Clinical Screening of Gene Rearrangements in Childhood Leukemia by Using a Multiplex Polymerase Chain Reaction-Microarray Approach


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ABSTRACT

Purpose: Currently, many forms of leukemia are considered potentially curable, with prognosis and clinical outcome strongly dependent on the underlying molecular pathophysiology. A substantial number of leukemia patients harbor nonrandom karyotypic abnormalities that define subgroups with unique biological and clinical features. For detection of these types of gene rearrangements, a combination of multiplex RT-PCR with hybridization on oligonucleotide microarray was presented previously, which identified five chromosomal translocations with fusion variants. In the present study, additional clinically relevant translocations were included in our analysis using a second generation of microarrays. We also expanded significantly on the clinical correlation of our findings.

Experimental Design: An oligonucleotide microarray was designed for hybridization with products of a multiplex RT-PCR to identify the following translocations: t(9;22)p190, t(4;11), t(12;21), t(1;19), typical for acute lymphoblastic leukemia; t(9;22)p210 for chronic myeloid leukemia; and t(8;21), t(15;17), inv16, typical for acute myeloblastic leukemia.

Results: To demonstrate the potential clinical application of the method, 247 cases of childhood leukemia were screened, and the above-mentioned gene rearrangements were found in 30% of cases. The sensitivity and specificity of the assay is comparable with the RT-PCR technique, so that it can be used to follow minimal residual disease. The feasibility of an additional refinement of the method, on-chip-multiplex PCR, has been successfully demonstrated by identifying a common translocation, t(9;22), in chronic myeloid leukemia.

Conclusions: Our data suggest that the microarray-based assay can be an effective and reliable tool in the clinical screening of leukemia patients for the presence of specific gene rearrangements with important diagnostic and prognostic implications. The method is amenable for automation and high-throughput analysis.

INTRODUCTION

The ability to detect fusion transcripts specific for leukemia cells has changed dramatically the clinical diagnostics of leukemia. Since the initial discovery of the fusion between the BCR and ABL genes in t(9;22), many other clinically relevant chromosome abnormalities have been identified that result in the juxtaposition and subsequent fusion of gene sequences (1). The fused genes involved in chromosomal aberrations that lead to leukemia play a major role in the development of cells along both myeloid and lymphoid lineages (2).

ALL, the most common childhood malignancy, is associated with chromosomal translocations t(9;22) (BCR/ABL), t(4;11) (MLL/AF4), t(1;19) (E2A/PBX1), and t(12;21) (TEL/AML1), which are used to risk-stratify patients in most large clinical trials. In AML, such translocations are represented by t(15;17) (PML/RARA), (8;21) (AML1/ETO), and inv16 (CBFB/MYH11; Ref. 3). Because of tremendous progress in diagnostics and therapy, leukemia is no longer invariably fatal. In particular, treatment with ATRA of acute promyelocytic leukemia patients with t(15;17)/PML/RARA and with STI571, the ABL-tyrosine kinase inhibitor, of patients with t(9;22)BCR/ABL, (4, 5) are two

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The abbreviations used are: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML, chronic myeloid leukemia; ATRA, all-trans retinoic acid; MRD, minimal residual disease; THR, threshold; PML, promyelocytic leukemia; RARA, retinoic acid receptor-alpha.
excellent paradigmatic examples. They both demonstrate the importance of molecular diagnostic in choosing targeted anti-leukemic therapy.

