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Rapid detection of clonality in patients with acute lymphoblastic leukemia

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Background and Objectives. Polymerase chain reaction (PCR) detection of clonal T-cell receptor (TCR) γ and δ gene rearrangements is widely used in clonality assessment of lymphoid leukemias and lymphomas and for detection of minimal residual disease of acute lymphoblastic leukemia (ALL). Standard analyses for clonality assessment include Southern blotting or PCR-based detection of clonal TCR gene rearrangements. The latter consist of heteroduplex PCR analysis by separation of PCR products on non-denaturing polyacrylamide gel (PAGE). We describe a rapid and sensitive method to identify specific clonal rearrangements in PCR fragments obtained by amplification of TCRγ and TCRδ genes.

Design and Methods. We applied a semi-automated electrophoretic technique (PhastSystem™, Amersham Pharmacia Biotech) and compared it with standard homo-heteroduplex analysis in 21 cases of childhood acute lymphoblastic leukemia (ALL).

Results. The results obtained for each sample analyzed by standard homo-heteroduplex detection were completely reproduced by the PhastSystem™ approach.

Interpretation and Conclusions. We conclude that heteroduplex analysis of TCR gene rearrangements using the semi-automated PhastSystem™ is a simple, rapid, cheap and highly reproducible method which can be used as an alternative to traditional analysis for detection of clonality.
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Key words: heteroduplex analysis, TCRγ and δ, polymerase chain reaction

Acute lymphoblastic leukemia (ALL) results from clonal expansion of a T- or B-cell at a different stage of differentation. The clonal cell population, carrying identical copies of rearranged T-cell receptor (TCR) and/or immunuglobulin (Ig) genes, can be identified in more than 90% of cases.[1] Therefore molecular analysis of TCR or Ig genes is used to prove or exclude clonality in human lymphoid tumors.[2] Many methods are able to identify clonal TCR or Ig rearrangements;[3] i.e. this can be achieved by Southern blot analysis[4] and proper use of probe/restriction enzyme combinations. However, this method requires a large amount of DNA, is time consuming and labor-intensive. More recently polymerase chain reaction (PCR) techniques have begun to be frequently used as alternatives. Although this technique is sensitive and fast, the risk of false-positive results due to amplification of similar rearrangements in polyclonal, or reactive lymphocytes, is one of the major drawbacks. To overcome this problem most laboratories apply homo-heteroduplex analysis.[5] In this method, PCR products are denatured at high temperature and subsequently renatured to induce homo- or heteroduplex formation. The presence of homoduplex and heteroduplex bands represents patterns of clonal T-cell population. Although this approach is simpler to apply in routine screening than Southern blotting, it still takes a long time for gel preparation and running (4 to 20 hours). We tested the applicability of PhastSystem™ technology (Pharmacia Biotech, Uppsala, Sweden) for detecting TCRγ and TCRδ gene rearrangements in a series of childhood ALL previously studied by conventional homo-heteroduplex analysis.

Design and Methods

Patients and cell samples

Twenty-one children with ALL (16 common ALL, 2 T-ALL, 3 pre-B ALL) were included in this study. All cases had been diagnosed according to standard morphologic and immunologic criteria.[6] Bone marrow mononuclear cells at diagnosis were isolated by Ficoll-Hypaque

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(Pharmacia, Uppsala, Sweden) gradient centrifugation. The percentage of BM blast cells was more than 80% in all the cases studied. High molecular weight DNA was extracted from bone marrow mononuclear cells using an automated DNA extractor [Applied Biosystems (ABI), Foster City, CT, USA].

PCR amplification analysis

Detection of the TCRγ VJ region and TCRγ V(D)J region was performed with the primers described elsewhere.[7] The reaction mixture for PCR in 50 μL contained 100 ng template DNA, 12.5 pmoles of 5′ and 3′ oligonucleotide primers, 200 nM dNTP, 20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 1 unit *Taq* polymerase. PCR conditions were 1.30 min at 94°C, 1 min at 60°C, and 1.30 min at 72°C, followed by 35 cycles of 1.30 min at 94°C, 30 s at 60°C, 1.30 min at 72°C, and a final extension phase of 7 min at 72°C. A 5 μL aliquot of each reaction was analyzed on 1.5% agarose gel after ethidium bromide staining. A negative (no DNA) control, DNA from peripheral blood of healthy donors and the appropriate positive control were included for all PCR reactions.

