

# Consultations in Molecular Diagnostics

## Comprehensive Characterization of a Novel Intronic Pseudo-Exon Inserted within an e14/a2 *BCR-ABL* Rearrangement in a Patient with Chronic Myeloid Leukemia

Nathalie Sorel,<sup>\*†</sup> Caroline Mayeur-Rousse,<sup>\*†</sup>  
Séverine Deverrière,<sup>\*</sup> Lydia Roy,<sup>‡§</sup>  
Elisabeth Brottier-Mancini,<sup>¶</sup> François Guilhot,<sup>‡§</sup>  
Ali G. Turhan,<sup>\*†</sup> and Jean Claude Chomel<sup>\*†</sup>

From the Services d'Hématologie et Oncologie Biologique,<sup>\*</sup> and  
d'Oncologie Hématologique et Thérapie Cellulaire,<sup>‡</sup> and the  
Centre d'Investigation Clinique,<sup>§</sup> INSERM CIC-P 802, Centre  
Hospitalier Universitaire de Poitiers, Poitiers; INSERM U935,<sup>¶</sup>  
Université de Poitiers, Poitiers; and the Service de Médecine  
Interne,<sup>¶</sup> Centre Hospitalier de La Rochelle, La Rochelle, France

**We identified a novel breakpoint cluster region-*ABL* rearrangement in a chronic myeloid leukemia (CML) patient. The e14/a2 (b3/a2) type *BCR-ABL* mRNA incorporated a 42-nucleotide intronic insertion of *ABL* intron 1b between *BCR* exon e14 and *ABL* exon a2. As we hypothesized that the rearrangement between *BCR* and *ABL* genes occurred near the inserted sequence and because of the relative small size of *BCR* intron 14, we determined the *BCR-ABL* breakpoint at the genomic DNA level. Using a PCR-based method, this analysis revealed that i) *BCR* intron 14 brought a potential lariat branch point and the polypyrimidine tract, ii) the *BCR-ABL* breakpoint created a chimeric acceptor site, and iii) the inserted sequence of *ABL* intron 1b carried at its 3' end a well-conserved donor splice site. Therefore, the inserted sequence was flanked by canonical consensus splice sites and recognized as a pseudo-exon (as shown by splice site prediction and exon finder software). Moreover, the insertion did not disrupt the reading frame between *BCR* and *ABL* and did not produce a premature stop codon. Instead, this novel *BCR-ABL* chimeric transcript encoded a functional oncoprotein with an in-frame insertion of 15 new amino acids. (*J Mol Diagn* 2010, 12:520–524; DOI: 10.2353/jmoldx.2010.090218)**

Philadelphia chromosome (Ph1) results from the reciprocal t(9;22)(q34;q11) translocation that fuses the *BCR* and *ABL* genes into a *BCR-ABL* chimeric gene, which encodes a constitutively activated tyrosine kinase oncoprotein.<sup>1</sup> Breakpoints in *ABL* generally occur upstream of exon a2 (rarely exon a3). Depending on the location of the breakpoint in the *BCR* gene, three main types of *BCR-ABL* transcripts can be produced.<sup>2</sup> In chronic myeloid leukemia (CML), most of the breakpoints in *BCR* occur in the 5.8-kb major breakpoint cluster region and result in the fusion at the mRNA level of *BCR* exon e13 (b2) or e14 (b3) to *ABL* exon a2, known as the e13/a2 or e14/a2 rearrangements. In Ph1-positive acute lymphoblastic leukemia, breakpoints in *BCR* are generally located in the minor major breakpoint cluster region and result in the e1/a2 *BCR-ABL* rearrangement. A third breakpoint in *BCR* ( $\mu$ -*BCR*) is observed in typical CML or neutrophilic leukemia and leads to the e19/a2 rearrangement. Finally, e6/a2 and e8/a2 *BCR-ABL* rearrangements are uncommonly observed in typical CML. Thus, in the great majority of CML cases, *BCR-ABL* rearrangements result in the joining of *BCR* exons e13 or e14 to *ABL* exon a2.

