Acta Haematologica

Case Report

Acta Haematol 2023;146:413–418
DOI: 10.1159/000531128

Received: February 7, 2023
Accepted: May 15, 2023
Published online: May 19, 2023

The Recurrent Atypical e8a2 *BCR::ABL1* Transcript with Insertion of an Inverted 55 Base Pair *ABL1* Intron 1b Sequence: A Detailed Molecular Analysis

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Keywords
BCR::ABL1 · Chromosomal translocation · Atypical transcript · Cryptic exon

Abstract
Atypical *BCR::ABL1* transcripts are found in approximately 2% of cases of chronic myeloid leukemia. It is important to detect them since affected patients also benefit from tyrosine kinase inhibitor therapy. In the rare e8a2 atypical *BCR::ABL1* transcript, two out-of-frame exons are fused, thus, interposed nucleotides are usually found at the fusion site to restore the reading frame. In approximately half of previously reported e8a2 *BCR::ABL1* cases, an inserted 55 bp sequence homologous to an inverted sequence from *ABL1* intron 1b was detected. The generation of this recurrent transcript variant is not obvious. This work describes the molecular analysis of such an e8a2 *BCR::ABL1* translocation from a CML patient. The genomic chromosomal breakpoint is identified, and the formation of this transcript is theoretically explained. The clinical course of the patient is reported, and recommendations are provided for the molecular analysis of future e8a2 *BCR::ABL1* cases.

Introduction

The chimeric *BCR::ABL1* gene is the molecular hallmark of chronic myeloid leukemia (CML). More than 95% of *BCR::ABL1*-positive CML patients reveal a *BCR::ABL1* transcript, showing a fusion of either *BCR* exon 13 or 14 and *ABL1* exon 2 (transcripts e13a2 and e14a2), indicating a chromosomal breakpoint in the so-called "major breakpoint cluster region" in *BCR*. Other "atypical" *BCR::ABL1* transcripts with different exon fusions are rarely found. One of these is the e8a2 *BCR::ABL1* transcript, first described by How et al. [1] and Branford et al. [2], of which only a handful of cases have been reported. The generation of this transcript type is more complex since *BCR* exon 8 and *ABL1* exon 2 are not in the same reading frame, thus, a simple fusion of the two exons should not lead to a functional BCR::ABL1 oncoprotein. In fact, the large majority of previously described e8a2 *BCR::ABL1* transcripts showed an insertion of additional nucleotides that were derived from intronic sequences of *ABL1*, *BCR*, or other genes, with only very few (difficult to explain) exceptions [3, 4]. Approximately half of the previously reported e8a2 *BCR::ABL1* transcript cases showed a 55 base pair (bp) insertion matching an inverted sequence

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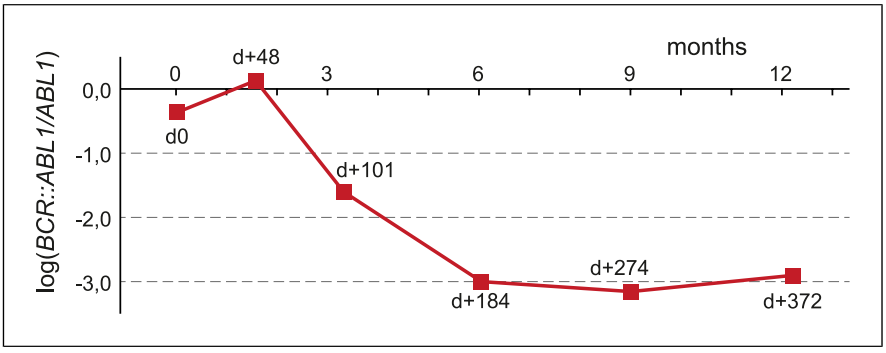

Fig. 1. Time course of the relative *BCR::ABL1* level during 12 months of therapy.

from *ABL1* intron 1b [2, 5–7]. How this intronic inverted sequence is incorporated into the final chimeric mRNA transcript is not obvious and has not been investigated previously.

