A New Polymerase Chain Reaction–Based Method for the Detection of T-Cell Clonality in Patients With Possible Cutaneous T-Cell Lymphoma

Joan Guitart, MD; Karen Kaul, MD, PhD

Objective: To evaluate a new, rapid, and sensitive method for the detection of T-cell clonality in patients with lesions suggestive of cutaneous T-cell lymphoma (CTCL).

Design: Skin specimens were obtained from 48 patients with possible CTCL. Polymerase chain reaction amplification of the T-cell receptor γ (TCRG) gene was performed using consensus primers to the V and J regions. Clonal populations having identical N-region sequences are detected by single-strand conformation polymorphism analysis using a semiautomated electrophoresis system with a silver-staining method for gel visualization. The results of clinicopathological assessment were compared with those of immunohistochemistry and polymerase chain reaction analysis.

Setting: Cutaneous lymphoma clinic at a university medical center.

The often-slow evolution of cutaneous T-cell lymphoma (CTCL) and the gravity of the diagnosis of malignant lymphoma pose a special diagnostic problem to dermatopathologists. Immunohistochemistry and molecular studies of the T-cell receptor γ (TCRG) gene are frequently used to corroborate the clinicopathological impression. For the past decade, the Southern blot analysis using T-cell receptor β probes has been the method of choice for the molecular demonstration of clonal T-cell populations. Southern blot analysis, however, is a labor-intensive, unwieldy procedure requiring several days for results. Its sensitivity in detecting a clonal population is at best in the range of 2% to 5% of total nucleated cells, and thus, small clonal T-cell populations in early lesions of mycosis fungoides (MF) can be difficult to detect.1,2 A new, rapid, and easy technique for the detection of T-cell clonality using polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) was recently developed.3 Our goal was to determine the value of the new PCR test compared with the clinicopathological correlation (CPC) and immunophenotyping.

RESULTS

The patient population included 33 men and 15 women with a mean age of 60.5 years. Details of the patients are listed in Table 1. Based on the CPC, 26 of the 48 patients were diagnosed as having CTCL. Of them, clonality was detected in 19 patients (73%) and an abnormal phenotype in 17 (68%) of 25 patients. Combining both tests, abnormal results were noted in 24 (92%) of 26 patients with CTCL. Clonality was also identified in 2 (12%) of 17 patients with presumably benign lesions on clinicopathological assessment.

Patients: Forty-eight patients with skin lesions suggestive of CTCL.

Results: Based on the clinicopathological assessment, 26 patients were diagnosed as having CTCL. Of them, clonality was detected in 19 patients (73%) and an abnormal phenotype in 17 (68%) of 25 patients. Combining both tests, abnormal results were noted in 24 (92%) of 26 patients with CTCL. Clonality was also identified in 2 (12%) of 17 patients with presumably benign lesions on clinicopathological assessment.

Conclusions: Polymerase chain reaction and single-strand conformation polymorphism analysis of the TCRG gene is a rapid and sensitive method that can contribute to the diagnosis of CTCL. The new method is especially useful when used in conjunction with immunophenotyping.

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SUBJECTS AND METHODS

SUBJECTS

We studied 48 consecutive cases of patients who presented at the Department of Dermatology of the Northwestern University Medical Center, Chicago, Ill, with lesions suggestive of CTCL from whom we were able to obtain tissue specimens for regular histological, immunohistochemistry, and PCR analysis. The CPC assessment was done by reviewing the clinical presentation, the histologic findings, and in some patients, the evolution of the lesions. The lesions were classified in 3 groups: CTCL, inconclusive, or benign. The inconclusive group included lesions that were clinically and histopathologically equivocal. Skin biopsy specimens from the inconclusive group were reported as “suggestive of MF” or “dermatitis with atypical lymphocytes,” and the biopsy specimens showed some atypical lymphocytes or other suspicious findings (ie, intraepidermal clusters of lymphocytes or wiry fibroplasia) but no definitive histological features of MF or other forms of CTCL. The results of PCR-SSCP analysis and immunohistochemistry were reached independently of the CPC.

IMMUNOHISTOCHEMISTRY

Immunohistological examination was performed on acetone-fixed frozen sections of the skin biopsy specimens using the avidin–biotinylated peroxidase (Vector Laboratories, Inc, Burlingame, Calif) method according to the manufacturer’s directions. Slides were counterstained with Harris hematoxylin. Frozen normal tonsillar sections were used as positive controls for each antibody, and equivalently diluted nonimmune mouse immunoglobulin was substituted for each antibody to serve as a negative control. The panel of monoclonal antibodies used includes CD2, CD3, CD4, CD5, and CD6 (Coulter Corp, Hialeah, Fla); CD7 (Becton Dickinson Immunocytological Systems, Mountain View, Calif); CD8 (Coulter Corp); CD15 (Becton Dickinson Immunocytological Systems); CD20 (Coulter Corp); CD25 (Becton Dickinson Immunocytological Systems); CD30 (Dako Corp, Carpinteria, Calif); CD45RB (Dako Corp); and normal mouse serum (Coulter Corp) (Table 1). The percentage of mononuclear cells expressing the different surface markers was estimated by 1 of us (J.G.). Antigen deletion was defined as less than 30% of the mononuclear cell infiltrate expressing a specific antigen.