V(D)J heteroduplex analysis

The TCR γ/δ PCR products were analyzed by standard homo-heteroduplex analysis as previously described.[5] Briefly: to favor homo-heteroduplex formation, 5 to 20 μL of each amplified PCR product were heated at 94°C for 5 min, rapidly cooled to 50°C and left at this temperature for 1 hour, then kept on ice until loading. Samples were run overnight at 10 mA on a 12% non-denaturing polyacrylamide gel (19:1, acrylamide:bis-acrylamide) in tris-borate EDTA (TBE) 1x buffer, at 4°C (Protean II Xi Cell apparatus; Bio-Rad, Hercules, CA, USA). Homo-heteroduplex patterns were shown by ethidium bromide staining. The homo-heteroduplex analysis using the PhastSystem™ apparatus was performed as follows: 0.5 μL of each amplified PCR product were heated at 94°C for 5 min, rapidly cooled to 50°C and left at this temperature for 1 hour, then kept on ice until loading as for the standard method. The automatic run consisted of a pre-run at 10 mA for 20 min (150 Vh) at 15°C and then 15 min at 10 mA (250 Vh) at 15°C in Native Strip buffer for migration of PCR product. Only 0.3 μL of PCR product were loaded on 12.5% non-denaturing polyacrylamide gel (PhastGel™ Pharmacia Biotech, Uppsala, Sweden). Silver staining was performed in the automatic development chamber by a modified protocol: 20% trichloroacetic acid for 7 min at 50°C, 50% ethanol, 5% glacial acetic acid for 4 min at 50°C, wash in water for 2 min at 50°C, 0.4% silver nitrate (Pharmacia Biotech, Uppsala, Sweden) for 10 min at 50°C, wash in water for 1 min, 2.5% sodium carbonate solution (Pharmacia Biotech, Uppsala, Sweden) for 5 min at 30°C, tris(hydroxymethyl)-aminomethane 0.3 M for 3.5 min at 30°C and 5% glycerol for 5 min at 30°C.

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Table 1. Heteroduplex analysis: PAGE and PhastSystem™.

Pt.	*Phenotype*	*Gene rearrangement*								
		TCRγ						*TCRδ*		
		VI-J1.3/2.3	*VII-J1.3/2.3*	*VIV-J1.3/2.3*	*VI-J1.1/2.1*	*VII-J1.1/2.1*	*VIV-J1.1/2.1*	*V1/J1*	*V2/D2*	*D2/D3*
1	Common	-	-	-	-	-	-	-	-	-
2	Common	Ho	-	-	Ho	-	-	-	He	-
3	Pre-B	-	He	-	-	-	-	-	Ho	Ho
4	Common	-	-	-	-	-	-	-	Ho	Ho
5	T	-	Ho	Ho	-	-	-	He	-	-
6	T	-	-	-	Ho	-	-	-	-	-
7	Common	-	-	-	-	-	-	-	-	-
8	Common	-	-	-	-	-	-	-	He	-
9	Common	Ho	-	-	-	Ho	-	-	-	-
10	Common	-	-	-	-	-	-	-	-	Ho
11	Common	He	-	-	-	-	-	-	He	-
12	Common	-	-	Ho	-	Ho	-	-	Ho	-
13	Common	-	-	-	-	-	-	-	He	-
14	Common	-	-	-	-	-	-	-	-	-
15	Common	-	-	-	-	-	-	-	-	-
16	Common	-	-	-	-	-	-	-	-	-
17	Common	-	-	-	-	-	-	-	-	-
18	Common	-	-	-	-	-	-	-	Ho	-
19	Pre-B	-	-	-	-	-	-	-	-	-
20	Pre-B	-	-	-	-	-	-	-	-	-
21	Common	He	-	-	He	-	-	-	-	-

Molecular analysis: Ho = homoduplex, He = heteroduplex, (-) = polyclonal PCR product. The types of rearrangement are listed in the box at the top of each column. All the results were obtained on BM-MNC at diagnosis analyzing every sample with both techniques: PAGE and PhastSystem™.

