DETECTION OF CLONALITY BY HETERODUPLEX ANALYSIS OF AMPLIFIED JUNCTIONAL REGION OF T-CELL RECEPTOR γ IN PATIENTS WITH T-CELL ACUTE LYMPHOBLASTIC LEUKEMIAS

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ABSTRACT

Background and Objective. Traditional gel electrophoresis of PCR amplification products of regions from T-cell receptor genes often does not differentiate monoclonal from polyclonal rearrangements. We used polymerase chain reaction (PCR) and heteroduplex analysis on polyacrylamide gels to improve the detection of monoclonal rearrangements.

Methods. We investigated heteroduplex analysis of the amplified Vγ-Jγ junctions of the rearranged T-cell receptor γ (TcR-γ) gene by electrophoretic separation on non-denaturing polyacrylamide gel (PAGE) in 8 T-cell acute lymphoblastic leukemia (T-ALL) patients analyzed at diagnosis.

Results. Clonal homoduplex and heteroduplex bands were present only in the T-ALL samples and not in controls. We confirmed clonality by direct sequencing of the Vγ-Jγ junction. In 2 instances the analysis was performed on samples obtained from the same patient at diagnosis and at relapse, respectively; the presence of the same clonal TcR-γ rearranged cell remained detectable during clinical progression of the disease.

Interpretation and Conclusions. Our heteroduplex analysis showed that separation of the PCR product by electrophoresis on non-denaturing PAGE is a rapid and convenient method for the detection of clonal TcR-γ rearrangements in T-ALL.

Key words: acute lymphoblastic leukemia, T-cell receptor γ gene, polymerase chain reaction

There are two types of antigen-specific T-cell receptors (TcR): the classical TcR-αβ, which is expressed in the majority (≥ 85%) of mature peripheral blood (PB) lymphocytes, and the alternative TcR-γ, which is expressed on a small fraction (≤ 15%) of PB lymphocytes.1-4 The TcR molecule consists of two different glycoproteins (TcR-α and TcR-β, or TcR-γ and TcR-δ).1-4 Each chain of the TcR heterodimer contains a variable antigen-recognition region and constant (C) region. The variable region is encoded by a variable (V) gene segment, a joining (J) gene segment and a junctional region linking the V and J gene segments together.1,3,4 This junctional region includes diversity (D) gene segments in the case of the TcR-β and TcR-δ genes.1,3,6

The TcR molecules from various T-lymphocytes differ from one another as a result of different combinations of V, D and J gene segments and different junctional regions that occur via gene rearrangement processes during early T-cell differentiation.1,3,5 TcR-γ contains only 6 V and 5 J functional gene segments.6,7 The available V and J gene segments determine the potentially different combinations. The junctional region determines the junctional diversity. It is formed by the presence of randomly inserted, so-called N-region nucleotides,8,11 and the deletion of nucleotides through trimming of the ends of the involved gene segments (P-region nucleotides).12 N-regions consist of nucleotides that are randomly inserted by enzyme terminal deoxynucleotidyl transferase (TdT) at the junctions of gene segments during the rearrangement process.

T-cell acute lymphoblastic leukemia (T-ALL) results from a clonal expansion of T-cells carrying identical copies of rearranged T-cell receptor genes. Therefore rearrangements of TcR genes are used as markers for clonality in human lymphoid tumors.12 Since the junctional regions of rearranged TcR gene segments are unique for each T-cell clone, DNA sequences at the junction of V and J segments can be used as clone-specific markers in individual patients.5,12-15