We report here a case of CML with an e14/a2 fusion mRNA containing a 42-bp sequence from *ABL* intron 1b inserted between *BCR* exon e14 and *ABL* exon a2. The insertion was characterized by sequencing at the mRNA level. The location of the *BCR-ABL* breakpoint was determined on genomic DNA, and the consensus donor and acceptor splice sites as well as a potential lariat branch point were identified. We demonstrated that, in this *BCR-ABL* rearrangement, the consensus splice sites were brought by *BCR* intron 14 (branch point and polypyrimi-

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Address reprint requests to Jean Claude Chomel, PharmD, Ph.D., Service d'Hématologie et Oncologie Biologique – INSERM U935, Centre Hospitalier Universitaire de Poitiers, 2, rue de la Milétrie, 86021 Poitiers Cedex, France. E-mail: j.c.chomel@chu-poitiers.fr.

dine tract), *BCR-ABL* junction (chimeric acceptor splice site), and *ABL* intron 1b (donor splice site).

## Materials and Methods

### Clinical Report

A 44-year-old man was admitted to the hospital for a screening colonoscopy to explore chronic mild rectal bleeding. The patient had no medical history and no symptoms except for a moderate fatigue and weight loss. During the visit preceding the endoscopic examination, a spleen easily palpable 18 cm below the left costal margin was observed. Blood cell count revealed white blood cells at  $214.0 \times 10^9/L$  (6% metamyelocytes, 22% myelocytes, 8% promyelocytes, and 4% myeloblasts), hemoglobin at 11.7g/dl, and platelets at  $427.0 \times 10^9/L$ . Moreover, renal and liver functions were normal with increased lactate dehydrogenase levels. Bone marrow aspirate revealed granulocytic hyperplasia consistent with chronic-phase CML. Some giant and abnormally shaped metamyelocytes were also observed on the bone marrow smear. The patient was classified as high risk according to Sokal prognosis score (1.67). He received hydroxyurea before entering a therapeutic trial using second generation tyrosine kinase inhibitor as first-line treatment after informed consent was obtained.

### RNA Isolation and Multiplex RT-PCR for BCR-ABL Transcript Detection

Total RNA was isolated from peripheral blood using RNable, according to the manufacturer's protocol (Eurobio, Les Ulis, France). Around 1  $\mu\text{g}$  of total RNA was retrotranscribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). *BCR-ABL* transcript detection was performed using a multiplex RT-PCR based on primers previously described in the literature<sup>3</sup> and the additional primer BCR-e17/18 (5'-CGCCATGAATGGGATCGAAGTA-3'), which enhances the detection of a rearrangement at the  $\mu$ -BCR locus. The PCR products were analyzed on a 1.2% agarose gel electrophoresis together with controls resulting from the amplification of e1/a2 (483 bp), e13/a2 (312 bp), e13/a3 (138 bp), e14/a2 (387 bp), and e19/a2 (545 bp) *BCR-ABL* rearrangements.

### Genomic DNA Isolation and Genomic Breakpoint Characterization

Genomic DNA was isolated from peripheral blood with BioSprint 15 DNA blood kit (Qiagen, Hilden, Germany) using a KingFisher instrument (Thermo Scientific, Hudson, NH). The genomic *BCR-ABL* breakpoint was characterized using five forward primers located in *BCR* introns 13 and 14, *BCR-a* (5'-CTTGTCACCTGCCTCCCTT-3'), *BCR-b* (5'-CACCTTCACCCCACAGCA-3'), *BCR-c* (5'-AGCAAGACTCCGCCTCAAA-3'), *BCR-d* (5'-CTTTGAGGGGCACCACCA-3'), *BCR-e* (5'-GGCTTCCCACA-