Although conventional cytogenetics allows the detection of most known chromosomal rearrangements, it cannot detect certain cryptic translocations such as t(12;21). In addition, it is a time-consuming and labor-intensive method with relatively low sensitivity. Fluorescence in situ hybridization is not a very efficient technique for simultaneous detection of several different translocations; in addition, it is less sensitive than PCR-based approaches. The RT-PCR-based techniques allow the identification of all clinically relevant aberrations in a fast and sensitive way. To date, the majority of RT-PCR screening programs have searched for each of the most common fusion transcripts individually, which is both time-consuming and costly. At present, several multiplex PCR systems with advantages over the aforementioned methods have been described, based on gel electrophoresis analysis of amplified fragments (6, 7), Southern blot or dot-blot hybridization (8), or fluorescence capillary analysis (9). In a previous study, we suggested the combined use of multiplex RT-PCR and hybridization with oligonucleotide microarrays for the precise identification of multiple fusion transcripts (10). In the case of translocations, the input of a hybridization step in the screening procedure increases the specificity of the assay, compared with the traditional analysis of PCR products by gel electrophoresis. Moreover, for some chromosomal translocations, such as t(4;11)MLL/AF4, the confirmation of gene rearrangement by hybridization technique was recommended previously (11).

The microarrays developed in our laboratory consist of an array of semispherical gel pads of 100 μm in diameter attached to a hydrophobic glass surface (12). The gel pads present a support for immobilized oligonucleotides and, at the same time, serve as nanoliter tubes to perform multiple sequential reactions including hybridization, PCR amplification, ligation, and mini-sequencing directly on microarrays. The gel-based microchips have been used previously for identification of bacteria and viruses, detection of mutations and nucleotide polymorphisms in human and bacterial genomes, and so on (13–15).

In this study, we describe: (a) improvement of the microarray-based method for the analysis of gene rearrangements to include a larger panel of translocations with prognostic value; (b) the application of microarrays as a screening/diagnostic tool in a clinical study of ~250 leukemia patients; (c) enhancement of the sensitivity of the test to the level of detection of a small number of residual leukemic cells (one blast per 10^3 to 10^4) during and after therapy (i.e., monitoring of MRD); and (d) development of a next generation of microarrays for direct on-chip-PCR and application of this procedure for the analysis of gene rearrangements with potential diagnostic value.

Patient Samples. The database references for genes involved in translocations were: MLL (L04731), AF4 (NM_005935), BCR (NM_021574), ABL (M14752), TEL (NM_001987), AML1 (X79549), ETO (D14289), CBFB (L20298), MYH11 (D10667), E2A (M1222), PBX (M86546), PML (M79462), and RARA (X06538).

Patient samples for the microarray testing were received from the Russian Children’s Clinical Hospital. Parental consent was obtained in all cases, and the study was approved by the Institutional Review Board of the Engelhardt Institute of Molecular Biology.

RNA Preparation, RT-PCR, and Preparation of DNA Targets for Hybridization. Leukocytes from the bone marrow aspirates were isolated after sample hemolysis in 0.8% NH4Cl. Total RNA was prepared by using RNAqueous kit (Ambion Inc., Austin, TX), according to the manufacturer’s recommendations. The RNA solution was treated with 0.1 units/μl RNase-free DNase I (Ambion, Inc.) at 37°C for 30 min in 100 μl of 50 mm Tris-HCl (pH 8.0) and 10 mm MgCl2. The reverse transcription was performed with 1 μl of total RNA derived from 10^6 cells using the following set of cdNA-specific primers: ABL:797L12, AF4:1664L12, AML1:1921L12, PBX1:526L11, ETO:350L12, MYH11:1552L12, MYH11:2271L12, and RARA:700L12 (6). Primer names include the name of the corresponding genes, the number of the 5’-nucleotide of the primer, U or L to indicate upper or lower strand, followed by the length of the primer.

To determine the sensitivity of the tests, RNA from leukemic cells was serially diluted in 10^{-1} steps up to 10^{-6} with RNA from HL-60 cells carrying no translocations. Diluted samples were then used in multiplex RT-PCR, followed by hybridization.

The PCR amplification of the ABL gene was performed in 100 μl of 1× PCR reaction buffer (Perkin-Elmer, Wellesley, MA) containing 2.5 units of TaqDNA polymerase and 50 pmol of each of the following oligonucleotide primers: ABL5' and ABL3'. Thirty cycles were performed: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 7 min. A 300-bp fragment of c-ABL mRNA served as an internal positive control for all mRNA samples to identify false negative results in the RT-PCR assay (8, 16).