We describe the case of a patient with CML and an e8a2 *BCR::ABL1* transcript with such an inverted interposed sequence. We molecularly identified the chromosomal breakpoint and provided a theoretical explanation for the generation of this transcript variant. Implications for molecular monitoring are discussed.

Case Presentation

Patient's History

The patient, a 74-year-old male from Germany presented with myeloproliferative neoplasm. He presented with a leukocyte count of 110 × 10^9/L, hemoglobin 15.6 g/dL, and 178 × 10^9/L platelets. The differential blood count showed 5% lymphocytes, 2% eosinophils, 3% basophils, 8% neutrophilic band forms, 57% segmented neutrophils, 10% metamyelocytes, 7% myelocytes, 5% promyelocytes, and 3% blasts. Clinically, he was largely asymptomatic. While the karyotype 46,XY,t(9;22)(q34;q11.2) [3] suggested a Philadelphia translocation, the initial RT-PCR analysis in an external laboratory failed to detect a *BCR::ABL1* transcript. RT-PCR analysis by multiplex PCR in our laboratory and subsequent sequencing of the PCR product revealed an e8a2 *BCR::ABL1* transcript with the aforementioned 55 bp insertion [8]. Bone marrow histology showed a myeloproliferative neoplasm with less than 2% blasts and a focal discrete fibrosis. The patient first received hydroxyurea for a few days and then 2 × 300 mg nilotinib daily. Molecular analysis by RT-qPCR using a quantitative PCR method with a newly designed primer in *BCR* exon 8 together with the standardized EAC cycling conditions, and *ABL1* as a housekeeping gene [9, 10] showed a reduction in the relative *BCR::ABL1* level to a value of 10^{-3} in the peripheral blood after 12 months of therapy (Fig. 1).

Genetic Analysis of the BCR::ABL1 *Break Region*

The procedures of the genetic analysis are illustrated in Figure 2. Technical details such as PCR methods and primer sequences are provided in the online supplement (for all online suppl. material, see https://doi.org/10.1159/000531128) to this report. All nucleotide positions refer to the human genome assembly GRCh38.p14 139608 bp *ABL1* intron 1 (NC_000009.12:130714456-130854063) and 10202 bp *BCR* intron 8 (NC_000022.11:23273775-23283976). After sequencing the mRNA transcript (Fig. 2a), all further molecular analyses were performed with genomic DNA. The break on chromosome 22 was assumed to be in the 10.2 kb *BCR* intron 8. Since the inverted intronic sequence was located near *ABL1* exon 1b (Fig. 2b), we hypothesized that one *ABL1* break occurred near the 5' end of *ABL1* intron 1b. Long-range PCRs with different PCR primers located in the inverted sequence and in various parts of *BCR* exon/intron 8 were performed which resulted in the identification of the 5' break in *BCR* intron 8 at nt 7894 and in *ABL1* intron 1b at nt 1072 (Fig. 2c). To obtain an estimate of the location of the second (3') break, i.e., the size of the inverted segment, various long-range PCRs were performed with one primer located in *BCR* intron 8 immediately 5' of the breakpoint and the second primer in (inverted) *ABL1* at various distances 3' to the inverted fragment. This revealed that a second break was located still in *ABL1* intron 1b and not further 5' upstream.

This 3' break in *ABL1* intron 1b was identified using a long-range inverse PCR approach with one primer located in the inverted fragment and the other in *BCR* intron 8 as shown in Figure 2c. Three different restriction enzymes were tested, and the enzyme *Taq*I led to the identification of the 3' break in *ABL1* intron 1b (Fig. 2c).

The 3' break in *ABL1* was located at nt 1415 of intron 1b. An 878 bp sequence fragment spanning from nt 195 to nt 1072 with 100% sequence identity to the *ABL1* reference sequence was inserted in reverse complement, while 342 bp of DNA sequence between nt 1073 and 1414 of *ABL1* intron 1b were missing (Fig. 3a).