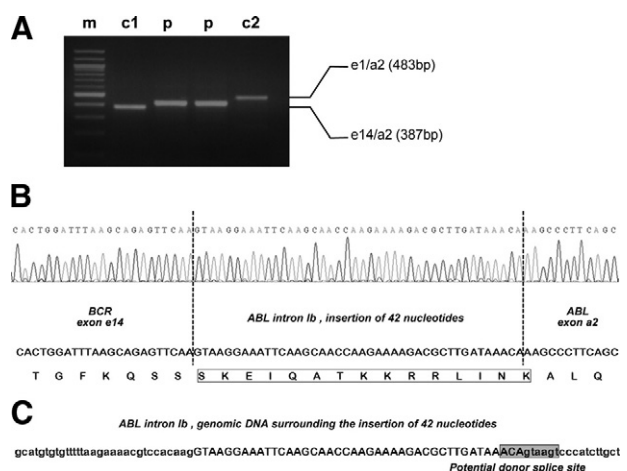
TCCCCCA-3'), and a reverse primer, *ABL-z* (5'-GCGTC-TTTTCTTGGTTGCTT-3'), located in *ABL* intron 1b.

### Direct Sequencing

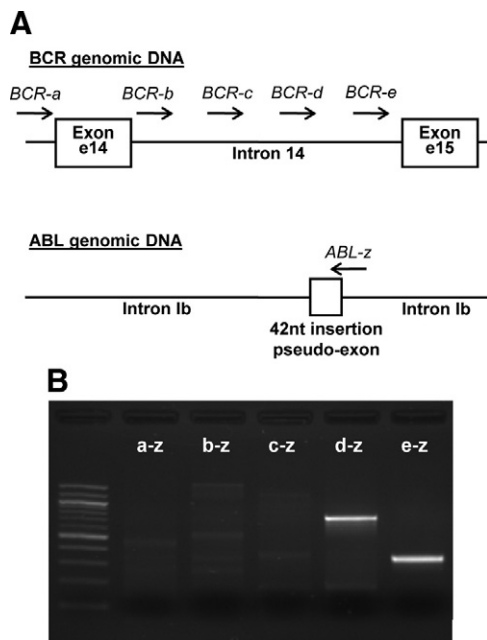
PCR products were purified using ExoSAP-it (USB, Cleveland, OH) and double strand sequenced with the Big Dye Terminator version 3.1 reagents (Applied Biosystems), according to the manufacturer's recommendations. Sequencing products were purified on DyeEx 2.0 Spin kit columns (Qiagen) and analyzed through an ABI 3100 Genetic Analyzer (Applied Biosystems).

## Results and Discussion

Cytogenetic and molecular analyses were performed at diagnosis. A classical t(9;22)(q34;q11) translocation without any additional abnormality was found, and a chimeric *BCR-ABL* transcript was identified by multiplex RT-PCR. However, the PCR fragment was distinct in size from those obtained from typical *BCR-ABL* rearrangements such as e1/a2, e13/a2, or e14/a2. As seen in Figure 1, the apparent size of this fragment in agarose gel electrophoresis was between 387 bp (e14/a2 amplicon) and 483 bp (e1/a2 amplicon). Direct sequencing of the PCR product revealed a 42-nucleotide insertion between *BCR* exon e14 and *ABL* exon a2. A basic local alignment search tool analysis (National Center for Biotechnology Information, Bethesda, MD) of the inserted sequence showed 100% identity with a genomic DNA sequence located near the middle of *ABL* intron 1b (GenBank accession number NG\_012034). Analysis of this sequence at the genomic DNA level by the Human Splicing Finder software (Human Splicing Finder; Montpellier University, Montpellier, France; version 2.4. 2008; available at <http://www.umd.be/HSF/>;



**Figure 1.** Detection of a novel intronic insertion within an e14/a2 *BCR-ABL* rearrangement. **A:** Using multiplex RT-PCR, an atypical *BCR-ABL* transcript was observed in the patient sample (p) as compared with e14/a2 (c1) and e1/a2 (c2) controls. **Lane m:** 100-bp ladder. **B:** Direct sequencing of the RT-PCR product revealed a 42-nucleotide insertion of *ABL* intron 1b within an e14/a2 *BCR-ABL* rearrangement. The resulting in-frame insertion of 15 amino acids was shown. **C:** A well-conserved donor splicing site was detected, at the genomic DNA level, in the 3' end of the insertion. Uppercase and lowercase nucleotides corresponded respectively to pseudo-exonic and intronic sequences.