Multiplex PCR assay for translocations was performed as two parallel nested (two-round) multiplex reactions. The 1/50 of reverse transcriptase reaction mixture (an equivalent of 20,000 cells) was added to each of two 25-μl multiplex mixtures containing 11 mM Tris-HCl (pH 8.3), 55 mM KCl, 1.5 mM MgCl2 (the concentration was optimized for each type of polymerase), 0.2 mM each of deoxyribonucleotide triphosphates, a mixture of primers (0.2 μM each), and 1.5 units of AmpliTaq-Gold polymerase (Perkin-Elmer) or Taq-polymerase (Biomas ter, Sileks, Russia). The primers used in the first round were: in tube 1: BCR:1698U19, BCR:3060U23, ABL:661L23, MLL: 3750U20, AF4:1636L29, TEL:871U23, AML1:1891L23, E2A:1051U21, PBX1:459L18; in tube 2: AML1:1863U21, ETO: 327L23, PML3:1211U19, PML3:861U19, RARA:540L19, CBFB:267U22, MYH11:2198L22, MYH11:1438L24. The volume of the mixture in the second round was 100 μl, and 4 μl of the first round product were added as a template. The following primers were used in the second round (0.125 μM each): in tube 1: BCR:1777U19, BCR:3128U22, ABL:642L23, MLL: 3751U20, AF4:1606L22, TEL:944U23, AML1:1772L21, E2A: 1051U21, PBX1:1436L21; in tube 2: AML1:1885U20, ETO: 116L22, PML3:1370U21, PML3:930U20, RARA:580L22, CBFB:267U22, MYH11:2198L22, MYH11:1438L24. Twenty-five cycles per each round were performed: 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min.
Gene Rearrangements with Multiplex PCR-Microarrays

Oligonucleotides were synthesized with an Applied Biosystems 394 DNA/RNA synthesizer using standard phosphoramidite chemistry. The oligonucleotide sequences are shown in Table 1. All oligonucleotides carry an amino group at the 5′ end for immobilization in the polyacrylamide gel. The oligonucleotide probes from both sense and antisense DNA strands were included. Microarrays of polyacrylamide gel pads were prepared using a copolymerization method (17).

Hybridization and Image Analysis. Hybridization was performed in 40–50 μl of 20% formamide, 6× saline-sodium phosphate-EDTA [0.06 M phosphate buffer (pH 7.4), 0.9 M NaCl, and 3 mM EDTA], and 1–2 μg of labeled DNA target. Before hybridization, the hybridization mixture was denatured at 94°C, briefly cooled on ice, and then applied on the microchip covered with hybridization chamber (Probe-Clip Press-SeaInubation Chamber; Sigma-Aldrich, St. Louis, MO) and left

Table 1 Oligonucleotides used for detection of translocations

<table>
<thead>
<tr>
<th>Type of translocation</th>
<th>Name of gene</th>
<th>Splice variants</th>
<th>Name of oligonucleotidea</th>
<th>Sequence from 5′ to 3′</th>
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<tr>
<td>Normal (1)</td>
<td>ABL</td>
<td>ABL1 (−)</td>
<td>TGTAGTTGCTTGGGACCCAGCTTG</td>
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<tr>
<td>t(8;21)</td>
<td>ETO</td>
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<td>t(15;17)</td>
<td>PML-RARA</td>
<td>bcr 1</td>
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<tr>
<td>inv(16)</td>
<td>CBFB</td>
<td>CBFB(+)</td>
<td>CGGGAATTTTAGAAGACCAAGTC</td>
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</tr>
<tr>
<td>t(1;19)</td>
<td>E2A-PBX</td>
<td>PBX(+)+</td>
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<tr>
<td>(5)</td>
<td>MLL</td>
<td>MLL(+)</td>
<td>GAGGAAATGGAGaatgaagttgag</td>
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<td>t(4;11)</td>
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<td>AF4(−)</td>
<td>ctcctaagcagctctgccaac</td>
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</tr>
<tr>
<td>(6)</td>
<td>BCR</td>
<td>ALL (9;22)(+)</td>
<td>GGCCTCCGAAGACCCGGCACATC</td>
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<tr>
<td>(7)</td>
<td>BCR</td>
<td>CML(+)</td>
<td>AAGCAGAGTCCTAACACCAAGATC</td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>AML1</td>
<td>TEL(+)</td>
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<td></td>
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<tr>
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<td>TEL-AML1</td>
<td>TEL1(+)</td>
<td>CTCCTTGCTTCCCTGGCGG</td>
<td></td>
</tr>
</tbody>
</table>

