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Multiplex PCR – a rapid screening method for detection of gene rearrangements in childhood acute lymphoblastic leukemia

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Abstract Chromosomal rearrangements in childhood acute lymphoblastic leukemia (ALL) play an important role in the identification of clinical relevant subgroups. For rapid and easy detection of the clinically most important gene rearrangements, a nested multiplex reverse transcriptase polymerase chain reaction (multiplex PCR) was developed. This multiplex PCR enables the detection of M-BCR/ABL, m-BCR/ABL, TEL/AML1, and MLL/AF4 fusion transcripts in one PCR reaction. However, the existence of splicing variants and different breakpoints on the DNA level hampers the discrimination of the rearrangements by their fragment size on an agarose gel. Therefore, one of the internal primers of each translocation (ABL-2, TEL-2, AF4–2) was labeled with a characteristic fluorescent dye, and an automatic fluorescence-based DNA fragment analysis was performed. The sensitivity of this multiplex PCR is in the same range as that of the corresponding single PCR reaction and allows a fast screening for the detection of therapy-relevant rearrangements, with a high turnover of samples.

Keywords Multiplex PCR · Chromosomal rearrangements · ALL · Genescan analysis

Introduction

Acute lymphoblastic leukemia (ALL), the most common childhood malignancy, is associated with chromosomal translocations allowing the identification of prognostically relevant subgroups [17, 26, 29]. These translocations or their molecular equivalents – t(9;22) (BCR/ABL), t(4;11) (MLL/AF4), and t(1;19) (PBX1/EA2) – are used to identify high-risk patients in most large therapy trials [8, 9, 13, 30, 31, 32, 37, 38, 39]. In the German multicenter trials ALL-BFM-95 and CoALL, genetic analysis is centralized and all bone marrow and/or blood samples of children with ALL are routinely screened for these rearrangements by PCR, with the exception of PBX1/EA2. In addition, all samples are screened for TEL/AML1 [t(12;21)] the most frequent fusion gene (25%) of childhood B-cell precursor ALL, which is supposed to have a good prognosis [3, 18, 25, 27, 36, 40, 41]. At present, the PCR of these rearrangements is performed in separate PCR assays, which is not only time and material consuming but also very expensive.

Based on these considerations, we developed a multiplex PCR that allows detection of the rearrangements BCR/ABL minor breakpoint (m), BCR/ABL major breakpoint (M), TEL/AML1, and MLL/AF4 in one step. A primer mix containing seven primers (M-BCR, m-BCR, ABL, TEL, AML1, MLL, AF4) was used for the multiplex PCR, in order to detect all four rearrangements in one assay. To improve sensitivity and specificity, a nested PCR protocol was performed.

Different breakpoints and/or splicing variants are described for BCR/ABL, TEL/AML1, and MLL/AF4 [1, 2, 4, 6, 7, 14, 15, 16, 20, 21, 24, 28, 35, 45] and, consequently, a broad range of different PCR products could originate in the multiplex PCR. The difference of a few basepairs will therefore complicate the determination of the rearrangements by fragment size on an agarose gel. Genescan analysis overcomes this problem by labeling one internal primer of each rearrangement on the 5′-end with a characteristic fluorescent dye (Fig. 1). Using this technique, the rearrangements can be identified not only by their fragment size, but also by their characteristic fluorescence emission.

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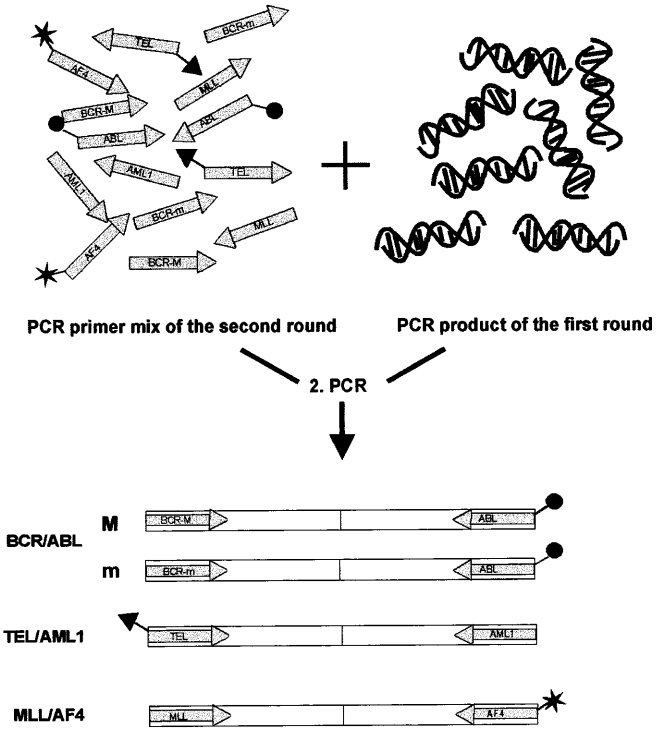