PRESENTATION AND DIAGNOSIS

Among the 26 patients having a diagnosis of CTCL, the PCR-SSCP assay revealed a clonal band in 19 patients (73%) (Table 2). An abnormal phenotype with surface antigen deletion was identified in 17 (68%) of 25 patients with CTCL. Of the 5 patients with CTCL with an abnormal phenotype but no detectable clonality, 3 had multiple antigens deleted, and hence, the immunophenotype was of great value (Figure 1). Only 2 patients with CTCL (8%) had a normal phenotype and no detectable clonality. A clonal band was also detected in 2 of the patients with CPC assessment of a benign lesion, presented with nonspecific dermatitis, possibly related to postirradiation xerosis in 1 of them.

PCR AMPLIFICATION

Polymerase chain reaction amplification was performed as previously described.1 Consensus primers to the V (V1.3) and J (J17) segments were used for PCR amplification, generating approximately a 268-base-pair fragment that included the N region.3 The reaction mixture consisted of a standard 1 × PCR buffer containing magnesium chloride, 1.5 mmol/L; 50 pmol of each primer; 200 µmol/L of each deoxynucleotide; DNA, 1 µg; and Taq DNA polymerase, 2.5 U, in a total volume of 50 µL. Amplification was performed in a thermal cycler (Perkin-Elmer 9600; Perkin-Elmer Corp, Norwalk, Conn) and consisted of an initial denaturation for 3 minutes, followed by 30 cycles at 94°C for 60 seconds, 61°C for 60 seconds, and 72°C for 60 seconds. Initial product analysis was performed using 0.7% agarose minigel containing ethidium bromide. Positive (1% CEM cells mixed with polyclonal lymphocytes) and negative (polyclonal lymphocyte DNA and DNA blanks consisting of sterile water) controls were used in each experiment. The dihydroxyuridine triphosphate/uracil-N-glycosylase system was used to control carryover contamination, along with spatially separate work areas and dedicated equipment at each area.

SSCP ANALYSIS AND SILVER STAINING

The SSCP analysis was performed using a semiautomated system (Phastgel; Pharmacia Biotech Inc, Uppsala, Sweden). One microliter of the amplified PCR mixture was diluted with an equal volume of formamide containing 5% bromophenol blue and was applied to preformed 12.5% homogeneous acrylamide gels. The electrophoretic run at 4°C using native buffer strips (Pharmacia Biotech Inc), consisted of a prerun of 400 V, 10 mA, 2.5 W, 100 volt-hours; an automatic sample application of 400 V, 1 mA, 2.5 W, and 2 volt-hours; and electrophoresis of 400 V, 10 mA, 2.5 W, and 80 volt-hours. The approximate prerun time was 30 minutes, and electrophoresis required 20 minutes. Silver staining was performed in the automated staining apparatus using directions and prepared reagents from the manufacturer’s kit (Phast system; Pharmacia Biotech Inc).

DNA EXTRACTION

High-molecular-weight DNA was obtained from frozen skin specimens using the Pure Gene kit (Gentra Systems, Inc, Minneapolis, Minn) according to the manufacturer’s directions.

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1% of the cells, in contrast to the sensitivity of the peripheral leukocytes showed detectable clones of less than 0.1% of the cells, in contrast to the sensitivity of the Southern blot, does not produce a PCR product. SSCP analysis were combined, the sensitivity of having clonality in T-cell or B-cell lymphocytic populations. The diagnosis of early lesions of MF is often difficult. This burden, shared by clinicians and pathologists, is partially due to the slow evolution of the lesions and the predominance of reactive lymphocytes over neoplastic cells in early MF. Immunophenotyping and gene rearrangement analyses are helpful tools to reach a prompt and decisive diagnosis. Because of its speed and sensitivity, PCR is rapidly becoming the preferred method for the detection of clonality in T-cell or B-cell lymphocytic populations.

