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Characterization of the Different *BCR-ABL* Transcripts with a Single Multiplex RT-PCR

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The diagnosis of chronic myeloid leukemia is based on detection of the Philadelphia (Ph) chromosome or the *BCR-ABL* gene. The junction present in the transcript may vary according to the reciprocal translocation t(9;22)(q34;11). Identification of the transcript (p190, p210 or p230) does not reveal the type of junction but this information is very important for classification of patients in clinical trials. Most identification kits do not explore p230 transcripts and are unable to determine exotic breakpoints. We have developed a clinical molecular diagnosis assay, able to identify all of the *BCR-ABL* transcripts and, by single assay, to characterize all of the possible transcript junctions. This technique is based on RT-PCR and PCR-capillary electrophoresis. For each patient sample, we performed RT-PCR with three different BCR primers each coupled to a specific different fluorochrome and a unique reverse ABL primer. Depending on the transcript, only one BCR primer was used for each RT-PCR. After capillary electrophoresis and fluorescence determination, we were able to identify both the transcript and its junction at the same time. *(J Mol Diagn 2004, 6:343–347)*

Chronic myeloid leukemia (CML) is a malignant clonal disorder of pluripotent hematopoietic stem cells resulting in an increase in myeloid, erythroid, and megacaryocyte lineage cells in peripheral blood, and myeloid hyperplasia in the bone marrow. CML is associated with a cytogenetic abnormality known as the Philadelphia (Ph) chromosome. This chromosome, a shortened chromosome 22, results from a t(9;22)(q34;11) reciprocal translocation, allowing the fusion of the 3′ region of the protooncogene *c-ABL* (9q34) with the 5′ region of the *BCR* (breakpoint cluster region) gene on chromosome 22q11.[1] The Philadelphia chromosome was the first chromosome abnormality to be associated with a specific human disease of malignant origin. The different fusion proteins encoded by *BCR-ABL* vary in size depending on the breakpoint in the *BCR* gene but share a high tyrosine kinase activity, in part responsible for the leukemogenesis.[2] Three breakpoint cluster regions in the *BCR* gene have been described to date: major (M-BCR), minor (m-BCR) and micro (μ-BCR)[3] (Figure 1).

More than 95% of Ph-positive CML patients present a breakpoint in the M-BCR region. Two major breakpoints are found, after the 13th exon resulting in a b2a2 (e13a2) fusion or after the 14th exon resulting in a b3a2 (e14a2) fusion. Both fusion mRNAs are translated into p210$^{BCR\text{-}ABL}$ protein.[4] Other junctions coding for p210$^{BCR\text{-}ABL}$ are rare. On the other hand, some p210$^{BCR\text{-}ABL}$ can be found in other types of leukemia.[5,6]

The breakpoint in the m-BCR region results in an e1a2 junction which is translated into a p190$^{BCR\text{-}ABL}$ protein. Some acute lymphoblastic leukemia (ALL) are induced by this protein.[7]

It is possible to observe a third BCR-ABL protein: p230$^{BCR\text{-}ABL}$. It consists of more than 90% of p160^{BCR} because the breakpoint is located in the 3′ end of the *BCR* gene, in the μ-BCR region, and its transcript contains a e19a2 junction. P230$^{BCR\text{-}ABL}$ has been associated with CML with maturation of neutrophils.[8]

There have been some case reports of CML patients having p190$^{BCR\text{-}ABL}$ or p230$^{BCR\text{-}ABL}$ fusion proteins. Finally, rare BCR-ABL variants involving other breakpoints have been described in patients with CML but never in patients with ALL.[9]

Several breakpoint regions in the *c-ABL* gene have been described.[10] The most frequent are 5′ of the second exon resulting in a2 junctions. Other breakpoints have been described between the second and the third exon, resulting in a3 junctions. The knowledge of such difference is critical in that case because the presence or the absence of an SH3 domain has clinical consequences.[11] Otherwise, these are still no information about differences between other types of junctions, but for scientific reasons, the availability of such information is indispensable and most of reference labs explore the position of breakpoint.