Fig. 1 The second round of multiplex PCR. One internal primer of each rearrangement is labeled with a characteristic fluorescence dye on the 5′-end: TEL-2 with FAM (▲ blue), AF4-2 with TAMRA (★ yellow), and ABL with JOE (● green)

Patients and methods

Patients and cell lines

Bone marrow or peripheral blood samples of patients with ALL were sent by mail from more than 70 pediatric oncology centers in Germany. The BCR/ABL major breakpoint was tested mainly with samples of CML patients. Ninety patients with ALL and four with CML consecutively enrolled in ongoing studies in Germany (ALL-BFM; CoALL, CML-päd) were screened by single PCR assays and multiplex PCR in parallel. Additionally, 60 patients (54 ALL and six CML) with a known rearrangement were analyzed by multiplex PCR retrospectively. For positive controls and sensitivity assays, cell lines (K562, SD1, MV4-11, RS4-11, REH, HL60) with the corresponding translocations were used [11, 12, 42, 44]. Mononuclear cells of patients were isolated by centrifugation using Nycoprep 1.077 (Nycomed, Oslo, Norway) and stored at –70 °C prior to use. Cell lines grown in suspension culture were centrifuged and stored at –70 °C.

For sensitivity studies 1 million cells of cell lines carrying one of the rearrangements (K562/M-BCR; SD1/m-BCR; REH/TEL/AML1; MV4-11/MLL/AF4) were serially diluted 1:10 with HL60 cells lacking any of these translocations.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted in a single-step method [5] and dissolved in 15 μl dH_2O. Three micrograms of cell-line RNA or 7 μl of patient RNA (about 1–5 μg) were reverse-transcribed with 200 units of SuperScript™ RNase H^- reverse transcriptase (GibcoBRL, Eggenstein, Germany). Following denaturation at 70 °C for 10 min, the cDNA synthesis was carried out at 37 °C for 45 min using random hexamer primers in a total volume of 20 μl. Subsequently, the cDNA was heated to 95 °C for 5 min to inactivate the reverse transcriptase and was then stored at –20 °C.

Maximum sensitivity and specificity were achieved by using a nested-PCR protocol. To verify the integrity of the isolated RNA and the correctness of the cDNA synthesis, the ubiquitously expressed ABL gene was amplified in a separate PCR. Primer sequences for the multiplex PCR assay and the amplification of ABL are given in Tables 1 and 2. The first round of PCR was done with the external primers. In the second round the internal primers ABL-2, TEL-2, and AF4-2 marked with a characteristic fluorescent dye at their 5′-end were used (Table 1). Amplification was performed with a Perkin Elmer Thermocycler 9600 (Perkin Elmer, Weiterstadt, Germany).

In the first round of PCR, 1 μl of cDNA was used for the ABL control assay, 1 μl for sensitivity assays, and 3 μl for the multiplex assay. The PCR was carried out in a final volume of 20 μl with 1×PCR-Buffer (GibcoBRL, Eggenstein, Germany), 1.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP (Boehringer Mannheim, Germany), 4% DMSO (only for the multiplex assay), 1.6 pmol of each primer, and 1 unit Taq polymerase (GibcoBRL, Eggenstein, Germany).