a The chimeric oligonucleotides from the splice sites are marked by the letter j (junction), and the two spliced regions are indicated with uppercase and lowercase letters.

for 30 s, and 72°C for 1 min; the second round was followed by an extension at 72°C for 7 min (6).

Individual PCR reactions for translocations used in comparative analysis of clinical samples were performed with individual sets of primers under conditions described for multiplex PCR. Ten microliters of the PCR reaction mix were analyzed on a 1.5% ethidium bromide-containing agarose gel. To prepare DNA target for hybridization with microchips, the amplified double-stranded DNA fragment obtained in the second round of PCR was mixed with the 300-bp product of the ABL gene and fragmented to ~50–100 bp with 0.01 units/μl DNeasy (Life Technologies, Inc.) for 10 min at 37°C in 40 mM Tris, 50 mM KCl, and 2.5 mM MgCl2. The fragmented DNA was labeled at 3′ ends using tetramethylrhodamine-6-dUTP (NEN) and terminal deoxynucleotidyl transferase (Promega, Madison, WI). Unincorporated nucleotides were removed by using Qiagen Nucleotide Removal kit (Qiagen Inc., Valencia, CA).
overnight at room temperature. The chamber was disassembled, and the microchip was washed for 10 min with 40 ml of 1× saline-sodium phosphate-EDTA at 37°C. Hybridization signals were monitored either with a Research Grade microchip analyzer consisting of a wide-field, high-aperture, long-working-distance fluorescence microscope coupled with a cooled charge-coupled device camera as described (18, 19), or with a portable chip analyzer (20) equipped with charge-coupled device camera as well. Data processing and image analysis were performed using dedicated software, Imageware version 1.1, developed at Engelhardt Institute of Molecular Biology.

**On-Chip-PCR Amplification.** A microchip for PCR amplification contained oligonucleotide primers immobilized through their 5' amino groups inside porous gel pads prepared as described previously (21). The oligonucleotide probe sequences were: ABL con 467(+), 5'NH2-GCCAAGGCTGGTGCCAAAGCAGCATTCCGCTGACCATC-3’; BCR1 1721(+), 5' NH2-GCAGATCTGGCACCACGTGCGGAG-3’; BCR2 3158(+), 5'NH2-GACTGTTCCAGCATCCTGAGATCTGGCCCAAGCAAC-3’; BCR3 3224(+) , 5'NH2-TCTGAATGTCATCGTCCACTCAGCCACTGG-3’.