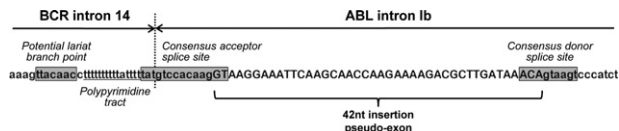


**Figure 2.** Identification of the *BCR-ABL* breakpoint on genomic DNA. **A:** As we hypothesized that the *BCR-ABL* breakpoint occurred near the 5' end of the inserted sequence, five primers located in *BCR* introns 13 and 14 and a reverse primer in *ABL* intron 1b (at the 3' end of the insertion) were designed. **B:** Specific PCR amplifications were observed with two pairs of primers (d-z and e-z).

accessed September 25, 2009)<sup>4</sup> revealed at the 3' end of the insertion a highly conserved donor site for splicing (ACAgtaagt). Neither lariat branch point nor polypyrimidine tract or complete acceptor site were identified in the *ABL* genomic DNA upstream of the insertion.

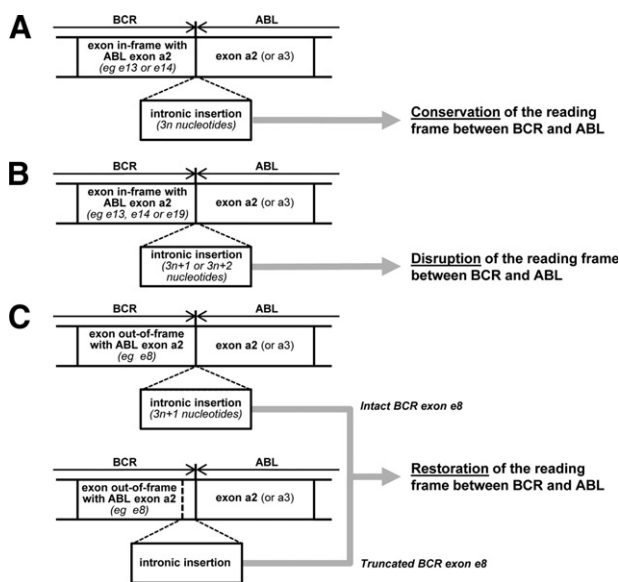
Because this sequence of 42 nucleotides was recognized as an exon, we hypothesized that potential branch point and acceptor splicing site were brought by *BCR* intron 14 during the translocation process and that the genomic breakpoint between *BCR* and *ABL* genes was located nearby the 5' end of *ABL* intron 1b inserted sequence. To examine this hypothesis and because of the small size of *BCR* intron 14 (2127bp), the precise location of the breakpoint in *BCR* was determined (Figure 2, A and B). For this, PCR experiments were performed using five forward primers (*BCR*-a to *BCR*-e) overlapping the *BCR* genomic region from the 3' end of intron 13 to the 3' end of intron 14 and a reverse primer (*ABL*-z) located in *ABL* intron 1b (at the 3' end of the 42-nucleotide insertion). Clear and specific PCR amplifications were obtained with primers *BCR*-e and *BCR*-d demonstrating that the rearrangement in *BCR* was located downstream primer *BCR*-e (at the 3' end of intron 14). Direct sequencing of PCR products revealed that the genomic *BCR-ABL* breakpoint occurred after the thymine at position 126,523 for *BCR* (95 bp upstream of *BCR* exon e15; GenBank accession number U07000) and before the guanine at position 75,101 for *ABL* intron 1b (GenBank accession number NG\_012034). The *BCR-ABL* breakpoint was located nine nucleotides upstream of the intronic inserted *ABL* sequence.