After an initial melting step (90 s at 95 °C), 35 amplification cycles of 15 s at 94 °C, 45 s at 64 °C, and 45 s at 72 °C were performed, followed by an extension step (6 min at 72 °C). One microliter of the first-round PCR product was subjected to the second round of PCR, differing by the annealing temperature (60 °C), the primer concentration (8 pmol), and the number of cy-

Table 1 PCR primers used in the multiplex assay. fd = fluorescent dye; JOE = green; FAM = blue; TAMRA = yellow

Multiplex PCR primer			
Primer	Position, orientation	Sequence 5′-NNN-3	fd
BCR-M-1	External, sense	CCTCTGACTATGAGCGTGCAGAGT	–
BCR-M-2	Internal, sense	AGAAGTGTTTCAGAAGCTTCTCCCT	–
BCR-m-1	External, sense	CAGCTCCAATGAGAACCTCACCTCCAGCG	–
BCR-m-2	Internal, sense	AACTCGCAACAGTCCTTCGACAGCAGCAG	–
ABL-1	External, antisense	CTCAGCGGATACTCAGCGGCATTGCGG	–
ABL-2	Internal, antisense	ACTGTTGACTGGCGTGATGTAGTTGCTTGG	JOE
TEL-1	External, sense	ACCAGGAGTCCTACCCTCTGTCAGTG	–
TEL-2	Internal, sense	CCGGCAGGAGAGCACACGCGTGATCCAG	FAM
AML1–1	External, antisense	GTAGGCAGCACGGAGCAGAGGAAGTTGG	–
AML1–2	Internal, antisense	AACGCCTCGCTCATCTTGCCTGGGCTCAG	–
MLL-1	External, sense	CTGAATCCAAACAGGCCACCACTC	–
MLL-2	Internal, sense	GGTCTCCCAGCCAGCACTGGTC	–
AF4–1	External, antisense	CTCACTGTCACTGAGCTGAAGGTCGTCTTCG	–
AF4–2	Internal, antisense	AGCATGGATGACGTTCCTTGCTGAG	TAMRA

Table 2 PCR Primers used in the ABL control assay. fd = fluorescent dye

ABL control PCR primer			
Primer	Position, orientation	Sequence 5′-NNN-3	fd
ABL-K1a	External, sense	CCAGTAGCATCTGACTTTGAGCCT	–
ABL-K1b	External, antisense	CCAGACTGTTGACTGGCGTGATGT	–
ABL-K2a	Internal, sense	TGAGTGAAGCCGCTCGTTGGAACT	–
ABL-K2b	Internal, antisense	TTCACACCATTCCCCATTGTGATT	–

cles (25). Ten microliters of the final PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining.

Genescan analysis

All amplification products which could not be exactly assigned to a rearrangement by fragment size were subsequently characterized by Genescan analysis. Depending on the concentration, the final PCR product was used undiluted or diluted with sterile water 1:10 or 1:30.

One microliter of the undiluted or diluted PCR product was mixed with 0.5 μl Genescan standard (Genescan-2500™ROX; Perkin Elmer, Weiterstadt) and 2.5 μl formamide. The samples were denatured at 90 °C for 2 min, subsequently chilled on ice, and subjected to electrophoresis using a 5% polyacrylamide gel in an automatic DNA-sequencer 373 A (Perkin Elmer, Weiterstadt). Gels were analyzed using an Apple Macintosh IIci computer and the Genescan software as supplied by the manufacturer.

Results

The multiplex PCR assay was able to detect all four rearrangements and their characteristic splicing variants or molecular breakpoints in the cell lines K562 (M-BCR/ABL), SD1 (m-BCR/ABL), REH (TEL/AML1), and MV4-11 (MLL/AF4) and in the patient samples (Figs. 2 and 3).

The sensitivity assays detected one cell harboring the rearrangement in the following dilutions: M-BCR/ABL (K562) 10^{-4}, m-BCR/ABL (SD1) 10^{-3}, TEL/AML1 (REH) 10^{-3}, MLL/AF4 (MV4–11) 10^{-4} (Fig. 4). Whereas the sensitivity for MLL/AF4 (10^{-4}) and m-BCR/ABL (10^{-3}) was of the same order of magnitude as the corresponding single PCR reaction, it was ten times lower for M-BCR/ABL (10^{-4}) and TEL/AML1 (10^{-3}).

PCR products of cell lines could be discriminated by their fragment size on an agarose gel (Fig. 2) and, additionally, they were analyzed by the Genescan method. With this technique, the PCR product of BCR/ABL showed a green signal with a fragment size of 447 bp (e1/a2) for SD1 and 470 bp (b3/a2) for K562. The REH cell line was detected by a blue double band of 306 and 345 bp, corresponding to the two variants of TEL/AML1 (e5/e2 and e5/e3), and for the MV4-11 cell line one yellow amplification product of 381 bp (e6/c) became visible (Fig. 3).

