Technical Advance

Detection of Clonal T-Cell Receptor γ Gene Rearrangements in Paraffin-Embedded Tissue by Polymerase Chain Reaction and Nonradioactive Single-Strand Conformational Polymorphism Analysis

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The diagnosis of T-cell lymphoproliferative disorders, which frequently involve the skin and other extranodal sites, is often problematic because of the difficulty in establishing clonality in paraffin-embedded tissue. To this end, we developed a simple, non-radioactive method to detect T-cell receptor γ (TCR- γ) gene rearrangements by polymerase chain reaction single-strand conformational polymorphism (PCR-SSCP) in paraffin-embedded tissue. Jurkat and HSB-2 cell lines and peripheral blood samples from normal individuals were used as monoclonal and polyclonal controls, respectively. DNA was extracted from 24 biopsies of T-cell lymphomas, 12 biopsies of reactive lymphoid infiltrates, and 2 biopsies of primary cutaneous large B-cell lymphomas. V γ 1–8, V γ 9, V γ 10, V γ 11, and J γ 1/J γ 2 consensus primers were used for TCR- γ gene rearrangement amplification and PCR products were analyzed by nonradioactive SSCP. Monoclonal controls yielded a well-defined banded pattern, whereas all polyclonal T-cell controls showed a reproducible pattern of smears. We detected monoclonality in 20/21 (95%) T-cell lymphoma cases, whereas no dominant T-cell clones were found in any of the reactive lymphoid infiltrates or B-cell lymphomas. Sensitivity of 1–5% was demonstrated by serially diluting Jurkat cells in mononuclear blood cells from normal individuals. We conclude that nonradioactive PCR-SSCP for TCR- γ gene rearrangement analysis is a useful adjunct to routine histological and immunophenotypic methods in the diagnosis of T-cell lymphoproliferative disorders in paraffin-embedded tissue. (*Am J Pathol* 1999, 154:67–75)

T-cell lymphoproliferative disorders frequently involve extranodal sites, including the skin.^{1,2} The diagnosis of these disorders is often difficult on the basis of clinical, histological, and immunohistochemical criteria and because tissue available for molecular assessment of clonality is often limited to archival paraffin blocks.

T-cell receptor (TCR) gene rearrangement analysis is a methodology used to detect clonality in a T-cell population.^{3,4} Clonality is not synonymous with malignancy because it can be detected in nonneoplastic lymphocytic infiltrates.⁵ Nevertheless, it is generally accepted that most neoplasms are clonal in origin. Therefore, the detection of a clonal population in an equivocal lymphoproliferative lesion can be a very useful diagnostic tool.

TCR gene rearrangements have been used in the detection of clonality by Southern hybridization.^{3,4} This technique requires a large amount of DNA that must be extracted from fresh or frozen tissue. More recently, polymerase chain reaction (PCR)-based methods have allowed the detection of T-cell clonality in paraffin-embedded tissue without the use of radioisotopes.^{6–9} Of the four TCR genes, α , β , γ , and δ , the TCR- γ gene consists of a relatively small set of variable (V) and joining (J) regions and is therefore particularly suitable for PCR amplification of DNA extracted from paraffin-embedded tissue using consensus primers.^{10–14} In addition, the TCR- γ locus is rearranged in the majority of normal and neoplastic T-lymphocytes. Unfortunately, the simple genomic organization of the TCR- γ locus and the presence of only one hypervariable N region in each joining segment are responsible for the small range of variability in length of the different rearrangements.¹⁵ As a consequence, the PCR

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Table 1. Summary of Cases

Diagnosis	No. of cases	Site	Clonal V γ 1-8	Clonal V γ 9	Clonal V γ 10	Clonal V γ 11
Large cell CTCL CD30+	6	skin	6/6	—	—	—
Mycosis fungoides (plaque/tumor stage)	9 samples/8 cases	skin	7/9	0/2	1/2	0/1
Large cell CTCL CD30—	1	skin	0/1	0/1	1/1	—
Precursor T-cell lymphoblastic lymphoma	2 samples/1 case	lymph node/effusion	2/2	—	—	—
$\gamma\delta$ T-cell lymphoma	1	subcutis	1/1	—	—	—
Peripheral T-cell lymphoma	1	lung	1/1	—	—	—
Peripheral T-cell lymphoma	2	lymph node	1/2	1/1	—	—
Enteropathy-associated T-cell lymphoma	2 samples/1 case	small bowel	2/2	—	—	—
Reactive lymphoid hyperplasia	6	lymph node	0/6	0/6	0/6	0/6
Lichen planus	2	skin	0/2	0/2	0/2	0/2
Chronic dermatitis	1	skin	0/1	0/1	0/1	0/1
Large B-cell lymphoma	2	skin	0/2	0/2	0/2	0/2
Lymphoid hyperplasia	3	skin	0/3	0/3	0/3	0/3