One method, which has been commonly used for assessing the clonality of lymphocytes, involves the...
detection of clonal antigen receptor gene rearrangements by Southern blot hybridization, and TcR-γ rearrangements have in fact been reported in all cases of T-ALL. Furthermore, polymerase chain reaction (PCR)-mediated amplification and subsequent direct sequencing or temperature-gradient gel electrophoresis (TGGE) have been used effectively to detect the clonal origin of junctional region sequences of TcR-γ in T-ALL. Nevertheless, the Southern blot method and the hybridization techniques all suffer from several technical disadvantages, including the long time needed to obtain results, the use of radiolabeled probes, and the need for a large amount of DNA. Moreover, both TGGE and the method employing PCR plus sequencing are susceptible to various artefacts, such as the use of long and CG-rich primers needed for cloning procedures. An alternative approach using heteroduplex analysis of amplified TcR-γ gene rearrangements has been developed and applied to the assessment of the clonality of cutaneous T-cell lymphomas. Furthermore, we tried to determine whether this approach could also offer significant advantages for diagnosis or monitoring of T-ALLs.

Materials and Methods

Patient and cell samples

Eight adult patients with a diagnosis of T-ALL were included in this study after giving informed consent. Cells were obtained from bone marrow samples from all patients at initial diagnosis after giving informed consent. Cells were obtained from bone marrow by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Immunophenotypic analysis was performed as previously described with a few modifications. The oligonucleotides were synthesized on a 391 DNA Synthesizer (Applied Biosystems, Foster City, CA, USA) using the solid-phase phosphotriester method in our laboratory. The oligonucleotide primers used are listed in Table 2. The primer pairs used in our amplification strategy were: P126 and P129, P126 and P130, P127 and P129, respectively; their positions are illustrated in Figure 1. We used the same primers as in ref. #16.

The PCR mixture was subjected to 35 serial cycles of denaturation (96°C for 1 min), annealing (60°C for 1 min) and extension to control the reactions (72°C for 1 min). A 15 µl aliquot of each reaction was analyzed on 2% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) and/or on 12% polyacrylamide gel visualized by ethidium bromide staining and further stained with silver.

V-J-Jy heteroduplex analysis

We analyzed the TcR-γ PCR products by heteroduplex analysis performed as previously described with a few modifications; 20 µl of each amplified product was heated at 96°C for 10 min, left to cool at room temperature for 15 min and then loaded. We called this procedure heteroduplex analysis. Samples were run for 4 hours on 12% non denaturing polyacrylamide gel electrophoresis. The type of rearrangement and the primers used for each PCR are given in the boxes at the top of each column.

Table 1. Results of immunophenotypic and molecular analysis by PCR of the T-ALL patients studied. Phase of disease, immunological phenotype assessment and PCR results of eight T-ALL patients at time of diagnosis (#1 to 8) and at relapse time (#9 and 10) are given. All the results are from bone marrow samples (BM); N.D. = not done. Immunological marker analysis: (+) means that 75% of all the cells are positive; (-) means that ≤25% of the cells are positive. Molecular analysis: (+) = clonal PCR product; (-) = polyclonal PCR product. The type of rearrangement and the primers used for each PCR are given in the boxes at the top of each column.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Immunophenotype</th>
<th>Heteroduplex analysis</th>
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<tbody>
<tr>
<td>No</td>
<td>Patient</td>
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<tr>
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<tr>
<td>1</td>
<td>C.D.</td>
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<td>2</td>
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<td>3</td>
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<td>4</td>
<td>B.R.</td>
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</tr>
<tr>
<td>5</td>
<td>B.E.</td>
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<tr>
<td>6</td>
<td>BE.FE.</td>
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<td>7</td>
<td>A.V.</td>
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<td>8</td>
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<tr>
<td>9</td>
<td>C.G.</td>
<td>relapse</td>
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<td>10</td>
<td>B.R.</td>
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(37.5:1 acrylamide: bisacrylamide) in Tris-Borate EDTA (TBE) 1 x buffer at 250 volts and 4°C (Protein II Cell apparatus, Bio-Rad Laboratories, Hercules, CA, USA), visualized by ethidium bromide staining and further stained with silver. Electrophoretic analysis on agarose gel gave rise to sharp bands in both leukemic and normal samples; by contrast, on polyclonal gel, when no clonal rearrangement was present in the sample subjected to heteroduplex analysis, the migration pattern appeared as a homogeneous smear. The absence of homoduplex and heteroduplex bands represents the pattern of a polyclonal T-cell population. In the presence of a major clone, when both TCR-γ alleles are rearranged in a given T-cell clone (biallelic) and often amplifiable in a single reaction (e.g. by VJ J1/2 primers), four double-strand chains predominate at the end of the PCR: of these, two are the original rearranged chains (homoduplexes; no mispairing at the VJ junction), and two originate from the exchange of the respective positive and negative strands, which result in mismatched V-J regions (heteroduplex). When only one clonal rearrangement is amplified by a given primer pair (monoallelic), only a single homoduplex fragment appears, without any heteroduplex.