Of the 60 patients (54 ALL and six CML) analyzed retrospectively by multiplex PCR the results were concordant in 52 cases (seven M-BCR/ABL, five m-BCR/ABL, 36 TEL/AML1, four MLL/AF4). Eight of them, however, showed no amplification in the multiplex assay but were positive for TEL/AML1 in the single PCR.

Ninety-four patients (90 ALL and four CML) were analyzed prospectively over a period of 2 months with single PCR reactions and multiplex PCR in parallel, and a complete concordance was found in 90 of them. In 67 patients none of the analyzed rearrangements was detected, whereas four showed M-BCR/ABL, five m-BCR/ABL, 13 TEL/AML1, and one the MLL/AF4 rearrangement. In the remaining four cases a TEL/AML1 amplification was visible in the single PCR assay, but no PCR product was observed by multiplex PCR.

The 12 TEL/AML1-negative patients of both the prospective and the retrospective study were additionally analyzed by the more sensitive Genescan technique. Ten of them showed a weak blue signal, two whereas remained negative.

Discussion

Identification of specific chromosomal aberrations or their molecular equivalents is an important tool for diagnosis and therapy stratification in childhood ALL [17, 26, 29]. Conventional cytogenetics allow the detec-

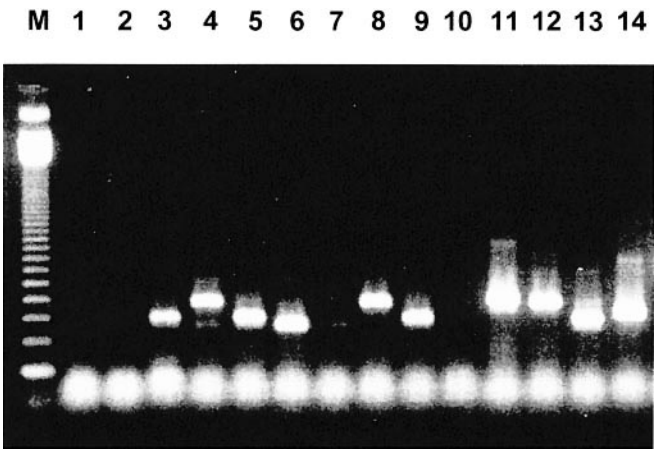

Fig. 2 Agarose gel analysis of different PCR products from patients and cell lines. *Lane M*: 123-bp marker; *lanes 1, 2, 7, 10*: patients with no rearrangement; *lanes 3, 5, 9*: patients with TEL/AML1 (e5/e2) rearrangement; *lane 6*: patient with TEL/AML1 (e5/e3) rearrangement; *lanes 4, 8*: patients with m-BCR/ABL (e1/a2) rearrangement. *Lanes 11–14,* cell lines used as positive controls – *lane 11*: K562 (M-BCR/ABL), *lane 12*: SD1 (m-BCR/ABL), *lane 13*: REH (TEL/AML1); *lane 14*: MV4–11 (MLL/AF4)