CTCL, cutaneous T-cell lymphoma

products cannot be optimally analyzed by polyacrylamide gel electrophoresis (PAGE), which separates DNA fragments on the basis of size. To overcome this problem, PCR products have recently been analyzed using procedures frequently used for point mutation detection, such as denaturing gradient gel electrophoresis (DGGE)^{5,16-18} and single-strand conformational polymorphism analysis (SSCP),¹⁹⁻²⁵ which separate DNA fragments according to nucleotide sequence in addition to size. The classic SSCP protocol²⁶ uses radioactive PCR products and large-formatted nondenaturing gels, although several nonradioactive SSCP protocols have now been developed.^{27,28} Nonradioactive PCR-SSCP of TCR- γ has been previously compared to TCR- β analysis by both the traditional Southern blot approach²³ and the reverse transcriptase PCR method²⁵ using available abundant frozen tissues, confirming the validity of this PCR technique in detecting clonality. Here, we describe a simplified, sensitive, highly reproducible, nonradioactive PCR-SSCP method for TCR- γ gene rearrangement analysis using precast minigels in a thermally controlled recirculation apparatus. This technique allows discrimination between clonal and polyclonal TCR- γ gene rearrangements in paraffin-embedded tissue and is suitable for routine use in diagnostic surgical pathology laboratories.

Materials and Methods

Controls

Jurkat and HSB-2 cell lines with known TCR- γ gene rearrangements were used as a source of monoclonal T cells for control experiments.¹⁷ The Jurkat cell line was used as monoclonal control for assays performed with V γ 1-8 and V γ 11 primers and the HSB-2 cell line was used as monoclonal control for V γ 9 and V γ 10 rearrangements. Peripheral blood samples from three normal individuals were used as polyclonal controls.

Patient Cases

A series of 24 biopsies from 21 patients was selected from the files of the Departments of Pathology of Beth

Israel Deaconess Medical Center and Istituto Dermatologico dell'Immacolata from 1994 to 1997 to include as many T-cell lymphoma subtypes as possible. The series included large cell cutaneous T-cell lymphoma (CTCL) CD30-positive (6 cases), mycosis fungoides (9 biopsies from 8 cases), large cell CTCL CD30-negative (1 case), subcutaneous $\gamma\delta$ T-cell lymphoma (1 case), peripheral T-cell lymphoma of the lung (1 case), nodal precursor T-cell lymphoblastic lymphoma and metachronous neoplastic pleural effusion (2 biopsies from 1 case), nodal peripheral T-cell lymphoma (2 cases), enteropathy-associated T-cell lymphoma of small bowel (2 biopsies from 1 case). CTCL represent the majority of the selected cases for two reasons. First, skin is the most frequent site for T-cell lymphoma in Western countries. Second, for most CTCL molecular assessment of clonality must be performed on paraffin-embedded tissue because frozen tissue is usually not available.

Fourteen cases of reactive nodal and cutaneous T-cell infiltrates were arbitrarily selected from the same files as controls. They included hyperplastic lymph nodes (6 cases), cutaneous lymphoid hyperplasia (3 cases), primary cutaneous large B-cell lymphoma (2 cases), lichen planus (2 cases), and chronic nonspecific dermatitis (1 case).

Thirty-one tissue samples and 1 pleural fluid were formalin-fixed and paraffin-embedded; the remaining six biopsies were frozen (Table 1). In all cases, the diagnosis was based on clinical, histological, and immunohistochemical criteria. T-cell immunophenotype was determined by immunohistochemical expression of one or more T-cell antigens (CD3, CD5, CD45RO) in neoplastic cells.

DNA Extraction

Cell lines were grown in RPMI medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (Gibco BRL) and 1% antibiotic-antimycotic (Gibco BRL). DNA was extracted from cell lines and peripheral blood cells using QIAamp Tissue Kit (QIAGEN, Chatsworth, CA). For paraffin-embedded tissues, 5- μ m sections were cut with disposable blades, collected on glass slides, deparaf-

Figure 1. Germline TCR- γ gene (top). Sets of primers used for PCR amplification of the rearranged TCR- γ gene (left). PCR/SSCP analysis of monoclonal and polyclonal controls (right).

finized with xylene, washed with ethanol, and rehydrated in deionized water. For frozen tissue, 5- to 10- μ m sections were cut, collected on glass slides, immediately fixed in cold 95% ethanol for 5 minutes, air-dried, and subsequently rehydrated in deionized water. The moist tissue was scraped off the glass slides with a sterile blade and digested in 50–150 μ l buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, 1% Tween 20, and 200 μ g/ml proteinase K. In 25 samples in which the infiltrate was localized only in circumscribed areas of the section, slides were lightly stained with hematoxylin and areas to be microdissected were visualized under the microscope and isolated on the slide, scraping the surrounding tissue with a sterile 30G1/2 needle. A new sterile 30G1/2 needle was then used to remove the target cells from the slide. These were placed in 30–50 μ l of digestion buffer. Proteinase K digestion was performed at 37°C for 12–36 hours. The samples were then heated at 94°C for 5 minutes to inactivate the enzyme and centrifuged and the supernatant was used as template for PCR amplification.