A microchip chamber for PCR amplification and hybridization was formed by placing a cover glass over the microchip glass slide. The glass and cover glass were separated by 18 × 18-mm plastic sticky spacer (Brinkmann Instruments, Westbury, NY). The reaction mixture (30 μl) contained 1X PCR buffer [11 mM Tris-HCl (pH 8.3), 55 mM KCl, and 1.5 mM MgCl2], 0.2 mM of each deoxynucleotide triphosphate, a mixture of primers (0.17 μM unlabeled primer and 0.85 μM labeled primer), and 1.5 units of AmpliTaq-Gold polymerase (PerkinElmer) or Taq-polymerase (Biomaster). The primers used in the multiplex on-chip-PCR reaction were: U-ABLCon(430), 5’NH2-CACAATGGGGAATGGTGTGAAGCCAAAC-3’; L-ABLCon(502), 5’-TexRed-NH2-GTACCAGGAGTGTTTCTCCGAGACTGTGTA-3’ for positive control gene ABL; and U-BCR1(+1697), 5’-NH2-CATCGTGGGGGTCCCGACG-3’; U-BCR2b(+3127), 5’-NH2-AGATGCTGACCACTCGTGTGAGAACTC-3’; L-ABL(232), 5’-TexRed-NH2-GTCAGATCTGCTGGCGGTAGA-3’ for t(9;22).

After preheating at 95°C for 120 s, 30–35 cycles of PCR amplification were performed at 94°C for 30 s, at annealing temperature 68–70°C for 30 s and at 72°C for 30 s. Thermo-cycling was performed using PCR-in situ block (Whatman Biometra GmbH, Gottingen, Germany). After amplification, the chamber was disassembled and the microchip was washed with 300 μl of water at 70°C for 10 min and air dried. The microchips were analyzed under a fluorescence microscope.

### RESULTS

**Microarray Design and Testing.** The diagnostic microarray system included one oligonucleotide probe for the c-ABL gene, which is expressed in normal cells and served as an internal positive control for the quality of DNA target prepared for hybridization, and twenty pairs of oligonucleotide probes 20–25 bp long for eight fusion genes: one probe in each pair from sense(+) strand and its counterpart from antisense strand (−) (Table 1). The ABL gene-specific probe was spotted horizontally in a lower row, whereas oligonucleotide probes specific for translocations were located in vertical rows as paired spots to control the reproducibility of hybridization and to enhance the reliability of results. The sense probe and its antisense counterpart were considered as one group of probes in automated image analysis. (Fig. 1A)

During the hybridization process, the amplified DNA fragments derived from c-ABL RNA or from chimeric sequences carrying the tetramethylrhodamine-6-dUTP label were binding with oligonucleotide probes on the array and, thus, the fluorescent label accumulated in the gel. The perfectly matched duplexes between DNA target and probe on the array were more stable than mismatched ones and gave the brightest fluorescence of the corresponding gel pads. In the absence of translocations, hybridization signals for the ABL gene only are seen (Fig. 1B).

Before using the microchips for clinical analysis, they were tested with samples of known genotypes to check the reliability of the results. Examples of such testing are shown in Fig. 2, A and B. Gene rearrangements were identified visually by finding gel pads with the brightest fluorescent signals, corresponding to perfect duplexes, and also using a special computer algorithm developed in our laboratory.

For most translocations, oligonucleotide probes corresponding to the sequence at the predominant fusion points were included. The t(4;11)MLL/AF4 can be represented by many different chimeric transcripts of similar prognostic value (22), and we used only one oligonucleotide probe from each gene partner, MLL and AF4, allowing the detection of the majority of possible transcripts derived from this rearrangement.

**Data Analysis.** The input data for the analysis were obtained from the measurements of fluorescence intensities $J_m = (C - B)/B$. Here, $C$ was the intensity per unit area in the internal region of a pad, $B$ was the counterpart background intensity, and $m$ numbered the pads on the chip (19).

In this algorithm, the signal intensities of the c-ABL gene probes served only to confirm the successful performance of the experiment and were excluded from the additional quantification.

The signal intensities of the translocation-specific probes that were spotted in duplicate were averaged. The higher value of these average signals selected from the sense (+) and anti-sense (−) probes was considered representative. The three most outlying signals were excluded, and the remaining values $J_e$ for oligonucleotide groups were defined as background signals. The mean $\langle J \rangle$ and SD $\sigma$ of the background signals were calculated, and THR = $\langle J \rangle + 3 \times \sigma$ was established as the cutoff level. We regarded any signal over the cutoff level to be a significant signal.