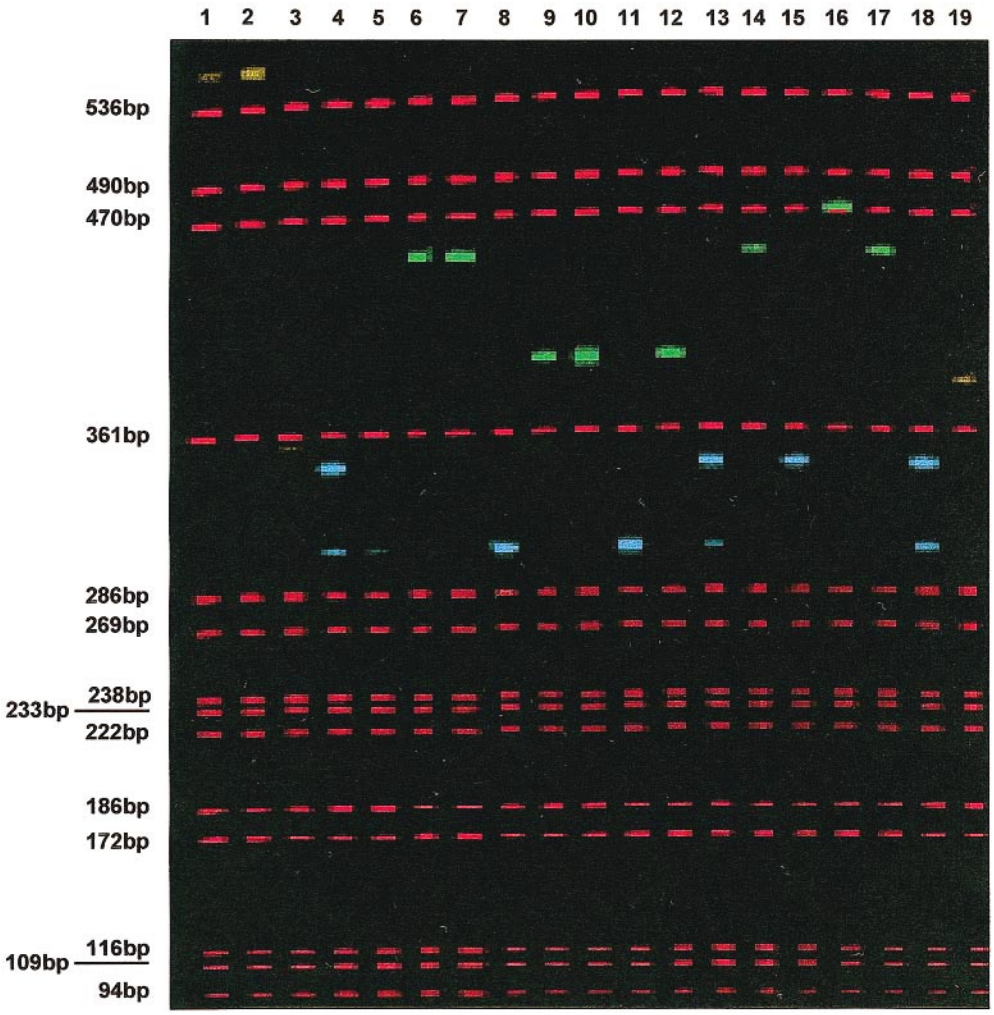

Fig. 3 Genescan analysis of different PCR products from patients and cell lines. BCR/ABL PCR products are labeled in *green,* TEL/AML1 in *blue,* MLL/AF4 in *yellow,* and the internal size marker in *red. Lanes 1, 2*: cell line RS4-11 (MLL/AF4) diluted 1:30 and 1:10, respectively; *lane 3*: patient with MLL/AF4 rearrangement; *lanes 4, 13, 15*: patients with two splicing variants of the TEL/AML1 (the small variant in *lane 15* detected only by computer); *lanes 5, 8, 11*: patients with the small variant of TEL/AML1; *lanes 6, 7, 14*: patients with m-BCR/ABL gene fusion; *lanes 9, 10, 12*: patients with b2a2 M-BCR/ABL rearrangement. *Lanes 16–19,* cell lines – *lane 16*: K562 (M-BCR/ABL); *lane 17*: SD1 (m-BCR/ABL); *lane 18*: REH (TEL/AML1); *lane 19*: MV4-11 (MLL/AF4)

tion of all these aberrations. However, it is very time consuming, the sensitivity is low, and, especially after mailing, the success rate is relatively low [19, 23]. In addition, cryptic translocations such as t(12;21) are hardly detectable [31, 34]. PCR techniques allow the identification of all clinically relevant aberrations in a fast and sensitive way. For screening, however, several PCR reactions have to be performed in order to detect one of these aberrations in a single patient. This time-consuming and expensive work could be overcome by a multiplex PCR that allowed the detection of all important rearrangements in one assay. Different multiplex PCRs have been reported for leukemias [10, 22, 33, 43], but none of them is able to detect the four rearrangements M-BCR/ABL, m-BCR/ABL, MLL/AF4, and TEL/AML1 in one assay.

Splicing variants and/or different breakpoints are known for these rearrangements. In the majority of patients two types of BCR/ABL exist. The major breakpoint (M-BCR) is found mainly in CML, whereas the minor breakpoint (m-BCR) can be detected in ALL patients [6, 20, 24]. The break of the ABL gene occurs mostly between exon a1 and a2 (rarely between a2 and a3), and in the BCR gene it is located behind exon e1 (m-BCR) or behind exon e13/b2 as well as e14/b3 (M-BCR) [4]. For TEL/AML1, two main forms are described, resulting in a fusion of TEL exon e5 and AML1 exon e2 or e3 [1, 14, 28, 35, 45]. The MLL/AF4 rearrangement shows the largest heterogeneity. In the MLL gene the breaks occur in an 8.3-kb breakpoint region, mainly after exons e6, e7, and e8. For AF4 three different breakpoints are described (b, c, d), and in addition alternative splicing is reported in MLL/AF4 [3, 7, 15, 16, 21].