PCR Amplification

β -globin

To assess the quality of the DNA extracted from paraffin-embedded samples, the human β -globin gene was amplified with specific primers (5'GAAGAGCCAAGGCG-

AGGTAC3' and 5'CAACTTCATCCACGTTCCACC3')²⁹ in a single-round PCR reaction for 35 cycles.

$V\gamma 1-8$

A seminested protocol involving two rounds of PCR was used for the amplification of the rearranged TCR- γ gene with $V\gamma 1-8$ primers. Consensus primers for $V\gamma 1$ family ($V\gamma 1-8/A$) and for $J\gamma 1$ and $J\gamma 2$ segments ($J\gamma 1/J\gamma 2$ consensus)²⁴ (Figure 1, left) were used in the first round (7 cycles). First-round products (1–10 μ l) were reamplified in a second-round (33 cycles) reaction using the same $J\gamma 1/J\gamma 2$ consensus downstream primer and an internal upstream consensus primer for $V\gamma 1$ family ($V\gamma 1-8/B$).¹⁷

$V\gamma 9$

Cases that demonstrated polyclonality with $V\gamma 1-8$ primers were reamplified with a single round of PCR using $V\gamma 9$ upstream primer with the $J\gamma 1/J\gamma 2$ consensus downstream primer for 40 cycles (Figure 1, left).¹⁷

$V\gamma 10$

Cases that demonstrated polyclonality with $V\gamma 1-8$ and $V\gamma 9$ primers were reamplified with a single round of PCR

using V γ 10 upstream primer with the J γ 1/J γ 2 consensus downstream primer for 40 cycles (Figure 1, left).¹⁷

V γ 11

Cases that demonstrated polyclonality with V γ 1–8, V γ 9, and V γ 10 primers were reamplified with a single round of PCR using V γ 11 upstream primer with the J γ 1/J γ 2 consensus downstream primer for 40 cycles (Figure 1, left).¹⁷

PCR amplification was performed in GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). The reaction mixture (50 μ l) contained PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3), 200 μ mol/L each of dNTP, 1.5 mmol/L MgCl₂, 0.2 μ mol/L of each primer, and 1.25 U of AmpliTaq Gold (Perkin Elmer). Either 80–120 ng of DNA or 1–10 μ l of paraffin-embedded or frozen tissue digests were used as template. To optimize PCR amplification of formalin-fixed paraffin-embedded samples, different aliquots of tissue digests were tested for each case and the concentration that produced the most efficient amplification was used for PCR/SSCP analysis. Each PCR cycle consisted of 94°C for 1 minute, 55°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds. Before each round, the PCR reaction mixture was heated to 94°C for 10 minutes to activate the AmpliTaq Gold. In all experiments, monoclonal (Jurkat and HSB-2 cell lines) and polyclonal (peripheral blood cells from normal individuals) controls were run in parallel with the test samples. To check the efficiency of the amplification, products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized on an UV transilluminator. SSCP analysis was performed only if a single band of the expected size was detected on the gel. DNA from each sample was amplified at least twice.

SSCP Analysis

SSCP analysis was performed using a thermostatically controlled electrophoresis apparatus. Briefly, 20% polyacrylamide TBE precast minigels (8.0 $\times$ 8.0 $\times$ 0.1 cm; 39:1 acrylamide:bis-acrylamide) were used with the ThermoFlow ETC System (Novex, San Diego, CA) filled with 1.5 $\times$ TBE buffer. The ThermoFlow ETC System consists of the ThermoFlow MiniCell, the ETC unit, and a high efficiency heat exchanger connected to an external thermostatically controlled circulating bath (Isotemp 1016 D, Fisher Scientific, Pittsburgh, PA). The buffer temperature is kept constant by recirculation through the heat exchanger at high flow rates. We optimized the SSCP, running the assay at different preset gel buffer temperatures (7°C, 10°C, 15°C, 20°C, 22°C, and 25°C) (data not shown). A constant buffer temperature of 22°C was used for V γ 1–8 PCR products and of 20°C for V γ 9, V γ 10, and V γ 11 PCR products. PCR products (5–10 μ l) were mixed with 0.4 μ l of 1 mol/L methylmercury hydroxide (Johnson Matthey Electronics, War Hill, MA), 1 μ l of 15% w/v Ficoll (MW 400,000) loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol, and 1 $\times$ TBE buffer to a total volume of 20 μ l. The mixture was heated to 95°C

for 5 minutes, chilled on ice for 1 minute, and loaded on the gel. Gels were run at 200 volts for 12 hours for V γ 1–8 products, 7 hours for V γ 9 products, and 5 hours for V γ 10 and V γ 11 products. Gels were subsequently stained with SYBR-Green II (Molecular Probes, Eugene, OR) diluted 1:10,000 in 1 $\times$ TBE for 30–45 minutes and destained in water for 10 minutes. Ultraviolet transillumination was used to visualize gels.