The genomic sequence surrounding the *BCR-ABL* breakpoint was then analyzed using Human Splicing Finder



**Figure 3.** Characterization of the consensus splice sites surrounding the 42-nucleotide insertion. Uppercase and lowercase nucleotides corresponded respectively to pseudo-exonic and intronic sequences. The *BCR-ABL* breakpoint occurred nine nucleotides upstream of the insertion. Well-conserved consensus splice sites were recognized in the sequence: donor site in *ABL* intron 1b, acceptor site at the *BCR-ABL* breakpoint, potential lariat branch point and polypyrimidine tract in *BCR* intron 14.

and SROOGLE (Splicing RegulatiOn Online Graphical Engine; Tel Aviv University, Ramat Aviv, Israel; 2008; available at <http://sroogle.tau.ac.il/>; accessed October 14, 2009)<sup>5</sup> software to identify splicing signals. In addition to the donor site already identified in *ABL* intron 1b, a consensus acceptor splice site (tatgtccacaagGT), a polypyrimidine tract and a potential lariat branch point (taccac) were identified (Figure 3). Finally, GeneMark (Eukaryotic GenMark.hmm. Georgia Institute of Technology, Atlanta, GA; version 3.0; 2005; available at <http://exon.gatech.edu/GeneMark/>; accessed October 14, 2009),<sup>6</sup> and FGENES (SoftBerry, Mount Kisco, NY; 2007; available at <http://linux1.softberry.com/berry.phtml>; accessed October 14, 2009) software clearly recognized the 42-nucleotide sequence of *ABL* intron 1b as an exon. Thus, in our observation, the *BCR-ABL* rearrangement was at the origin of a chimeric splicing acceptor site. The *BCR* genomic DNA sequence (intron 14) contributed to the polypyrimidine tract and an upstream potential branch point, whereas the *ABL* DNA sequence (intron 1b) brought a part of the acceptor site and a well-conserved splicing donor site. Consequently, the *BCR-ABL* rearrangement generated the recognition of an intronic sequence of 42 nucleotides derived from *ABL* intron 1b as a



**Figure 4.** Consequences of an intronic insertion between *BCR* and *ABL* exons. **A:** The insertion conserves the reading frame between *BCR* and *ABL* exons. **B:** The inserted sequence disrupts the reading frame and leads to a premature stop codon. **C:** The insertion restores a reading frame with intact or truncated last *BCR* exon e8.

pseudo-exon. At the mRNA level, the rearrangement consisted in the following sequence: *BCR* exon e14/ins 42 nucleotides from *ABL* intron 1b/*ABL* exon a2. This insertion maintained the reading frame between *BCR* and *ABL* mRNA and did not generate a premature stop codon. Therefore, it encoded an in-frame novel 15-amino acid sequence (SKEIQATKKRRLINK) inserted between amino acid 927 of *BCR* and amino acid 28 of *ABL* type 1a (Figure 1), leading to a functional protein slightly larger than the classical *BCR-ABL* oncoprotein of 210 kDa.