Genetic Analysis of the ABL1::BCR *Breakpoint Region*

No expression of a chimeric *ABL1::BCR* transcript was detected by RT-PCR using PCR primers located in *ABL1* exon 1b and *BCR* exon 9.

Bioinformatic Analysis of the Breakpoint Region

The complete breakpoint region in *ABL1* and *BCR* was sequenced from a remission sample of the patient to analyze possible sequence variations that could have facilitated the chromosomal break event. Genomic repeats were analyzed with *RepeatMasker* version 4.0.9, *RSSsite* and the *Tandem Repeats Finder* [11–13]. No repetitive element was identified in the vicinity of the breakpoint in *BCR*, while four elements were identified in the first 1500 bp of

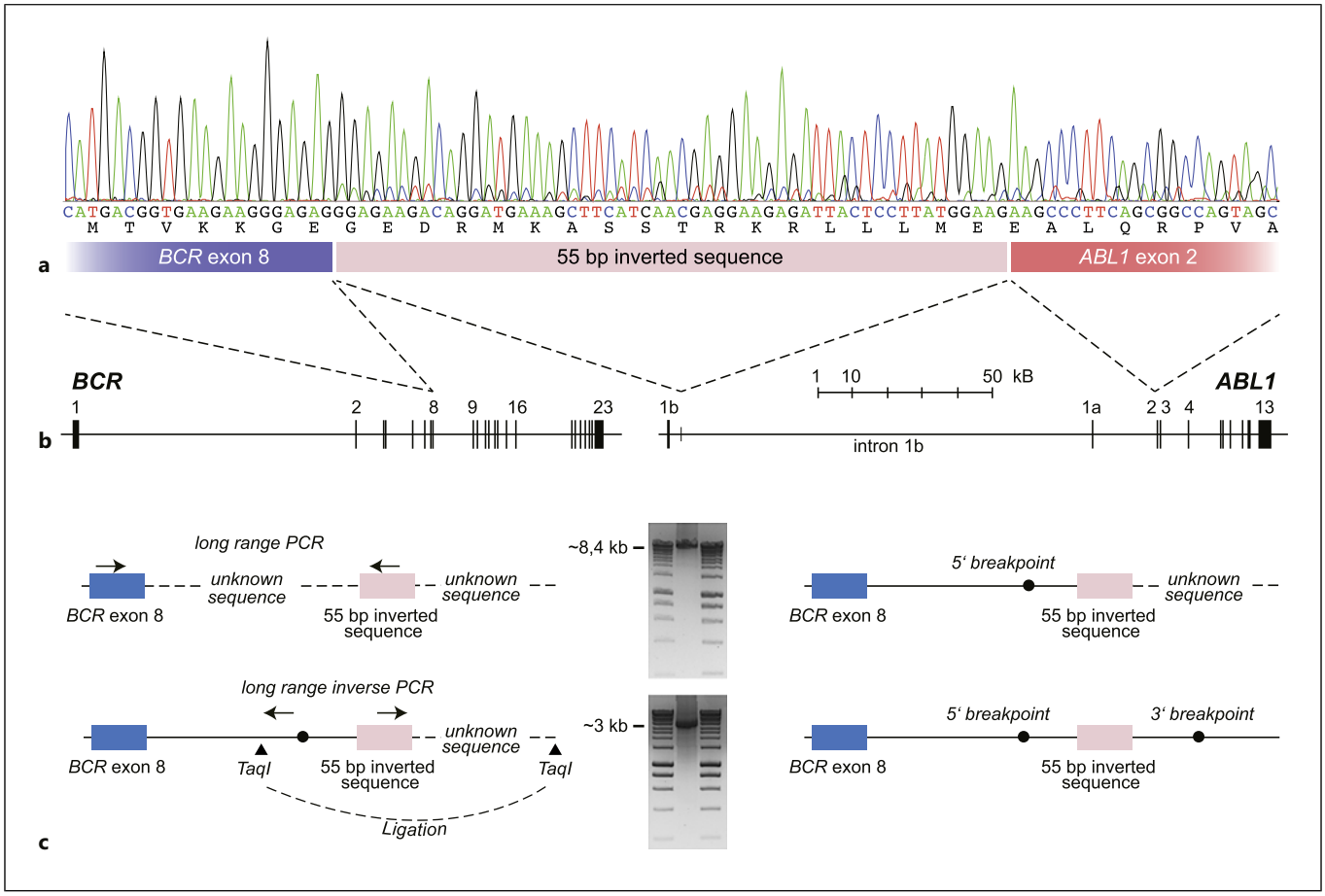

Fig. 2. e8a2 transcript and the identification of breaks on chromosomes 9 and 22. **a** Sequence chromatogram of the *BCR::ABL1* e8a2 transcript. **b** Exon organization of *BCR* and *ABL1* with the location of the 55 bp insert in *ABL1* intron 1b. **c** PCR and sequencing strategy to identify the breaks in *BCR::ABL1*. Upper row: long-range PCR for identification of the 5' break. Lower row: long-range inverse PCR for identification of the 3' break(s). Arrows indicate PCR primer locations and orientations. Gel images show PCR products.