Sequencing of SSCP Bands

One dominant SSCP band from two cases of T-cell lymphoma (1 large cell CTCL CD30-positive and 1 mycosis fungoides) was excised from the 20% polyacrylamide gel using a sterile blade. In both cases, SSCP bands were localized at the level of the uppermost smear present in the polyclonal controls. To isolate DNA, the gel slice was crushed against the wall of a microcentrifuge tube and incubated for 16 hours in elution buffer (0.5 mol/L ammonium acetate, 10 mmol/L magnesium acetate, 1 mmol/L EDTA, pH 8, 0.1% sodium dodecyl sulfate) at 37°C on a rotating wheel.³⁰ DNA was subsequently precipitated in ethanol and resuspended in sterile water. The eluted DNA was reamplified using V γ 1–8/B and J γ 1/J γ 2 consensus primers. PCR products were cleaned with the GeneClean II kit (BIO 101, Vista, CA) and sequenced using dye terminator fluorescence on an ABI 373 automated sequencer. One hundred nanograms of PCR products were used with 3.2 pmol/L V γ 1–8/B upstream primer. Sequencing was performed under the following cycling conditions: 96°C for 15 seconds, 55°C for 2 minutes, and 73°C for 1 minute.

Sensitivity Test

To assess the sensitivity of the technique, Jurkat cells were serially diluted in normal peripheral blood mononuclear cells. Jurkat cells were pelleted and resuspended in sterile phosphate-buffered saline at a concentration of 10³ cells/ μ l. Peripheral blood mononuclear cells were isolated by sedimentation in gradients of Ficoll-Paque (Pharmacia, Upsala, Sweden), pelleted, and resuspended in sterile phosphate-buffered saline at the same concentration as Jurkat cells. Serial dilutions of Jurkat cells in mononuclear blood cells from a normal individual (1:2, 1:10, 1:20, 1:100, and 1:1000) were prepared and DNA extraction was performed by boiling the samples for 15 minutes. Ten microliters of each boiled sample were subsequently used as template for PCR amplification using V γ 1–8 and J γ 1/J γ 2 consensus primers.

Results

Separation of PCR Products by Size (Agarose Gel)

Aliquots of PCR products were resolved on 2% agarose gel. All tested cases showed the presence of a single band of 268 bp for β -globin products (not shown). PCR

Figure 2. A: V γ 1–8 PCR products run on 2% agarose gel. Lane M: 100 bp molecular weight marker. Lanes 1 and 6: Jurkat cell line (monoclonal control). Lanes 2 and 7: peripheral blood cells from a normal donor (polyclonal control). (Lane 3): subcutaneous $\gamma\delta$ T-cell lymphoma. Lane 4: large cell CTCL CD30+. Lane 5: mycosis fungoides. Lane 8: hyperplastic lymph node. Lane 9: primary cutaneous large B-cell lymphoma. Lane 10: chronic nonspecific dermatitis. All cases produce a single band of approximately 240 bp. B: V γ 1–8 PCR products run on SSCP gel. Monoclonal controls (lanes 1 and 6) and T-cell lymphoma cases (lanes 3–5) produce a pattern of bands. The uppermost band in lane 4 splits into two bands when the gel runs for a longer period of time, producing a pattern of four predominant bands, interpreted as a biallelic rearrangement. Polyclonal controls (lanes 2 and 7), reactive cases, and B-cell lymphomas (lanes 8–10) produce a pattern of three smears (arrows).

amplification with specific primers for TCR- γ gene demonstrated a single band of approximately 240 bp for V γ 1–8 products (Figure 2A), 180 bp for V γ 9 products (Figure 3A), 160 bp for V γ 10 products, and 140 bp for V γ 11 products (Figure 4A).

Separation of PCR Products by Sequence (SSCP)

V γ 1–8 PCR Products

SSCP analysis of V γ 1–8 PCR products resulted in two distinct patterns. All polyclonal T-cell controls produced a pattern of three smears of variable intensity occurring at reproducible locations and distances from one another (Figure 1, right, and Figure 2B, Lanes 2 and 7). In contrast, the monoclonal control (Jurkat cell line) yielded a well-defined banded pattern at the level of the lowest smear observed in the polyclonal samples (Figure 1, right, and Figure 2B, Lanes 1 and 6). Occasionally, a slow migrating band above the uppermost smear could be observed in both polyclonal and monoclonal samples and was thus regarded as an artifact (not shown).