The identification of chimeric transcript was performed stepwise. At the first step, the type of translocation was determined comparing the signals $J_e$ from groups 8, 9, and 10 for AML translocations and from groups 18, 19, 20, 21, and 22 for ALL translocations with the THR value according to the rule $J_g > THR$. At the second step, a particular chimeric transcript was found by comparing the group signals in corresponding vertical rows.

As an example, we consider here an analysis of a clinical sample from an AML patient. The DNA target was prepared and hybridized with an AML fragment of the chip including probes for translocations t(8;21), t(15;17), and inv16 (Fig. 2A). After hybridization and fluorescence signal measurement, the outlying signal was detected in group 8, indicating the presence of
Next, the sequence at the fusion point was verified by detecting an outlying signal in group 5 (Fig. 3). Thus, the patient was defined as having t(8;21) AML1/ETO.

The algorithm was derived from the processing of >50 experiments and then applied to the analysis of other clinical samples.

The Sensitivity Studies. To estimate the sensitivity of our approach, a serial dilution was performed for each translocation using bone marrow samples from primary patients before treatment. Then multiplex PCR, followed by hybridization with microarray, was performed for each dilution step. The results were considered positive when the type of chimeric transcript was correctly identified by computer analysis of the hybridization pattern. For different translocations, the sensitivity assays detected one cell harboring the rearrangements in the following dilutions: BCR/ABL p190 and p210 $10^{-4}$; MLL/AF4 $10^{-3}$; TEL/AML1 $10^{-3}$; E2A/PBX1 $10^{-4}$; PML/RARA $10^{-4}$; AML1/ETO $10^{-3}$; CBFB/MYH11 $10^{-3}$. An example of detection of the sensitivity limit for CBFB/MYH11 transcript is presented in Fig. 4, A and B.
This sensitivity is sufficient to detect MRD. An example of MRD analysis for a patient carrying translocation t(9;22) BCR/ABL p190 during his induction therapy is shown in Fig. 5. The fluorescence signals from corresponding probes were 2- to 3-fold above the threshold of statistical significance, so the presence of chimeric transcript detected by microarray analysis looked unambiguous. When examined by semiquantitative RT-PCR, the same sample showed the presence of BCR/ABL fusion transcript at $10^{-4}$ dilution.

**Clinical Screening.** Our approach proved to be able to detect all eight rearrangements and their characteristic splicing variants or molecular breakpoints in different clinical samples. In total, samples from 247 patients (156 ALL, 15 CML, and 76 AML) were analyzed over a period of 2.5 years using a microarray-based approach and single PCR reactions in parallel. The data are summarized in Table 2.

**Translocations Detected in AML Patients.** All cases of gene rearrangements found in AML patients using microarrays were confirmed by individual RT-PCR reactions. The t(8;21) was found in 9%, t(15;17) in 13%, and inv(1) in 8% of cases. One patient with biphenotypic leukemia carried t(4;11). Taken together, these translocations represented 31% of our AML patients. Among 10 patients with PML/RARA, 7 had bcr1 type of fusion and 3 had bcr3 transcript. Most AML patients, except those with the M3 variant, received treatment according to the protocol AML-2000 (Moscow-Minsk) developed in the Research Institute of Pediatric Hematology (see below for details of therapeutic protocols). Some patients were treated according to the protocol AML-BFM-83/87 (Germany; Ref. 23). The AML patients with the M3 variant carrying t(15;17) PML/...
were treated with ATRA differentiation therapy according to the APL-98 protocol of the Research Institute of Pediatric Hematology.