Although infrequent, several nonclassical *BCR-ABL* rearrangements incorporating intronic sequences recognized as a pseudo-exon have been previously reported in CML patients at presentation. In most of the cases, the inserted intronic sequence derived from *ABL* intron 1b (120.1 kb),<sup>7-11</sup> from *ABL* intron 1a (18.5 kb),<sup>12,13</sup> and more rarely from *BCR* intron 8 (10.2 kb).<sup>14</sup> The insertion of intronic sequences within a *BCR-ABL* rearrangement may have three major consequences: the conservation, the disruption, or the restoration of an open reading frame depending on the location of the insertion and the number of nucleotides inserted (Figure 4). *BCR* exons e1, e6, e13, e14, and e19 (and theoretically exons e12 and e20) can be fused to *ABL* exon a2 (rarely a3) without a reading frame shift generating a functional oncoprotein. Because molecular breakpoints in *BCR* and *ABL* genes generally occurred in introns, an inserted sequence should be a multiple of three nucleotides to conserve the reading frame (Figure 4A). Concerning the e13/a2 and e14/a2 rearrangements characteristic of CML, very few cases of in-frame insertion of a pseudo-exon between coding *BCR* and *ABL* sequences were described. Apart from the case mentioned here, an insertion of 12 nucleotides between *BCR* exon e13 and *ABL* exon a2 was reported previously.<sup>15</sup> Moreover, it should be noted that several studies described, in addition to a classical e13/a2, e14/a2, or e19/a2 mRNA, aberrant *BCR-ABL* transcripts harboring a frameshift insertion (3n + 1 or 3n + 2 nucleotides) disrupting the reading frame and leading to a premature stop codon (Figure 4B).<sup>7,16,17</sup> Concerning rearrangements involving *BCR* exons out-of-frame with *ABL* exon a2 (or a3), an intronic insertion could restore the reading frame between the two coding sequences (Figure 4C). As a consequence, the resulting *BCR-ABL* protein could then be functional. The *BCR* exon 8 was most closely involved in such rearrangements with the identification of e8/intronic insertion/a2 *BCR-ABL* transcripts. To restore an artificial reading frame between *BCR* and *ABL* sequences, two mechanisms were mainly described: i) an intronic insertion of 3n + 1 nucleotides between *BCR* exon e8 and *ABL* exon a2,<sup>10,14</sup> or ii) an intronic insertion within exon 8 (formation of an e8 partial/insertion/a2 rearrangement).<sup>11-13</sup> Concerning the e8/a2 rearrangement, the same *ABL* intron 1b inverted insertion has been reported in two CML cases.<sup>10,18</sup>

From a technical standpoint, the very low incidence of small sequences inserted within typical e13/a2 or e14/a2 rearrangement in CML could be the consequence of a misidentification of these types of transcript in agarose gel electrophoresis (because of a too small difference in size). Conversely, a rearrangement involving *BCR* exon 8

(in whole or in part) and an intronic insertion (restoring the reading frame) should be easier to detect because of a theoretical size clearly distinguishable from classical amplicons. Regarding the *BCR-ABL* rearrangement described here, the insertion of 42 nucleotides, increasing the amplicon length (190 bp as compared with the 148 bp of the typical e14/a2 transcript), could affect the efficiency of standard quantitative RT-PCR.<sup>18</sup> Consequently, to correctly monitor the response to tyrosine kinase inhibitor therapy, PCR primers (especially the forward primer in *BCR*) should be redesigned to reduce the amplicon size.

In conclusion, we identified a novel *BCR-ABL* transcript produced by the fusion of *BCR* exon e14 to *ABL* exon a2 with a 42-bp insertion from *ABL* intron 1b. Because of the rearrangement process, this pseudo-exon was flanked by canonical consensus splice sites, did not disrupt the reading frame between *BCR* and *ABL*, and did not generate a premature termination codon. The encoded *BCR-ABL* protein contains an in-frame insertion of 15 amino acids. However, the full biological and clinical implications of the rearrangement reported here remain unknown. Therefore, no reliable relationship between this unusual *BCR-ABL* transcript and an apparent more aggressive form of CML can be established. These results highlight the usefulness of a robust method to theoretically detect all types of *BCR-ABL* mRNA. Finally, the detection of an atypical transcript (as the *BCR* exon e14/ins 42 nucleotides from *ABL* intron 1b/*ABL* exon a2 rearrangement reported here) requires the adjustment of quantitative RT-PCR protocols for an optimized monitoring of the residual disease.

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