Figure 3. A: V γ 9 PCR products run on 2% agarose gel. Lane M: 100-bp molecular weight marker. Lane 1: HSB-2 cell line (monoclonal control). Lane 2: peripheral blood cells from a normal donor (polyclonal control). Lane 3: peripheral T-cell lymphoma of lymph-node. All cases produce a single band of approximately 180 bp. B: V γ 9 PCR products run on SSCP gel. Monoclonal control (lane 1) and T-cell lymphoma case (lane 3) produce a pattern of bands. Polyclonal control (lane 2) produces a pattern of three smears (arrows).

Results of the tissue samples study are summarized in Table 1. By SSCP analysis, 20 of 24 biopsies of T-cell lymphomas produced a banded pattern characteristic of monoclonality, using V γ 1 (V γ 1–8) family consensus and J γ 1/J γ 2 consensus primers. Clonality was thus confirmed in 17 of 21 (81%) T-cell lymphoma cases analyzed with this set of primers. The bands were localized at the level of one (or rarely two) of the three smears characteristic of the polyclonal controls (Figure 2B, Lanes 3–5). Each banded pattern consisted of two stronger bands corresponding to the single DNA strands of the clonal TCR- γ rearrangement. For each sample, the banded pattern was identical when the products of at least two distinct PCR amplifications were run in the same gel (Figure 5). In two T-cell lymphoma cases (2 large cell CTCL CD 30-

Figure 4. A: V γ 10 and V γ 11 PCR products run on 2% agarose gel. Lane M: 100-bp molecular weight marker. V γ 10 PCR products (lanes 1–4) produce a single band of approximately 160 bp. Lane 1: HSB-2 cell line (monoclonal control). (Lane 2): peripheral blood cells from a normal donor (polyclonal control). Lane 3: large cell CTCL CD30-. Lane 4: mycosis fungoides. V γ 11 PCR products (lanes 5–7) produce a single band of approximately 140bp. Lane 5: Jurkat cell line (monoclonal control). Lane 6: peripheral blood cells from a normal donor (polyclonal control). Lane 7: mycosis fungoides. B: V γ 10 and V γ 11 PCR products run on SSCP gel. Monoclonal controls (lanes 1 and 5) produce a pattern of bands. Polyclonal controls (lanes 2 and 6) produce a smear. Large cell CTCL CD30- monoclonal with V γ 10 primers (lane 3). Mycosis fungoides case polyclonal with V γ 10 (lane 4) and V γ 11 (lane 7) primers.

Figure 5. A: Mycosis fungoides: atypical lymphoid cells with hyperchromatic and convoluted nuclei in papillary dermis, and epidermotropism without significant spongiosis. B: PCR/SSCP analysis of two synchronous lesions from a patient with mycosis fungoides shows identical SSCP banded patterns. **Lane 1:** Jurkat cell line (monoclonal control). **Lane 2:** peripheral blood cells from a normal donor (polyclonal control). **Lanes 3–5:** three separate PCR reactions of lesion from the face. **Lanes 6–8:** three separate PCR reactions of lesion from the back.

positive), SSCP analysis produced a banded pattern consisting of four predominant bands, most likely corresponding to biallelic rearrangements (Figure 2B, Lane 4). When the PCR products of these two cases were run on a 10% polyacrylamide gel, each produced two bands, confirming the rearrangement of both alleles (not shown). In three T-cell lymphoma cases, in which samples from synchronous (1 mycosis fungoides) and metachronous (1 precursor T-cell lymphoblastic lymphoma and 1 enteropathy associated T-cell lymphoma) lesions were studied, identical banded patterns were observed on the SSCP gel for samples from the same case (Figure 5). Using the same set of primers, 6/6 hyperplastic lymph nodes, 4/6 inflammatory conditions of the skin, and 2/2 B-cell lymphomas showed three smears identical to those obtained with polyclonal controls (Figure 2B, Lanes 8–10). Two cutaneous lichen planus cases and one T-cell lymphoma case (mycosis fungoides) were characterized by the presence of faint multiple bands located at all three levels of smearing described for polyclonal cases (not shown). However, in each of these cases the banded pattern was not reproducible in the multiple amplifications of the same DNA sample. In one of the two lichen planus cases, DNA was re-extracted using a smaller amount of digestion buffer to obtain a higher DNA concentration and the assay was repeated. This time a smear characteristic of a polyclonal population was observed.