ABL1 intron 1b (elements with intronic location: MIR3 218–322 bp, Charlie4z 400–452 bp, L1ME3G 1226–1333 bp, Charlie1a 1345–1501 bp). No tandem repeats or microhomologies were detected at the break sites. There was one potential 12 bp cryptic recombination signal sequence (cRSS) at 279–306 bp and two potential 23 bp cRSSs at 385–423 bp and at 1242–1280 bp. All three cRSSs were on the (-) strand. No single-nucleotide polymorphisms were detected.

Discussion

Various e8a2 *BCR::ABL1* transcript variants have been reported in the literature. Many of them were singular cases showing interposed short nucleotide sequences derived from different chromosomes and genes [14–16]. The 55 bp *ABL1* insert e8a2 *BCR::ABL1* variant is of particular theoretical interest since it has been recurrently observed. None of the previously reported e8a2 *BCR::ABL1* cases was analyzed on the genomic level.

Genetic Analysis

Previous work has located *BCR::ABL1* chromosomal breaks in the first 1.5 kb of *ABL1* intron 1b. Linhartova et al. [17] analyzed the der(22) chromosomal breaks of CML patients with e13a2/e14a2 transcripts. Three of the analyzed 46 breaks in *ABL1* intron 1b were located in this region (with GenBank accession numbers): at nt 866 (KR092020), nt 841 (KR091994), and nt 604 (KR092015). Krumbholz et al. identified the der(22) and/or der(9) break locations in 59 pediatric CML