The *BCR-ABL* gene can be detected using several molecular methods, ie, Southern blotting, fluorescence *in*

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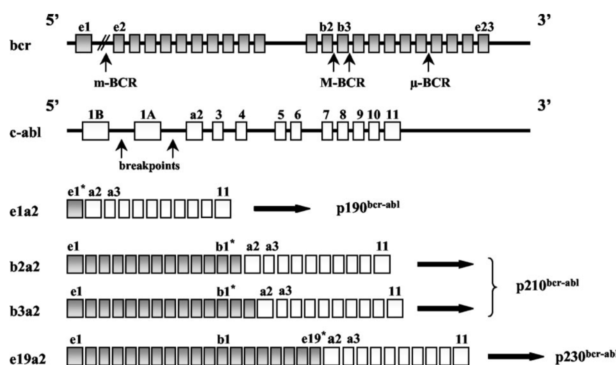

Figure 1. Molecular structure of *BCR* and *c-ABL* genes and their main translocated transcripts. **Arrows** indicate breakpoints in *BCR* and *c-ABL*. **Asterisks** show sense primers which are used for the detection of different transcripts (e1, Tet; b1, Fam; e19, Hex). The reverse primer is systematically a3.

situ hybridization (FISH) and reverse-transcription polymerase chain reaction (RT-PCR). For its simplicity, rapidity, and sensitivity, RT-PCR is one of the most common techniques used for this purpose.[12–16] Due to the long introns in which DNA breaks occur, a classical PCR using genomic DNA is difficult to carry out. Characterization of the transcript is not necessary for the diagnosis but permits follow-up at the molecular level. We have thus developed a technique based on the principle of PCR-capillary electrophoresis to identify all types of transcripts in a rapid and precise manner.

Briefly, our technique consists of performing multiplex PCR using primers coupled to different fluorochromes. Then, the optical system of a sequencer (ABI PRISM 310, Perkin-Elmer, Courtaboeuf, France) is used to identify the transcript. Depending on the fluorescence and the size of the PCR product, we can identify at the same time both the transcript (fluorescence) and the type of junction that it contains (size). The method is simple, rapid, and does not require complex equipment. Here, we report on the theoretical aspects and practical validation of this technique.

Materials and Methods

Primers

The sequences of the oligomers are listed in Table 1. PCR fragment analysis by capillary electrophoresis (CE) can be performed on fragments from 50 to 500 bp in length. We wanted to have only one sense primer associated with one transcript. We therefore chose a primer specific for exon b1 (e12) of the BCR gene able to amplify all variants of M-BCR. Specific primers for e1 and e19 were chosen for detection of breakpoints respectively in minor BCR and micro BCR regions. The ABL primer was specific for exon a3 and can thus detect junctions in a2 and a3 according to the shortness of the a2 exon (174 bp). Each BCR primer was coupled with a different fluorochrome in 5′.

RNA Extraction, cDNA Synthesis, and PCR Amplification

Total RNA was extracted from 5 ml of blood using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and was then dissolved in 50 μl of sterile water. cDNA synthesis was carried out at 37°C for 1 hour after adjustment of the mixture to contain 5 μl buffer 5X - Gibco-BRL (250 mmol/L Tris-HCl pH 8.3, 375 mmol/L KCl, 15 mmol/L $MgCl_2$), 10 mmol/L DTT (Gibco-BRL, Cergy-Pontoise, France), 1 mmol/L dNTP (Roche Diagnostics, Meylan, France), 2.4 μg of random hexamer primers, 2 μl of sterile water, 10 μl of RNA, 40 U of RNAguard (Amersham Biosciences, Orsay, France), and 400 U of reverse transcriptase of Moloney murine leukemia virus (MMLV). The reaction medium was made up to 50 μl with sterile water. Finally, the reaction was stopped by a 2-minute incubation at 100°C.