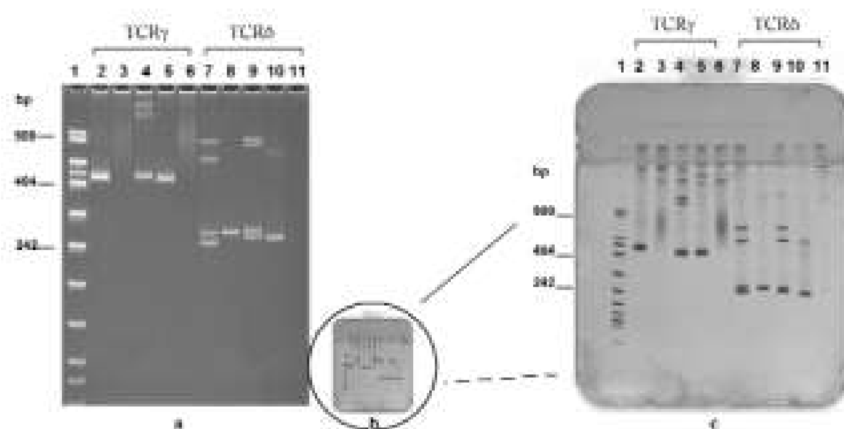

Figure 1. Homo-heteroduplex analysis of TCR rearrangements. (a) PAGE analysis and ethidium bromide staining; (b) PhastSystem™ analysis and silver staining: the presented sizes of the gels are proportional; (c) detail of the Phastgel™. The same PCR amplified products, after denaturation and renaturation were run in both systems. In each panel, lane 1: molecular weight marker. Panel (a) and (c) lanes 2, 3, 4, 7, 8 and 9: patients #2, #6, #21, #8, #12 and #13, respectively. Lane 5: positive control for Vγ I-Jγ 1.3/2.3, lane 6: polyclonal MNC DNA. Lane 10: positive control Vδ2-Dδ3, lane 11: polyclonal MNC DNA.

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Results

The aim of this study was to compare the reliability of the rapid heteroduplex analysis method with the standard method in this population of patients with ALL. The results of TCR γ and δ gene rearrangement analyses by both conventional homo-heteroduplex analysis and rapid PhastSystem™ methods were completely concordant and are given in Table 1. Clonality of TCRγ gene rearrangement was seen in 8 out of 21 cases. TCRδ was found to be clonal in 10 out of 21 patients. Figure 1 shows the results of TCR clonality analysis obtained for the same cases with the two different techniques. Panel a) shows standard homo-heteroduplex analysis and panel b) shows PhastSystem™ analysis of TCR rearrangements. Patient #2 (lane 2) exhibited Vγl–Jγ1.3/2.3 monoallelic rearrangement; patient #6 (lane 3) was found to be polyclonal for Vγl-Jγ1.3/2.3 while patient #21 (lane 4) showed biallelic Vγl-Jγ1.3/2.3 rearrangement. Patient #8 (lane 7) and patient #13 (lane 9) were found to have biclonal Vδ2-Dδ3 rearrangement. Patient #12 (lane 8) exhibited monoallelic rearrangement for Vδ2-Dδ23. As shown in Table 1, the parallel (PAGE and PhastSystem™) homo-heteroduplex analysis of our patients series showed identical results. Figure 1 shows that the two methods are also comparable in terms of band resolution (see heteroduplex cases, lanes #4, 7, and 9).

Discussion

PhastSystem™ technology was successfully used for clonality assessment of ALL by homo-heteroduplex analysis. As compared to standard non-denaturing gel electrophoresis (PAGE), PhastSystem™ was faster, producing results in a few minutes as compared to the 4-24 hours required by PAGE. Furthermore the use of ready-made gels made it possible to obtain reproducible results of good quality, independently from the experience of the investigators and their technical laboratory skills. Interestingly PhastSystem™ gels can be stained even for DNA, with the same silver staining commonly used for protein staining.

The importance of minimal residual disease (MRD) detection for diagnosis and monitoring of large series of ALL patients enrolled in clinical studies prompted several groups, including ours, to devote some effort to achieving standardized and reproducible methods for Ig and TCR gene recombination analysis. In this context the proposed technique, albeit not sensitive enough for MRD analysis, could represent an important step forward in the directions of saving time, reducing cost and increasing reproducibility in the assessment of clonality by homo-heteroduplex analyses in acute and cronic lymphoproliferative diseases.[8,9] Comparing the two techniques we observed a 50 % saving in reagents and a 70 % saving in time/researcher. The automated coloration system avoids the use of ethidium bromide and reduces biological hazards for the operator. Another great advantage in using minigels is the lesser quantity of chemicals used, decreasing the amount of waste materials.

The technique reached a sensitivity of $10^{-1}/10^{-2}$ in serial dilution experiments of patient's leukemic DNA in polyclonal mononuclear cells.[10] The levels of sensitivity are adequate for the screening of initial diagnostic samples, but not for MRD detection,[11-13] a higher sensitivity ($10^{-4}/10^{-6}$) being required for this latter. When patient-specific oligonucleotide complementary to the junctional region of TCRδ chain gene was used, a sensitivity of $10^{-3}/10^{-4}$ in serial dilution experiments was

achieved (data not shown).

In conclusion our findings indicate that the PhastSystem™, reliable, reproducible and time-saving, can substitute the PAGE technique in the screening of clonality of Ig and TCR recombinations.

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GG and SS were responsible for designing the study and writing the paper. GB and AB critically revised the manuscript and gave the final approval for its submission.

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Disclosures

Conflict of interest: none.

Redundant publications: no overlapping with previous publications.

Manuscript processing

This paper was peer-reviewed by two external referees and by Prof. Francesco Lo Coco, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Prof. Lo Coco and the Editors. Manuscript received January 15, 2001; accepted February 28, 2001.

Potential implications for clinical practice

Rapid screening of clonality of all lymphoproliferative diseases both acute and chronic. Results are available in a short time, thus accelerating further analyses such as minimal residual disease evaluation.

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