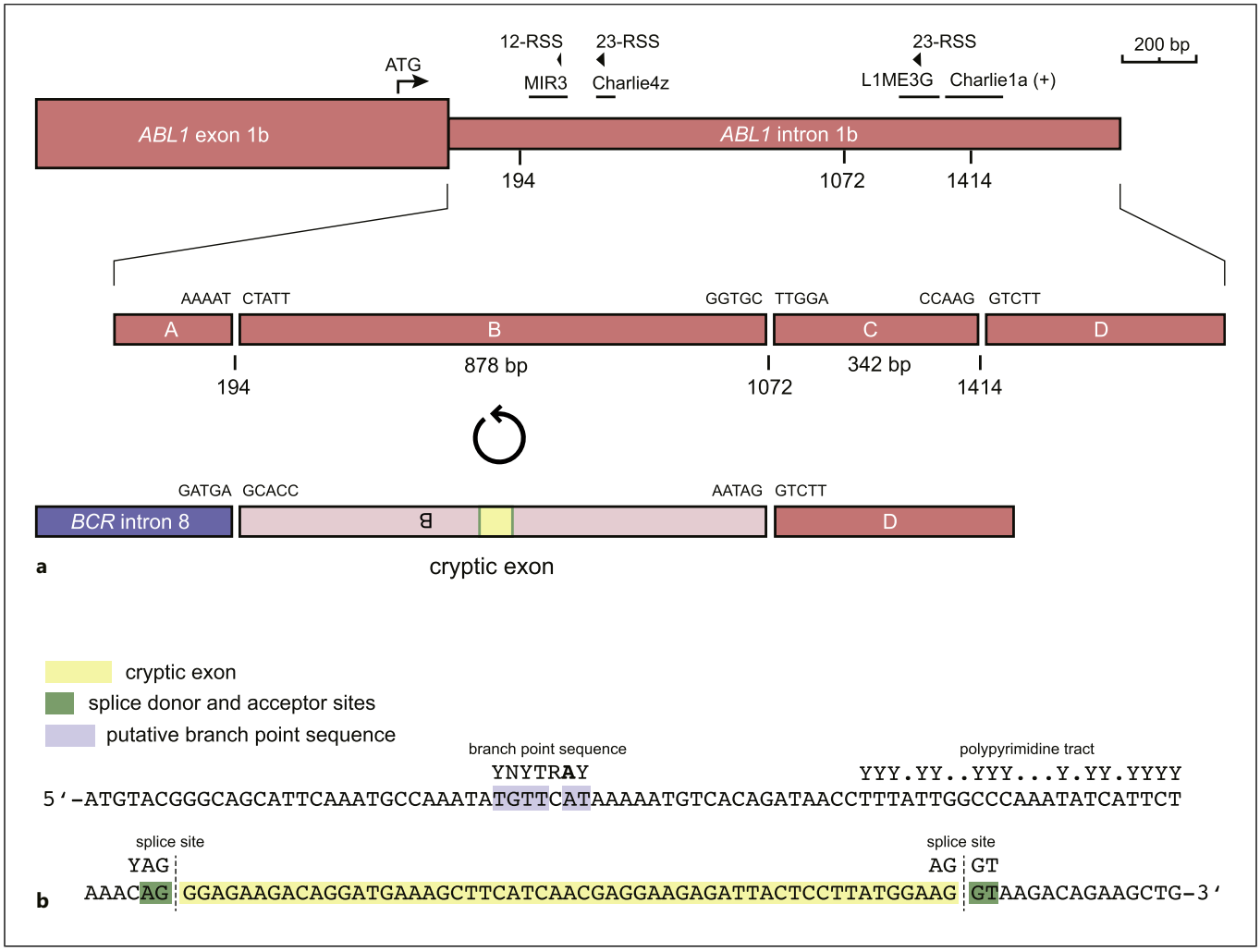

Fig. 3. Hypothetical mechanism leading to the generation of the observed transcript. **a** Break region in *ABL1* intron 1b with the three break positions and formation of the *BCR::ABL1* fusion. The position of repetitive DNA elements and cryptic recombination signal sequences are indicated as arrows or bars. **b** Nucleotide sequence of the cryptic exon with adjacent regions.

patients [18]. Six of them mapped to the break region at 575 bp (JQ425201), 698 bp (JQ425193), 1154 bp (JQ425167), 1276 bp (JQ425228), 1280 bp (JQ425229), and 1435 bp (JQ425168). In the author's previous work, however, none of the 64 identified der(22) and der(9) chromosomal breaks of e13a2/e14a2-*BCR::ABL1*-positive patients were located in this region [19]. Thus there was a higher density of *BCR::ABL1* break events in this region than could statistically be expected. When analyzing the breakpoint junctions in the current case, no microhomologies, repetitive DNA elements, or cRSS) sites were found that were likely involved in the chromosomal break. The question of whether those elements play a role in the formation of the *BCR::ABL1* translocation has been controversially discussed [17–22].

A reciprocal der(9) or *ABL1::BCR* fusion breakpoint could not be characterized. Since no expression of an *ABL1::BCR* mRNA transcript was detectable by RT-PCR, it is possible that the translocation led to a partial or complete deletion of the reciprocal allele. Such deletions of der(9) are frequently observed in CML [23], and the abovementioned studies also did not detect reciprocal *ABL1::BCR* breakpoints in a significant percentage of cases.