**Translocations Detected in ALL/CML Patients.** The t(1;19)PBX/E2A was found in 1.6%, t(4;11)MLL/AF4 in 4%, t(9;22)BCR/ABL p190 in 2.4%, and t(12;21)TEL/AML1 in 17.5% of ALL cases. In total, these translocations represented 26% of all patients with ALL. The ALL patients were treated according to the protocols ALL-BFM-90 (Germany) or ALL-MB-91/02 (Moscow-Berlin) (24). A relatively large subset of patients (n/H11005 15) was children with CML at diagnosis. Some of them were in clinical remission but had low levels of BCR/ABL transcript p210; in total, BCR/ABL 210 was found in 12 of them (80% of all CML cases). After molecular diagnosis confirmed the presence of BCR/ABL rearrangement, targeted therapy of Imatinib (Gleevec, STI 571) was prescribed to four patients.

**Comparison of Multiplex-Microarray Analysis and Standard PCR Analysis.** Concordance between the two methods was found in all but two cases. One clinical case was not correctly identified by the standard PCR method: in a patient with t(1;19), the length of an amplified PCR fragment was too short and did not correspond to the fusion variant known for this translocation. These data were reproducible and suggested an unusual splice variant of the corresponding transcript. However, after microarray analysis, the point of fusion was found to be located as expected (25). After sequencing of the suspicious PCR fragment, the validity of microarray data were confirmed.

Another patient was found to be false positive for t(9;22)BCR/ABL when analyzed by both methods. This case had to be verified during a MRD study, and no translocation was found in the original sample of the patient. It is likely that the false identification of translocation occurred because of crossover contamination during the cDNA preparation step, because both
the microarray data and standard PCR analysis gave the same incorrect result.

**On-Chip-PCR Amplification.** The on-chip PCR approach was a modification of multiplex PCR, in which two PCR steps were performed inside the hybridization chamber. During the first cycles, the first step of multiplex PCR with one of the primers carrying fluorescent label occurred in the liquid phase over the chip. While accumulating in the reaction mixture, this first round product was beginning to anneal to the complementary primers immobilized inside the gel pads, and these primers were extended. Long perfect duplexes formed during this on-chip primer elongation produced gradually increasing fluorescent signal in corresponding gel pads. This approach was tested on the t(9;22) translocation, and a corresponding microarray was designed (Fig. 6A). In the absence of chimeric transcript BCR/ABL, the only positive signal was seen for the ABL control gene (Fig. 6B). When the chimeric transcript BCR/ABL was present in the clinical sample, the fluorescence signal appeared in gel pads in which corresponding oligonucleotide probes were immobilized (Fig. 6C).

**DISCUSSION**

Compared with cytogenetic diagnostics and fluorescence in situ hybridization, RT-PCR techniques for detection of gene rearrangements in leukemia are increasingly being used, because they are considered quicker and more sensitive. However, for screening purposes, a number of PCR reactions have to be performed to detect one of these aberrations in a single patient. This time-consuming and expensive work could be replaced by multiplex PCR, which allows the detection of all relevant rearrangements in one assay. Numerous multiplex PCR protocols have been reported for leukemias, which represent a combination of PCR and gel electrophoresis, dot-blot hybridization, or related techniques (6–9). Because of miniaturization of the experimental format and possibility to adapt the system for high-throughput automated clinical analysis, hybridization-based microarray assays have been suggested for use in screening of chromosome translocations (10, 26). The introduction of microarray technology to analyze the products of multiplex PCR increases the specificity and reliability of the analysis, confirming the molecular sequence of PCR product. This approach allowed us to avoid common errors in the detection of translocations associated with nonspecific annealing of primers, which can sometimes occur during PCR, as we have demonstrated for one clinical case in the course of screening. Individual RT-PCR reactions were performed for all cases in our screening study, and a high concordance between the two techniques was observed. Because of the high sensitivity of the microarray-based approach, the same precautions, as for other PCR techniques, must be made to avoid false positive results because of contamination (6). Contamination seems to be the main source of false positive results for both methods.