The PCR reaction medium consisted of 5 μl cDNA, 2.5 μl 10X buffer, 2 mmol/L $MgCl_2$, 250 μmol/L of each dNTP, 10 pM of each primer, and 3 units of *Taq* polymerase in a final volume of 25 μl. PCR was performed using a 9700 GeneAmp Thermo Cycler (Perkin Elmer) under the following cycling conditions: initial denaturation at 94°C for 2 minutes, followed by 30 cycles at: 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by a final 5-minute extension step at 72°C. To exclude a false negativity, RT-PCR for G6PD mRNA was performed, in parallel, under the same conditions as for *BCR-ABL* RT-PCR. The reaction was verified by agarose gel electrophoresis.

PCR Product Analysis

Fluorescent-labeled PCR products were detected using the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) and GeneScan Collection software (3.7 version). The following CE run parameters were used: Genescan Short Tandem Repeat Performance Optimized Polymer (GS STR

Table 1. Oligonucleotide Primers

Sequence	Fluorochrome	Use
GCTTCACACCATTCCCCATT		a3 primer (ABL)
TGGAGGAGGTGGGCATCTAC	Tet	e1 primer (BCR)
GCAGAGTGGAGGGAGAACAT	Fam	e12 (b1) primer (BCR)
CCTCGCAGAACTCGCAACAG	Hex	e19 primer (BCR)
GATGCCTTCCATCAGTCGGA	Tet	G6PD-S
GCTCACTCTGTTTGCGGATG		G6PD-R

G6PD-S, Glucose-6-phosphate dehydrogenase sense; G6PD-R, Glucose-6-phosphate dehydrogenase reverse; Tet, 4, 7, 2′, 7′-tetrachloro-6-carboxyfluorescein; Fam, 6-carboxyfluorescein; Hex, 4, 7, 2′, 4′, 5′, 7′-hexachloro-6-carboxyfluorescein.

Table 2. Theoretical Size, Fluorochrome Correspondence, and Number of Patients Observed for Each PCR Product

P210 *BCR-ABL*			P230 *BCR-ABL*			P190 *BCR-ABL*		
Color	Size (pb)	Transcript/quantity observed	Color	Size (pb)	Transcript/quantity observed	Color	Size (pb)	Transcript/quantity observed
Fam (blue)	92	b1a3	Hex (black)	127	e19a3	Tet (green)	223	e1a3
	197	b2a3		262	e20a3		397	***e1a2***/1
	272	b3a3/1		301	***e19a2***/3		865	e6a3
	371	***b2a2***/51		436	e20a2			
	446	***b3a2***/96						

The detected fluorescence permits characterization of the type of transcript: blue for p210, black for p230, and green for p190. The size of the PCR product allows, at the same time, identification of the junction between BCR and c-ABL. The most frequent transcripts are indicated in bold. The e1a2 transcript was also observed on the pGD190 plasmid. The number of patients with each transcript is indicated. Fifty-six patients showed negative amplifications. Four patients showed b3a2 and b2a2 in the same sample (not mentioned in the table).

POP) 4 (1 ml) C module, GS POP 4 polymer, 5- to 10-second injection time at a voltage of 15 kV, a 15 kV electrophoresis voltage with a resultant 7 to 9 ampere current at a column temperature of 60°C, and a 30-minute electrophoresis time. GeneScan data analysis parameters were: matrix C virtual filter, 2800 to 6000 analysis range, base-lining, multi-component data processing, light peak smoothing, a minimum peak, detection limit of 50 relative fluorescent units (RFU), size call range of 50 to 500 bp using the local Southern size calling method, Tamara-labeled GS 500 size standard, and no split peak correction.

Results

Our laboratory is responsible for following up CML patients by molecular study. For this purpose, we need to know which type of junction(s) each patient carries. We have developed the present technique to facilitate the identification of these junctions. From now on, 212 CML patients will be investigated using this method. The large number of patients allowed us to validate our protocol. Theoretical results for the transcripts observed in these 212 samples are presented in Table 2.