Because of this molecular heterogeneity, agarose gel analysis might be not sufficient in some cases to identify the specific rearrangement exactly. However, Fluorescent labeling of the internal primer overcomes this problem. With Genescan analysis the existing rearrangement can be determined by its characteristic fluorescence color and fragment size. Furthermore, the Genescan analysis allows the exact size calculation of each PCR product by application of an internal ROX-labeled standard. Therefore, it is possible to determine the splicing variants or breakpoints of the rearrangements.

A complete concordance was observed for the rearrangements M-BCR/ABL, m-BCR/ABL, and MLL/

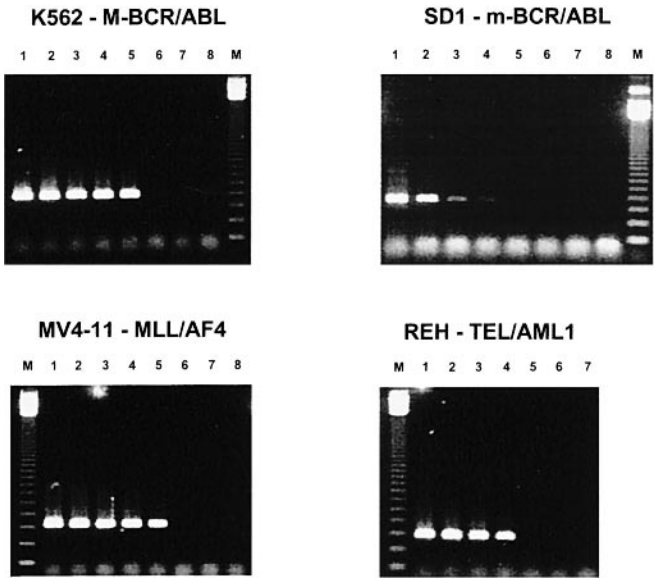

Fig. 4 Sensitivity assays for the rearrangements M-BCR/ABL, m-BCR/ABL, TEL/AML1, and MLL/AF4 with the corresponding cell lines. *Lane M*: 123-bp marker; *lane 1*: undiluted; *lane 2*: diluted 10^{-1}; *lane 3*: diluted 10^{-2}; *lane 4*: diluted 10^{-3}; *lane 5*: diluted 10^{-4}; *lane 6*: diluted 10^{-5}; *lane 7*: cell line HL60 undiluted; *lane 8*: negative control

AF4. All patients positive for one of these rearrangements by single PCR are positive in the multiplex assay as well. For the TEL/AML1 rearrangement, however, a discrepancy became obvious. Of 61 patients with a TEL/AML1 rearrangement in routine single PCR assay, 12 (19.7%) showed no amplification in the multiplex PCR. This could be due to the sensitivity, which is ten times lower (10^{-3}) in the multiplex PCR. The sensitivity of m-BCR/ABL is in the same range as that of TEL/AML1, but no difference to the single PCR reaction is noticeable. It is possible that expression of the TEL/AML1 fusion transcript in patients is lower than that of m-BCR/ABL, whereas in the two cell lines REH (TEL/AML1) and SD1 (m-BCR/ABL) the same expression rate exists.

On the other hand, patients' bone marrow or blood samples sent to our laboratory by mail are 24–48 h old, whereas the RNA samples from cell lines were prepared directly. Cells harboring the TEL/AML1 rearrangement might be more sensitive, and in consequence the amount of RNA could be lower. Possibly, the sensitivity of 10^{-3} achieved by this multiplex assay is too low, whereas the detection level of the single PCR (10^{-4}) is sufficient.

Furthermore, PCR inhibitors of blood and bone marrow samples (e.g., heparin, hemoglobin) could be carried and therefore influence the PCR effectiveness of patient samples, especially in the case of TEL/AML1.

Ten of the 12 TEL/AML1 false-negative cases showed a weak blue signal in the Genescan analysis, which is more sensitive than a normal agarose gel. In consequence, all samples should be analyzed with the Genescan technique, and other PCR methods, e.g., hot start PCR, should be be tested in parallel to optimize the assay. Nevertheless, the multiplex PCR is a powerful tool for detecting the therapy-relevant rearrangements in a short time with a high sample turnover.

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