**DNA sequencing analysis**

The homoduplex bands were cut out, minced, left overnight at 37°C in 400 mL of elution buffer [500 mM (NH₄)CH₃COO, 10 mM Mg(CH₃COO)₂, 1 mM Na₂EDTA, 0.1% SDS], and centrifuged for 5 min at 12,000 rpm in a microfuge. The DNA was then precipitated in ethanol and resuspended in Tris-EDTA (TE), buffer pH 7.6. Twenty ng of DNA were employed for the sequencing reaction with an AmpliCycle Sequencing Kit (Perkin Elmer, Cetus, Norwalk, CT, USA). The sequencing primers used were Jy1/2 for the positive strand and Vy1 and Vy11 for the negative strand.

**Results**

**T-ALL phenotypes**

All cases were regarded as unequivocally malignant by morphologic standards and immunophenotypic studies (Table 1). Seven samples (#1-3, 5-8) were TdT+ and four (#1, 3, 5, 7) CD1+. The patients differed in their CD4/CD8 phenotype. Four T-ALL demonstrated a CD4+/CD8- phenotype (#2, 3, 6, 7), 3 a CD4+/CD8- phenotype (#4, 5, 8), and one was CD4+/CD8+ (#1). Interestingly, the phenotypes of patients #3 and 4 were the same at relapse as at diagnosis.

**Electrophoretic analysis of the Vγ-Jγ PCR product**

The PCR products were approximately 300 bp long, depending on the type of primer pair used. After electrophoresis on 2% ethidium bromide-stained agarose gel, the PCR products obtained with different primer combinations appeared as intense sharp bands not only with the products derived from T-ALL DNA samples, but also with those from healthy DNA controls (Figure 2). Moreover, the samples were positive with all the primer pairs used.

When we analyzed the same TcR-γ PCR products on non-denaturing polyacrylamide gel electrophoresis (without previous denaturation = heteroduplex analysis) following silver staining, the polyclonal Vy-Jy junctions of non-neoplastic cells were again indistinguishable from the monoclonal ones, with amplification producing sharp bands for both (data not shown), even though the polyclonal PCR products in the polyacrylamide gels were generally broader and less sharp than the monoclonal counterparts.

When we followed the PCR-based approach, which takes advantages of the heterogeneity of the Vy-Jy junctional sequences and of the different migration properties on polyacrylamide gel of the Vy-Jy segments containing a few mismatches (heteroduplex) as compared to fully matched Vy-Jy junctions (homoduplex), polyacrylamide gel analysis of PCR products from specimens lacking clonal TcR-γ gene rearrangements persistently revealed a homogeneous smear without the presence of any band. By contrast, each PCR sample containing a clonal rearrangement showed one (monoallelic Vy-Jy rearrangement) or 2 (biallelic Vy-Jy rearrangements) prominent bands in either ethidium bromide or silver staining (homoduplex pattern) (Figure 3). When a bi-allelic rearrangement was present and amplified with the same primer pairs, two additional bands became visible in the upper part of the gel (heteroduplex bands).