PCR products were analyzed as described above. The Genescan Collection software adjusts the sensitivity automatically so that the peak of fluorescence corresponding to the transcript is perfectly visible and distinguishable. The resolution of the CE is largely sufficient to obtain a reliable distinction between all types of transcripts, including those differing by only a few bases. Typical electropherogram data generated by the described assay for every type of transcript are shown in Figure 2.

Discussion

The existence of three breakpoints in the BCR and the random aspect of the reciprocal translocation between chromosomes 9 and 22 render the identification of the transcript difficult. Techniques used for the characterization of the transcript were incomplete because they did not allowed the identification of the junction at the same time,[17] or, like the Southern blot, they were not able to identify every possible transcript. We therefore decided to develop a new method to obtain a reliable and complete diagnosis in one step.

In this report, we show that standard techniques such as multiplex PCR and capillary electrophoresis can be used for a fast, complete, and reliable diagnosis. Primers were selected so that the mRNA (coding for p190$^{BCR\text{-}ABL}$, p210$^{BCR\text{-}ABL}$, or p230$^{BCR\text{-}ABL}$) can be identified without any doubt. The reverse primer is unique (a3 in *c-ABL*), because this exon is present in all transcripts resulting from a t(9;22)(q34;11) reciprocal translocation. Indeed, the use of exon a2 for primer would give false-negative results in the rare cases with breakpoints between exons a2 and a3 of the *ABL* gene. Sense primers were selected to permit the characterization of the different breakpoint regions in *BCR* (m-BCR, M-BCR, μ-BCR). Moreover, every sense primer has been coupled with a different fluorochrome so it can be detected by the optic system of an ABI PRISM 310 sequencer. Of course, exon e1 is present in each transcript, and b1 in those coding for p210$^{BCR\text{-}ABL}$ and p230$^{BCR\text{-}ABL}$. M-BCR and μ-BCR transcripts could thus be theoretically amplified and detected using the e1 primer and the μ-BCR transcripts using the b1 primer. However in the case of a positive RT-PCR, the products obtained would be too long to be identified by this method. The analysis, as described in Materials and Methods, can be performed on fragments ranging from 50 to 500 bp in length.

Characterization of the junction is based on the size of the products detected after PCR. For example, the result from a PCR performed on a transcript coding for p210$^{BCR\text{-}ABL}$ will be different depending on whether the junction is b2a2 or b3a2. Moreover, every PCR product is systematically verified by agarose gel electrophoresis and sequencing.

The G6PD product is used as positive control. We had samples where G6PD was observed but not *BCR-ABL*. It was confirmed by absence of Philadelphia chromosome. In rare cases where the G6PD product did not amplify, a mistake in the RT-PCR process was suspected and a new study was performed on a new sample. We have had 11 failures during development. Moreover, during development of this technique, we used healthy patient as negative controls. Subsequently, we did not use cDNA controls.