V γ 9 PCR Products

SSCP analysis of V γ 9 PCR products resulted in two distinct patterns. All polyclonal T-cell controls produced a pattern of two smears of variable intensity occurring at reproducible locations and distances from one another (Figure 1, right and Figure 3B, Lane 2). In contrast, the monoclonal control (HSB-2 cell line) yielded a well-defined banded pattern at the level of both smears observed in the polyclonal samples (Figure 1, right; Figure 3B, Lane 1).

Four T-cell lymphoma cases, twelve reactive lymphocytic infiltrates, and two primary B-cell lymphoma cases that did not show a monoclonal result with the first set of

primers were also amplified using V γ 9 and J γ 1/J γ 2 consensus primers and submitted to SSCP analysis. One of four T-cell lymphomas analyzed showed a monoclonal V γ 9 rearrangement (Figure 3B, Lane 3), whereas the remaining three cases produced polyclonal smears. Polyclonal V γ 9 rearrangements were also observed in all reactive lymphocytic infiltrates and B-cell lymphomas analyzed. As observed for V γ 1–8 PCR products, each banded pattern, consisting of two predominant bands, was reproducible when different PCR amplifications of the same sample were run on the same SSCP gel (not shown).

V γ 10 and V γ 11 PCR Products

SSCP analysis of V γ 10 and V γ 11 PCR products resulted in two distinct patterns. All polyclonal T-cell controls produced a smear (Figure 1, right; Figure 4B, Lanes 2 and 6), whereas monoclonal controls (HSB-2 and Jurkat cell lines) yielded a well-defined banded pattern (Figure 1, right; Figure 4B, Lanes 1 and 5). Three T-cell lymphoma samples, all reactive T-cell infiltrates, and B-cell lymphomas, which did not show a monoclonal result with either V γ 1–8 or V γ 9 primers, were also amplified using V γ 10 and J γ 1/J γ 2 consensus primers and submitted to SSCP analysis (Figure 4B, Lanes 3 and 4). Two of three T-cell lymphomas analyzed showed monoclonal V γ 10 rearrangements (Figure 4B, Lane 3). The remaining T-cell lymphoma case, all reactive T-cell infiltrates, and B-cell lymphomas previously tested for V γ 10 rearrangements (Figure 4B, Lane 4) were amplified using V γ 11 and J γ 1/J γ 2 consensus primers and submitted to SSCP analysis (Figure 4B, Lane 7). The T-cell lymphoma case showed polyclonality. Polyclonal V γ 10 and V γ 11 rearrangements were observed in all reactive T-cell infiltrates and B-cell lymphomas.

In summary, monoclonality was detected in 20/24 biopsies using V γ 1–8 primers, in 1/4 using V γ 9 primers, in 2/3 using V γ 10 primers, and in 0/1 remaining samples using V γ 11 primers. Thus, using V γ 1–11 primers, monoclonality was established in 23/24 samples from 20/21 patients.

Sequencing of SSCP Bands

Results of sequencing SSCP bands confirmed that the bands were derived from clonal TCR- γ gene rearrangements, in both T-cell lymphoma cases analyzed. Sequencing analysis showed that both rearrangements used the V γ 2 variable region and each one was characterized by a unique N hypervariable segment (large cell cutaneous T-cell lymphoma CD30-positive: TCAACGCG-TAAATTATT, mycosis fungoides: CCTATATGGATCC).

Sensitivity Test

To assess the sensitivity of the technique, Jurkat cells were serially diluted in normal peripheral blood mononuclear cells. A banded pattern was still observable on the SSCP gel when the Jurkat cell line represented 1–5% of the sample (Figure 6).

Figure 6. Sensitivity test performed on serial dilutions of Jurkat cells in mononuclear blood cells from a normal donor. **Lane 1:** 100% Jurkat. **Lane 2:** 100% mononuclear blood cells. **Lane 3:** blank. **Lane 4:** 50% Jurkat. **Lane 5:** 10% Jurkat. **Lane 6:** 5% Jurkat. **Lane 7:** 1% Jurkat. **Lane 8:** 0.1% Jurkat.

Discussion

T-cell lymphoproliferative disorders are often difficult to diagnose on the basis of clinical, histological, and immunophenotypic criteria. In the absence of a reliable immunohistochemical marker of clonality for T-lymphocytes, genetic analysis of TCR is the simplest technique to assess the presence of a dominant T-cell clone in a lymphocytic infiltrate.^{3,4} Because T-cell lymphoproliferative disorders frequently involve the skin and other extranodal sites,^{1,2} tissue available for molecular assessment of clonality is often limited to archival paraffin blocks. As a result, microdissection/PCR techniques are required.

Because the junctional sequence of TCR- γ gene rearrangements shows relatively small variation in length but significant diversity in nucleotide composition,^{15,17} only techniques that separate PCR products on the basis of nucleotide sequence can reliably detect clonal TCR- γ gene rearrangements. SSCP²⁶ analysis and DGGE^{31,32} are procedures that allow separation of DNA fragments of identical length but different nucleotide sequence. These methods are based on the principle that secondary structures affect DNA mobility through a polyacrylamide gel.

