosystems), followed by a quick denaturation step at 95°C during 3 minutes stopped by chilling on ice. Samples were loaded onto the 3130xl Genetic Analyzer with POP7 (Applied Biosystems), a capillary electrophoresis system with fluorescence detection. The size and height of the peak were determined using GeneMapper Software.

The PCR and melting analysis for abldelexon7 form

On bcr-abl PCR products, we did a nested PCR lead on the abl component with the same primers as used in HRM analysis. The only difference was the use of a forward 5′ fluorescence-labeled one (Eurofins MWG Operon). Amplified cDNA (1 μL) was mixed with 2.5 μL of 10× Buffer, 5 μL of SolutionQ, 1.5 μL of 25 mmol/L MgCl₂, 1 μL of 5 mmol/L dNTP mix, 10 pmol of 10 μmol/L forward primer and 15 pmol of reverse primer; 0.5 μL of HotStarTaq (5 U/μL; Qiagen), and distilled water in a final volume of 25 μL. Thermal cycling conditions were as follows: 94°C for 7 minutes for denaturation, then 95°C for 60 seconds, 59°C for 40 seconds, 72°C for 90 seconds, repeated for 20 cycles, followed by 72°C for 5 minutes. After amplification, 2 μL of PCR product were mixed with 12 μL of deionized formamide and 0.4 μL of GeneScan 500 ROX Size Standard (Applied Biosystems), followed by a quick denaturation step at 95°C during 3 minutes stopped by chilling on ice. Samples were loaded onto the 3130xl Genetic Analyzer with POP7 (Applied Biosystems), a capillary electrophoresis system with fluorescence detection. The size and height of the peak were determined using GeneMapper Software.

**Quantification of bcr-abldelexon7 form**

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The wild-type transcript, bcr-ablwt, was sized to 352 bp, and the bcr-abldelexon7 was sized to 167 bp (exon 7 size, 185 bp). bcr-abldelexon7/bcr-abl proportion was assigned, making the ratio of the height of the peak of bcr-abldelexon7 sum of the height of the peak.

**Sequencing analysis of fragment analysis products**

After migration of amplicons on agarose gel, PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer’s protocol and directly sequenced in forward and reverse directions with the same primers used for the nested PCR onto the 3130xl Genetic Analyzer after purification with the BigDye XTerminator (Applied Biosystems).

**Quantification of abldelexon7 form**

**Plasmid construct.** A PCR with a forward primer overlapping abl exons 6 and 8, in excess of Taq polymerase to add additional A at the 5′ and 3′ extremities, was used to produce an amplicon carrying the abldelexon7 with a 309-bp size. After checking PCR specificity, this amplicon was inserted in a vector and then in a chemically competent Escherichia coli (Invitrogen). After 2 days of culture on isopropyl-l-thio-B-d-galactopyranoside/
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ampicillin medium, white colonies were isolated; after overnight culture in Luria-Bertani medium, plasmids were extracted and then sequenced with the same protocol as previously described. In parallel, DNA dosage was done using a NanoDrop and concentration (X) was converted into plasmid copy number/µL using the following formula: X g µL⁻¹ DNA/[plasmid length in base pairs x 660] x 6,022.1023 = Y molecules/µL. In this case, the plasmid length was 4,177 bp.

Quantitative PCR analysis. Quantitative PCR was done on a LightCycler 480. For that, 2 µL of cDNA were mixed with 10 µL of SYBR Green Master Mix (Roche Diagnostics), 1 µL of relevant primers (10 µmol/L), and 8 µL of H₂O. All samples for each gene (i.e., abl as reference gene and abldelexon7 as target gene) were made in duplicate. Standard curves were obtained for abl using three known dilutions of abl plasmid (Ipsogen) and for abldelexon7 with six dilutions of our plasmid, concentrations spreading between 10⁸ and 10³ copies/µL.

Statistical analysis
To estimate whether the difference in the ratio bcr-abldelexon7/bcr-abl and abldelexon7/abl was statistically significant between different groups (patients and healthy donor), we did a nonparametric test (Wilcoxon), and a P value of <0.05 was considered statistically significant. χ² or McNemar χ² test (for matched samples) was used for comparisons of frequencies.