Activation of a Cryptic Exon

The incorporation of the 55 bp sequence between *BCR* exon 8 and *ABL1* exon 2 in the detected mRNA transcript can be theoretically explained by the activation of a cryptic exon (Fig. 3). This has already been hypothesized by Tchirkov et al. [24] but the authors did not investigate it on the genomic level. The 55 bp cryptic exon possessed intact splice donor and splice acceptor sites, a small polypyrimidine tract and putative splice branching site, which however did not fully match the consensus YNYTRAY motif [25]. Whether this cryptic exon was activated by some kind of abnormality or variant in the splicing apparatus of our patient remains unclear since there is no known transcriptional unit on the minus strand of the *ABL1* gene locus, thus, it cannot be decided if this exon is spliced in healthy individuals. As can be seen from the sequence chromatogram in Figure 2a, a transcript with direct fusion of *BCR* exon 8 and *ABL1* exon 2 is also transcribed with low efficiency, but this should not result in a functional BCR::ABL1 protein. The cryptic exon was also predicted with some probability by bioinformatic web-based tools, albeit with only moderate probability: NetGene2-2.42 (p = 0.83 donor, p = 0.77 acceptor splice site) [26] and GENSCAN (p = 0.304) [27].

Molecular Monitoring

Molecular monitoring of atypical *BCR::ABL1* transcripts poses a diagnostic problem since no international guidelines exist for this process. In the case of the typical e13a2/e14a2 *BCR::ABL1* transcripts the EAC PCR primers are widely used, and detailed recommendations for molecular monitoring have been issued [28], but no such guidelines exist for atypical transcripts. Recently, Schäfer et al. [29] addressed this problem and designed and tested PCR primers for several atypical *BCR::ABL1* transcripts, including e8a2, using a relative patient-specific scale, i.e., the *BCR::ABL1* transcript level at diagnosis was set to 100% and the relative reduction was calculated during the time course. We adopted this approach, albeit with different PCR primers and probe and our patient showed a significant reduction in the relative *BCR::ABL1* level to about below 10^{-3} (0.1%) after 9 months of therapy.

Conclusions

This report investigated and elucidated the molecular background of the recurrent e8a2 *BCR::ABL1* transcript variant with an interposed 55 bp inverted *ABL1* sequence. The generation of this *BCR::ABL1* variant is explained by a complex chromosomal translocation involving three chromosomal breaks with partial inversion of and deletion in the *ABL1* gene locus and subsequent activation of a cryptic exon on the minus DNA strand. Although this transcript variant is recurrent, i.e., repeatedly experimentally observed, it does not imply that the underlying chromosomal break events are identical in different 55 bp insert e8a2 *BCR::ABL1* cases. To obtain a better understanding of the generation of this variant we suggest the molecular investigation of future e8a2 cases also at the genomic level. The steps undertaken in our work could serve as a model for such an analysis.

Acknowledgments

The authors wish to thank Ms. Daniela Gröger for her skillful technical work and Ms. Thekla Schwarzer for organizational assistance.

Statement of Ethics

Our research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from the patient for publication of this case report and any accompanying images. This retrospective review of patient data did not require ethical approval in accordance with local and national guidelines.

Conflict of Interest Statement

T.B. received speakers' honoraria from Novartis and Pfizer. L.B. received honoraria from Sanofi, Astellas, Amgen, consultancy fee from Gilead, Hexal, and Menarini, consultancy fee and honoraria from AbbVie, BMS/Celgene, Daiichi Sankyo, Janssen, Jazz Pharmaceuticals, Novartis, and Pfizer and research funding from Bayer and Jazz Pharmaceuticals. P.C. received speakers' honoraria from Novartis, BMS, Incyte, and Pfizer.

Funding Sources

The work was funded by institutional research support.

Author Contributions

T.B. designed research and drafted the manuscript. L.B. and P.C. treated the patient and contributed to the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The nucleotide sequence of the chromosomal break sequence has been submitted to the GenBank/EMBL/DDBJ database and is available under accession number OP797408. Further inquiries can be directed to the corresponding author.

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