Reverse transcriptase PCR/SSCP analysis of TCR- β transcripts has been successfully used to detect dominant TCR gene rearrangements within a population of T lymphocytes.³³⁻³⁶ However, because RNA cannot be efficiently extracted from paraffin-embedded tissue, this methodology is not applicable to archival samples. Both PCR/SSCP and PCR/DGGE have recently been used in the analysis of TCR- γ gene rearrangements either for diagnostic purposes or to follow the evolution of a T-cell malignancy of a given patient.^{5,16-25} In our opinion, both techniques, used as reported in the literature thus far, have limitations for routine diagnostic use.

PCR/DGGE has been extensively used for the detection of clonal TCR- γ gene rearrangements. However, in our experience and according to several authors,^{9,23,25} DGGE is a complex technique that requires both a specialized apparatus and refined technical skills (eg, in casting denaturing gels with reproducible gradients) and is thus not readily adaptable to the routine clinical laboratory. In addition, Offermans et al,³⁷ after testing DGGE and SSCP as tools for the detection of junctional diversity in rearranged TCR sequences, pointed out that although both methodologies are suitable, SSCP is a relatively simple and rapid procedure when compared to DGGE.

Volkenandt and Koch used a cRNA-SSCP methodology to analyze rearranged TCR- γ genes in acute lymphocytic leukemia, gastrointestinal lymphomas, and cutane-

ous lymphomas.¹⁹⁻²¹ cRNA-SSCP is based on multiple different conformations of RNA (generated by *in vitro* transcription from PCR products) that give rise to an individual fingerprint. Although this methodology appears to have a higher sensitivity in point mutation detection compared to DNA-SSCP, when this methodology is applied to TCR- γ gene rearrangement analysis, the presence of multiple bands on the SSCP gel prevents the discrimination between monoclonal and oligoclonal T-cell populations. In fact, as pointed out by Koch et al,²⁰ biallelic TCR gene rearrangements or the simultaneous presence of additional clones cannot be reliably distinguished. Finally, cRNA-SSCP technique has the disadvantage of requiring an additional time-consuming and expensive *in vitro* transcription step.

Baruchel and co-workers studied clonality by PAGE analysis followed by assessment of clonal evolution at Ig/TCR loci (both γ and δ) in acute lymphoblastic leukemia by SSCP.²² Because all relevant gene rearrangements were screened by PCR-PAGE analysis and a radioactive SSCP technique was used only to compare rearranged junctional sequences at presentation and relapse, it is difficult to compare this study with ours, which has a strictly diagnostic intent.

Two additional recent articles describe TCR- γ rearrangements using nonradioactive PCR-SSCP.^{23,25} Although their results confirm the utility of this technique, the authors have used predominantly nonarchival tumor samples. In addition, Kaul and co-workers²³ used consensus primers for the V γ 1 (V γ 1-8) family only, whereas Lynas et al²⁵ did not adequately address the issue of the sensitivity of their technique.

Our goal was to develop a PCR-SSCP technique that is simple, reproducible, nonradioactive, and sensitive, as well as a valid alternative to the DGGE technology. In addition, because most T-cell lymphomas that present a diagnostic dilemma are extranodal and thus almost invariably formalin-fixed and paraffin-embedded, the methodology was developed in archival tissues, often microdissected from stained slides.

In devising our methodology, we chose to address the parameters that, in the traditional approach, were responsible for the lack of reproducibility of the SSCP technique.^{26,27} Gel temperature is the most critical parameter influencing SSCP band resolution and reproducibility. To avoid heat-induced conformational changes in the secondary structure, gel temperature should be constant. This, however, is quite difficult to achieve in large gels. It has been recently shown that the use of a thermostatically controlled circulator which accurately maintains a predetermined buffer temperature within the gel unit allows reproducible separation of single strands of nonradioactive PCR products on polyacrylamide precast minigels.²⁷ The ideal temperature for nonradioactive SSCP varies among individual PCR products and requires empirical trials to obtain optimal results for each product. The temperatures of 22°C for V γ 1-8 products and of 20°C for V γ 9, V γ 10, and V γ 11 products were chosen because they produced a well defined banded pattern for monoclonal controls and a smear for polyclonal controls.

In addition, to maximize the sensitivity of detection, SSCP gel staining was performed using SYBR-Green II (Molecular Probes), which has been shown to give better results in staining ssDNA when compared to ethidium bromide.²⁸

In summary, there are five novel aspects of the technique described here. First, it is applicable to paraffin-embedded tissue. Second, it is simple, based on the use of commercially available precast minigels. Third, it is reproducible, based on standardization of all parameters including PCR settings, running time, temperature (using a recirculating apparatus capable of maintaining constant buffer temperature), and voltage. In fact, the same banding pattern is obtained when the same sample is amplified and run on different SSCP gels (data not shown). Fourth, it offers high sensitivity of detection for a nonradioactive technique with the use of SYBR Green II. Fifth, it uses microdissection before DNA extraction.