Results
bcr-abldelexon7 rate increases with the sensitivity of techniques used for screening
Sequences were analyzed with sequencing analysis in the two reading directions. Deletion of exon 7 leads to a frameshift. On a cohort of 63 resistant patients, 15 patients
harbored a point mutation and only 2 patients (3.2%) harbored the *bcr-abl*delexon7 mutation. All patients harboring *bcr-abl*delexon7 show in fact a mixture with wild-type kinase domain and the deleted one.

To improve the detection of *bcr-abl*delexon7, we developed an HRM assay for its screening. The normalized melting curves of each genotype are shown in Fig. 1. The slope of the melting curve of patients harboring *bcr-abl*delexon7 was markedly reduced, and so, the deleted allele could be easily distinguished from the wild-type one. Analysis was actually done on second derivative melt curve plots, which plot the rate of change in fluorescence against the temperature because it is easier to visualize the different melting domains. The presence of *bcr-abl*delexon7 leads to a shift in the Tm from 87.5°C to 84.3°C (P < 0.0001). Method specificity was confirmed by sequencing HRM products in the two reading directions with the same primers used for HRM analysis. After agarose migration, patients with two peaks in HRM analysis show two bands. The upper one corresponds to *bcr-abl*delexon7 and the lower one to *bcr-abl*wt.

Using HRM analysis, among the 63 patients tested, 34 (54%) show a shift in Tm, interpreted as the presence of *bcr-abl*delexon7. The two patients found to be deleted in sequencing analysis were indeed confirmed by HRM analysis. As in sequencing method, all patients harboring *bcr-abl*delexon7 show in fact a mixture of *bcr-abl*delexon7 and *bcr-abl*wt. Moreover, the height of the peak of *bcr-abl*delexon7 is not constant, suggesting variations in *bcr-abl* delexon7 amount.

On the other hand, 12 patients with *bcr-abl*delexon7 associated a point mutation (35.3%), as well as 7 of 29 patients with *bcr-abl*wt. No significant difference in the number of detected mutations between patients harboring *bcr-abl*delexon7 or *bcr-abl*wt was detected (P = 0.3362; Table 1).

*bcr-abl*delexon7 is a splice variant of native *abl*

Specificity of our *abl*delexon7 cDNA was proved using a plasmid harboring no *abl*delexon7, which showed no amplification, allowing us to quantify only *abl*delexon7 transcripts without interferences. The plasmid *abl*delexon7 standard curve is characterized by a slope of −3.361 and a Y intercept of 37.81. Ratio was normalized to *abl* gene, showing a curve with a slope of −3.475 and a Y intercept of 38.93.

### Table 1. Frequencies of *bcr-abl*delexon7 by direct sequencing and HRM analysis and its association with point mutation

<table>
<thead>
<tr>
<th>Technique for screening</th>
<th>Genotype</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>bcr-abl</em>wt†</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>bcr-abl</em>delexon7‡</td>
<td></td>
</tr>
<tr>
<td>Sequencing analysis</td>
<td>61 (96.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (3.2)</td>
<td>&lt;0.05§</td>
</tr>
<tr>
<td>HRM analysis</td>
<td>29 (46)</td>
<td>34 (54)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Point mutation associated</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>No point mutation associated</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>

* †*bcr-abl*wt: patients with no exon 7 deletion with or without point mutation.
† † † *bcr-abl*delexon7: patients with exon 7 deletion with or without point mutation.
‡ † † † Patients with *bcr-abl*delexon7 detected by HRM analysis versus direct sequencing by McNemar χ² test.
§ † † † † Patients with *bcr-abl*delexon7 (HRM) associating a point mutation versus ones with no point mutation by χ² test.

(23.8%) harbored a point mutation and only 2 patients (3.2%) harbored the *bcr-abl*delexon7 mutation.

All patients harboring *bcr-abl*delexon7 show in fact a mixture with wild-type kinase domain and the deleted one.