Using this method and these combinations of primers, we were able to identify TcR-γ rearrangements in all samples; the TcR-γ gene rearrangements occurred in 16 out of 20 alleles. All 16 rearranged alleles contained at least one rearranged gene of the VJ family; twelve had the Jy1/2 gene and 4 the JyP1/P2 gene. Two patients (#3 and 8), who had both alleles rearranged to the Jy1/2 gene, showed two fast migrating bands (homoduplex) accompanied by two fainter, slower migrating fragments (heteroduplex).

In order to confirm the finding that it is possible
to distinguish accurately between monoclonal and polyclonal TcR rearrangements with non denaturing PAGE, we sequenced some samples showing sharp bands. From two samples (#4 and 5) we directly sequenced the homoduplex fragments once they were dissolved in the polyacrylamide gel. This sequencing approach confirmed the specificity of PCR, showing V/I2/H9253/8-V/I2/H9253/12 (case #2) and V/I2/H9253/2-J/I2/H9253/12 (case #5) junction sequences in the samples analyzed.

**Discussion**

Amplification by PCR of the rearranged TcR-γ gene has been described for the detection of clonal T-cell populations. We and other investigators have selected universal consensus Vγ primers corresponding to homologous regions in the Vγ1-8 genes, in the Vγ9 and in the Vγ10 genes. While our sequencing results clearly showed that these consensus primers successfully amplify the rearranged genes, we also found that the presence of distinct bands after electrophoresis of TcR-γ PCR products on agarose gel alone cannot be interpreted as a reliable marker of clonality.

In our experience, heteroduplex analysis on PAGE was capable of separating identical (i.e. clonal) PCR fragments in a mixture of partially homologous (i.e. polyclonal) PCR products. It therefore appears to be an ideal method for identifying clonal PCR products. Because of the diversity of TcR-γ junctions, with heteroduplex analysis on PAGE a mixture of multiple heteroduplexes bearing extensive mismatch regions should be formed by denaturation (96°C) and reannealing of the TcR-γ PCR product from a polyclonal sample. This mixture of multiple heteroduplexes will migrate and appear as a diffuse smear. On the other hand, TcR-γ PCR...
products containing a significant percentage of monoclonal (i.e. identical) sequences after denaturation and reannealing should result in a high proportion of matched homoduplexes running as sharp single or double bands, with monoallelic and biallelic rearrangements, respectively.

Our strategy for detecting monoclonal TcR-\(\gamma\) junctions presents several practical advantages. It requires less work than other approaches (Southern blot, subcloning of PCR products); it is also less time-consuming than Southern blot analysis, providing results in just a few days. PCR products can be obtained even from partially degraded DNA, whereas degradation can be a major problem in Southern blot hybridization. Moreover, heteroduplex analysis does not require radioactivity, thus reducing costs and eliminating the need for special disposal of radioactive and contaminated materials. Finally, the heteroduplex system appears to be less susceptible to the artefacts often associated with Southern blot hybridization.

With regards to sensitivity, PCR may again have advantages over Southern blot analysis of antigen receptor gene rearrangements. In particular, Southern blot seems to have an absolute threshold of detection of 1-5% neoplastic cells per sample, while PCR followed by heteroduplex analysis and silver stain achieved a sensitivity of 0.1-1%. 23-27 In addition, our procedures could be further shortened and simplified by a more rational use of primers. In fact, most one-rearranged-allele samples can be amplified using a single pair of primers (i.e. V\(\gamma\)1 and J\(\gamma\)1/2) because of non-random V-J gene usage.

Heteroduplex analysis of the TcR-\(\gamma\) gene also has several advantages over other methods employing PCR, such as agarose gel electrophoresis analysis 28 or TGGE.29 In the former, a nested PCR with internal sequence specific primers is usually necessary, while in the latter a long stretch of GC-rich base tail is needed inside the primer for better resolution of the amplified primer. By contrast, heteroduplex analysis is relatively simple and rapid.

In conclusion, we think that the heteroduplex analysis approach described herein could represent a step forward in the evaluation of T-cell clonality in T-ALL.

References