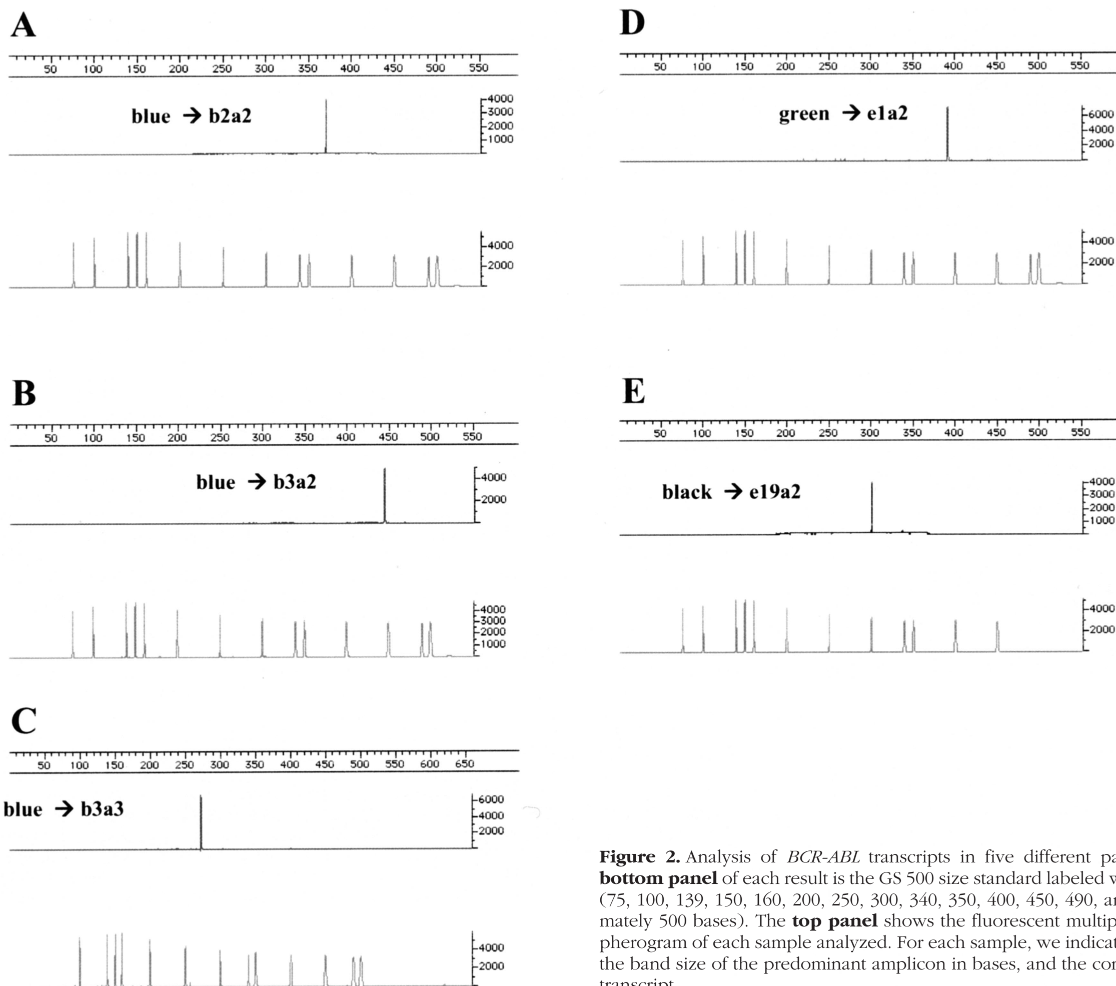

Figure 2. Analysis of *BCR-ABL* transcripts in five different patients. The **bottom panel** of each result is the GS 500 size standard labeled with Tamara (75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and approximately 500 bases). The **top panel** shows the fluorescent multiplex electropherogram of each sample analyzed. For each sample, we indicate the color, the band size of the predominant amplicon in bases, and the corresponding transcript.

In our samples we did not find any patient carrying the b1a3 or the e1a3 junctions. This fact is probably not due to a failure of our technique, as theoretically these transcripts should be identified. The only limitation of our technique concerns rare and atypical transcripts, which are either too long to be amplified or too long to be detected by CE. However, in case of a negative result, such transcripts are identifiable with agarose gel electrophoresis and sequencing. These latter techniques will also be used for the very rare detectable transcripts such as e6a2,[9] e8a2[18] or e2a1.[19]

Conclusions

The fluorescence multiplex fragment length assay presents many advantages over conventional methods. It does not require a heavy implementation, is fast and precise, and gives at the same time both the breakpoint region and the junction. This information is critical not for molecular residual disease following, but for scientific purpose. It is inconceivable to ignore the breakpoint in a clinical following way, as new molecules appear which could have different effects depending on the breakpoint. Finally, our technique is highly reliable, except in the case of rare transcripts for which specific primers could be developed in the future.

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