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**Abfdelexon7 is a splice variant of native abl**

Specificity of our *abl*delexon7 cDNA was proved using a plasmid harboring no *abl*delexon7, which showed no amplification, allowing us to quantify only *abl*delexon7 transcripts without interferences. The plasmid *abl*delexon7 standard curve is characterized by a slope of −3.361 and a Y intercept of 37.81. Ratio was normalized to *abl* gene, showing a curve with a slope of −3.475 and a Y intercept of 38.93. First, all samples, including negative control CML

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**Table 2. Expression of *abl*delexon7 and *bcr-abl*delexon7 compared with diagnosis and resistance status**

<table>
<thead>
<tr>
<th>Type of transcript</th>
<th>Status</th>
<th>n</th>
<th>Normalized ratio (%)</th>
<th>P*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>abl</em>delexon7/abl</td>
<td>CML at diagnosis</td>
<td>17</td>
<td>1.81</td>
<td>0.0743</td>
<td>0.0130</td>
</tr>
<tr>
<td></td>
<td>CML at resistance</td>
<td>32</td>
<td>1.37</td>
<td>0.1286</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>20</td>
<td>1.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bcr-abl</em>delexon7/</td>
<td>CML at diagnosis</td>
<td>11</td>
<td>21.3</td>
<td>0.2815</td>
<td></td>
</tr>
<tr>
<td>CML at resistance</td>
<td>12</td>
<td>29.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* †CML at diagnosis or at resistance versus negative control.
† † CML at diagnosis versus CML resistant.
‡ † † Eleven and 12 among 17 and 18 patients, respectively, for whom *bcr-abl*delexon7 was detectable.

CML patients at diagnosis show a median ratio at $1.81\%$, resistant CML at $1.37\%$, compared with negative controls with a ratio of $1.63\%$. No statistically significant difference was found neither between CML at diagnosis and negative control ($P = 0.0743$) nor between resistant CML and negative control ($P = 0.1286$). However, the difference was found between CML at diagnosis and resistant CML ($P = 0.0130$) due to a higher mean for CML at diagnosis and a lower mean for resistant CML compared with negative control (Table 2).

**bcr-abldelexon7 is not related to occurrence of resistance**

Quantification of bcr-abldelexon7 after a nested PCR (long-range PCR for selection of bcr-abl transcript) with the same technique as the one used for quantification of abldelexon7, namely, use of SYBR Green, which incorporates in any double-strand DNA, risked to quantify not desired amplicons. That is why we chose a fragment analysis method.

The presence of bcr-abldelexon7 was not more common in resistant patients than in patients at diagnosis ($P = 0.8149$). Indeed, among the 17 patients at diagnosis, 11 (64.7%) show a peak corresponding to bcr-abldelexon7 in mixture with bcr-ablwt (Fig. 2) and, for resistant patients, we detected a bcr-abldelexon7 in 12 patients (18 patients could be tested because, for others patients, the quantity amount of bcr-abl was too low to do fragment analysis).

This technique allowed us to know if there was a correlation between expression level of bcr-abldelexon7 and resistance (Table 2). When the deleted form was present, with a mean expression for patients at diagnosis of $21.3\%$, there was no difference in expression for resistant patients ($29.11\%$, $P = 0.2815$). Moreover, for six resistant patients, samples at diagnosis were available. Two patients showed no bcr-abldelexon7 neither at diagnosis nor at resistance, and one showed appearance of the deleted form at time of resistance. On the contrary, in two patients harboring bcr-abldelexon7 at diagnosis, one experienced a complete disappearance of this transcript, and the other decreased of $>75\%$ at resistance. So, bcr-abldelexon7 and its expression did not seem to be involved in development of resistance against tyrosine kinase inhibitors.

**Discussion**

Occurrence of point mutations is the main mechanism of acquired resistance in CML (18–20), and bypassing them is the major challenge for the next years. In addition, different deleted or inserted forms have been described recently and several studies focused on their incidence and putative deleterious potential. bcr-abldelexon7 was first described by Curvo et al. (14) who found it in five resistant patients and hypothesized that it could be related to imatinib resistance. In a large cohort of resistant patients, Sherbenou et al. reported 2 cases of this deletion among 101 samples. Using more sensitive techniques, such as HRM (21) or PCR fluorescent fragment analysis, we show in the present study that bcr-abldelexon7 is not so rare. Indeed, its incidence is $\sim 54\%$ in our cohort, whereas by direct sequencing we could detect only $3.2\%$ cases, a result very similar to what was previously published ($1.98\%$). This is not surprising because sensitivity of direct sequencing is roughly $20\%$ (22), and we found a median expression of $27.4\%$ of bcr-abldelexon7, ranging from $6.4\%$ to $55.2\%$. So, by direct sequencing, a minor mutated population cannot be separated from
background signal, hence a clear underevaluation of deletion frequency.