Using this technique, we were able to demonstrate clonality in 20/21 (95%) T-cell lymphoma cases. A very high percentage of our cases (81%) showed at least one clonal TCR- γ gene rearrangement involving V γ 1-8 and J γ 1/J γ 2 regions. Three additional cases showed clonality when subsequently analyzed with V γ 9, V γ 10, V γ 11, and J γ 1/J γ 2 primers.

It has been previously shown that V γ 1-8 and J γ 1/J γ 2 segments are involved in approximately 60-70% of clonal TCR- γ gene rearrangements detected by Southern blot analysis in various T-cell malignancies^{14,38} and in 79% of clonal TCR- γ gene rearrangements detected by Southern blot and PCR/heteroduplex methodology in CTCL.³⁹ Clonality has been demonstrated in 90% of CTCL cases investigated by PCR/DGGE, using primers for V γ 1-9 and J γ 1/J γ 2 segments.⁵ Our study confirms the frequent use of V γ 1-9 and J γ 1/J γ 2 regions in neoplastic clones of CTCL, representing the majority of T-cell lymphoma cases included in our study. However, the true frequency of V γ 1-9 and J γ 1/J γ 2 segments in clonal gene rearrangements of CTCL needs to be assessed by further studies.

One of 21 (5%) T-cell lymphoma cases showed polyclonality with all sets of primers used in our study. We can speculate that the TCR- γ genes of the neoplastic clone were in germline configuration, deleted, or, most likely, contained rearrangements involving one of the JP, JP1, and JP2 regions that have not been investigated. Several authors who recently studied TCR- γ gene by PCR found that approximately 10% of T-cell lymphoma cases contained clonal TCR- γ gene rearrangements exclusively involving JP, JP1, and JP2 pseudogenes.^{25,40} Therefore, we estimate that about 5-10% of clonal TCR- γ gene rearrangements are not detectable by our technique, which does not include primers for JP, J1, and JP2 segments. We did not detect clonality in any of the reactive lymphocytic infiltrates, hyperplastic lymph nodes, or B-cell lymphomas with any of the sets of primers used (V γ 1-8, V γ 9, V γ 10, V γ 11, and J γ 1/J γ 2 primers). However, two lichen planus cases and one T-cell lymphoma case analyzed using V γ 1-8 primers produced faint, non-reproducible bands on the SSCP gel. The T-cell lymphoma case showed monoclonality when subsequently

analyzed with V γ 10 primers. In one of the two lichen planus cases, in which the assay was repeated using a higher DNA concentration, a reproducible smear characteristic of a polyclonal population was observed. The banded pattern obtained with lower amounts of template in this lichen planus case is most likely related to the preferential random amplification of TCR- γ rearrangements of reactive T-lymphocytes present in samples with low DNA concentration (so-called pseudoclonality).^{41,42} Accordingly, in the T-cell lymphoma case, we hypothesize that only the TCR- γ gene rearrangements of benign tumor infiltrating lymphocytes were randomly amplified using V γ 1-8 and J γ 1/J γ 2 primers, whereas the rearrangements of the neoplastic clone (shown to be clonal with V γ 10 primers) were not. To avoid pseudoclonality, products from at least two different PCR amplifications should be run on the same SSCP gel and only if an identical banded pattern is obtained should the sample be considered clonal for TCR- γ gene rearrangement. In addition, seminested PCR is used with V γ 1-8 and J γ 1/J γ 2 consensus primers to increase the sensitivity and specificity of the amplification. Finally, SSCP should be run only if a single band of the expected size and with an intensity comparable to that of controls is present on the 2% agarose gel.

The SSCP banded pattern is closely related to the nucleotide sequence of the DNA fragments and is, therefore, highly specific for each monoclonal TCR- γ gene rearrangement. Therefore, the PCR-SSCP technique has the advantage of allowing direct comparison between results of analyses performed on multiple samples. More specifically, it may be used to evaluate synchronous and metachronous lesions from the same patient. Furthermore, clonal TCR- γ gene rearrangements can be confirmed by sequencing dominant SSCP bands, without the need for subcloning techniques.

In conclusion, we describe a simple, sensitive, reproducible, nonradioactive technique for the assessment of T-cell clonality in archival paraffin-embedded tissue. This technique is amenable to use in routine diagnostic pathology. Based on the results of this study, we are currently using TCR- γ PCR-SSCP as an adjunct to the tools commonly used to diagnose T-cell lymphoma.

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