For most translocations, the positive signals on microarray were obviously stronger than negative signals, so that the identification of chimeric transcripts could be performed visually. However, in some cases, the background signals may interfere with true signals because of cross-hybridization. To overcome this problem and to increase the specificity of the analysis, we used a set of probes from different parts of chimeric genes for each translocation. In combination with a computerized algorithm, this allowed us to reach an unambiguous interpretation of the hybridization patterns even when background signals were distributed nonrandomly. The image analysis of the t(8;21) AML1/ETO was paradigmatic, whereas the stepwise algorithm allowed us to exclude the nonspecific cross-hybridization signals from group 6, particularly probes specific for PML/RARA (Figs. 2A and 3). The threshold values were counted based on the overall fluorescence of individual microarray, and this made the procedure more adaptable to individual samples and less dependent on experimental variations. The microarray approach was tested successfully with many clinical samples.

The role of molecular diagnosis is becoming increasingly significant in choosing the appropriate protocol for leukemia treatment. The AML-2000 (Moscow-Minsk) stratifies patients

<table>
<thead>
<tr>
<th>Translocation</th>
<th>AML patients</th>
<th>ALL patients</th>
<th>CML patients</th>
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<tr>
<td>(1) t(8;21) AML1/ETO</td>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>(2) t(15;17) PML/RARA</td>
<td>10</td>
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<tr>
<td>(3) inv(16) CBFB/MYH11</td>
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<tr>
<td>(4) t(1;19) E2A/PLX1</td>
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<tr>
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<td>6</td>
<td>3</td>
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<td>(6) t(9;22) BCR/ABL p190</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>(7) t(9;22) BCR/ABL p210</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>(8) t(12;21) TEL/AML1</td>
<td>52</td>
<td>125</td>
<td>20</td>
</tr>
<tr>
<td>No translocations</td>
<td>76</td>
<td>156</td>
<td>3</td>
</tr>
<tr>
<td>Total no. of patients</td>
<td>76</td>
<td>156</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 2** Summary of translocations detected by multiplex PCR-microarray method and confirmed by standard PCR analysis
into two treatment arms with differentiation therapy using high-dose cytarabine for patients carrying inv(16) and alternative therapy for others during the induction step (27). The APL-98 protocol prescribes ATRA in combination with other drugs for patients with t(15;17). The protocols for ALL treatment, ALL-BFM-90 and ALL-MB-91/02, commit the blocks of high-dose chemotherapy to patients with t(9;22) p190 and t(4;11) (24, 28). Thus, reliability and accuracy are crucial in identification of such molecular markers as translocations for risk stratification. In our study, we aimed to develop the optimum assay that would fulfill these requirements.

The characteristics of a multiplex PCR-microarray assay suggest that its application to routine clinical screening can significantly improve the ability of clinical laboratories to diagnose leukemia patients accurately and rapidly. This approach can be also extended to the detection of minimal residual disease, which will improve clinical follow-up of patients with translocations and other genetic alterations (29). The limits of detection, which can be achieved using this method (one leukemic blast per 10^3 to 10^6 normal cells), are considered to be critical in defining the effectiveness of therapy and risk of relapse (Fig. 4). Generally, this range of sensitivity is comparable with the sensitivity of the most advanced PCR-based methods of MRD detection (30). At present, we consider our method as a qualitative assay, but it has the potential for quantitative analysis analogous to semi-quantitative RT-PCR assay.

The real advantage of gel-based microarrays can be the possibility of performing multiplex PCR reactions directly on a chip. This could diminish a number of handling steps and thereby minimize the risk of contamination. In addition, the time of analysis is decreased from 14–16 h (the time necessary for hybridization) to 2–3 h (the time for on-chip-PCR amplification). We expect that additional development of on-chip-PCR directed to the extension of PCR multiplicity can additionally simplify clinical analysis.

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We dedicate this publication to the memory of our esteemed colleague Andrei Mirzabekov.

REFERENCES


Clinical Screening of Gene Rearrangements in Childhood Leukemia by Using a Multiplex Polymerase Chain Reaction-Microarray Approach