Different studies hypothesized that bcr-abl^del exon7 could be correlated with a high degree of resistance against tyrosine kinase inhibitor, only based on its presence in a resistant patient cohort, without comparison with a normal control population. Our results lead to a quite different interpretation. First, studying abl transcript, we found this exon 7 deletion in all samples tested, among bcr-abl negative as well as positive donors, suggesting that abl^del exon7 probably results from an alternative splicing of the abl gene. Roughly 90% of human protein-coding genes have alternative splicing form, this mechanism being a key regulator of gene expression (23, 24). For the abl gene, one alternative variant has already been described and studied (25), and four others have been annotated in different databases such as Havan a or Ensemble genebuild, but none of them includes an exon 7 deletion while retaining normal exons 6 and 8. This exon skipping is not random, some exons being more sensitive to alternative splicing, first due to the presence of elements in DNA sequences enhancing splicing as ESE (exonic splicing enhancer), ISE (intronic splicing enhancer), or Alu elements but also to the localization of the exon in tertiary structure of the protein. Indeed, exons out of the membrane or in a loop are more sensitive to alternative splicing. Exon 7 of abl is located in the activation loop, surrounded by four different Alu elements in intron 7 (http://transpogene.tau.ac.il; ref. 26), and seems to contain various ESE (http://genes.mit.edu/burgelab/rescue-ese/; ref. 27) that could explain why this exon is preferentially spliced. Whereas some authors showed that alternative splicing patterns are changed in cancer (28, 29), this mechanism does not seem to exist in the abl component for insertion or deletion. Indeed, as we showed for abl^del exon7, the insertion of 35 bp between exon 8 and exon 9, another big rearrangement of abl, first described in CML-resistant patients, has been reported in normal population (30), whereas its bcr-abl component has been related to resistance (31).

To know whether bcr-abl^del exon7 is related to resistance, we decided to focus on its expression. bcr-abl^del exon7 is found not only in resistant but also in newly diagnosed patients with an equivalent frequency. Moreover, the level of its expression does not seem to be correlated with occurrence of resistance because some patients, for example, show this transcript at diagnosis but lost it at resistance occurrence. bcr-abl^del exon7 leads to a codon stop in exon 8 at position 382, resulting in a truncated protein with no activation loop, this loop being phosphorylated that is necessary for a full activation of the kinase, stabilizing an active conformation for substrate binding and catalysis (32). A truncated protein would probably be inactive and undergo degradation or, alternatively, its RNA would disappear through nonsense RNA decay mechanisms. Using a quantitative microarray technique, it has been evaluated that 35% of alternative splicing events lead to a premature termination codon, and these splice variants are targeted by the process of nonsense RNA decay (33). Sherbenou et al. focus in another deletion in kinase domain, the exon 4 deletion. As for bcr-abl^del exon7, they found it in a large number of patients under imatinib therapy (80%), with no correlation between the presence of this deletion and outcome, and showed that this deletion mutant does not exhibit a dominant-negative effect with respect to bcr-abl activation (34).

Our result confirmed that bcr-abl deletions of exon 7 are quite frequent in patients under imatinib therapy, probably resulting from an alternative splicing mechanism. The use of sensitive techniques allows to easily detect this variant, frequently passed over by direct sequencing. There is no correlation between the detection of this peculiar form and resistance to tyrosine kinase inhibitor. Additional studies are required to know, first, if the resulting truncated protein is indeed synthesized in the cell or if its RNA goes to RNA decay mechanism and, second, to explain why only some patients at diagnosis exhibit this rearrangement, whereas all patients carry abl^del exon7.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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