Polymerase chain reaction offers substantial advantages over the widely used Southern blot technique for the detection of clonal T cells in MF.\(^1,4\) Whereas the Southern blot analyzes DNA from each of the cells of a specimen, the PCR technique effectively analyzes only those cells with T-cell-receptor rearrangement because amplification only occurs for the rearranged loci. Therefore, DNA from non-T cells, which dilutes the clonal DNA in the Southern blot, does not produce a PCR product. Serial dilutions of the neoplastic T-cell line CEM into peripheral leukocytes showed detectable clones of less than 1% of the cells, in contrast to the sensitivity of the Southern blot technique of approximately 5% (Figure 2).\(^{1,2}\) The PCR results are available in as little as 1 day, compared with up to 2 weeks for completion of the Southern blot. Polymerase chain reaction requires less DNA, making it ideal for small skin biopsy specimens and, furthermore, can be performed on paraffin-embedded tissues. Most PCR-based assays used for the detection of clonal T-cell populations have targeted the TCRG gene. Whereas the TCRB (T-cell receptor β) gene is commonly examined by the Southern blot technique, the size and complexity of this locus makes the design of a PCR assay somewhat difficult. Although the TCRG gene product is not expressed on the surface of most mature T cells, rearrangement of the TCRG gene takes place concomitantly with the TCRB gene in most T cells.\(^3\) The small and simple structure of the TCRG gene, which contains only 11 variable segments, in contrast to the hundreds of V segments for the TCRB gene, makes it a suitable target for PCR amplification. Regrettably, the TCRG gene is also rearranged in some B-cell and myeloid leukemias, making it a good indicator of clonality but a poor lineage marker.\(^6,7\) Assessment of lineage, however, is less critical than the detection of clonality in the diagnosis of CTCL.

This study used consensus primers directed toward the V and J regions of the TCRG gene.\(^6\) The primer set used in this study was chosen because it had homology to most of the variable regions actively used in rearrangement of the TCRG gene and was expected to be the best single pair of primers possible. These primers amplify a fragment of DNA, including the N region, a segment between the V and J regions in which individual nucleotides are added or deleted during the normal rearrangement process by the enzyme terminal deoxynucleotide transferase.\(^9\) Functionally, the N region serves to increase the variability of the antigen-recognition or variable portion of the T-cell receptor. It is also a unique marker useful in the assessment of clonality; clonal cells will have identical N regions.

Other PCR methods targeting the N region of the TCRG gene have been reported.\(^{10-14}\) Some\(^15\) have analyzed the PCR products based on size alone by gel electrophoresis. Amplified fragments containing the N regions of rearranged genes from polyclonal populations appear as a smear, whereas clonal populations yield a distinct band composed of amplicons of identical size. Others\(^13,15,16,17\) have used chemical or temperature-gradient

### Table 1. Summary of Case Results (N = 48)

<table>
<thead>
<tr>
<th>Clinicopathological Correlation</th>
<th>No. of Patients</th>
<th>Age, Mean (Range), y</th>
<th>Sex Distribution, M/F</th>
<th>CD4/CD8 Ratio, Mean</th>
<th>Histopathological Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>17</td>
<td>60.7 (37-87)</td>
<td>12:5</td>
<td>5.5</td>
<td>Pseudolymphomas (HIV- and drug-related (n = 2); atypical dermatitis, C/W actinic reticuloid (n = 3); nonspecific dermatitis (n = 5); dermatitis with atypical lymphocytes (n = 6); and psoriasis (n = 1)</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>5</td>
<td>59.6 (43-69)</td>
<td>4:1</td>
<td>7.8</td>
<td>Suggestive of MF (n = 3); suggestive of MF, folliculotropic type (n = 1); and dermatitis with atypical lymphocytes (n = 1)</td>
</tr>
<tr>
<td>Malignant</td>
<td>26</td>
<td>60.5 (36-87)</td>
<td>17:9</td>
<td>6.6</td>
<td>MF (n = 14); C/W MF (n = 4); C/W lymphomatoid papulosis (n = 2); CTCL, nonepidermotropic or large cell (n = 3); and C/W Sézary syndrome (n = 3)</td>
</tr>
</tbody>
</table>

* HIV indicates human immunodeficiency virus; C/W, consistent with; MF, mycosis fungoides; and CTCL, cutaneous T-cell lymphoma.

### Table 2. Statistical Analysis Summary*

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-SSCP</td>
<td>73 (19/26)</td>
<td>88 (15/17)</td>
<td>90 (19/21)</td>
</tr>
<tr>
<td>Immunophenotype</td>
<td>68 (17/25)</td>
<td>93 (14/15)</td>
<td>94 (17/18)</td>
</tr>
<tr>
<td>PCR plus immunophenotype†</td>
<td>92 (24/26)</td>
<td>82 (14/17)</td>
<td>89 (24/27)</td>
</tr>
</tbody>
</table>

* Data are given as percentage (number of positive findings/number of patients tested). PCR indicates polymerase chain reaction; SSCP, single-strand conformation polymorphism.† Positive results by PCR clonality, abnormal phenotype, or both.

1 with actinic reticuloid and 1 with a history of MF treated with electron-beam irradiation. Both patients responded well to psoralen UV-A therapy.

When the results of immunophenotyping and PCR-SSCP analysis were combined, the sensitivity of having CTCL and an abnormal result on 1 or both tests was 92% (24/26).
gels for analysis; clonal amplicons will denature and stop migrating, forming a band in an electrophoretic gel. Other investigators have used nucleotide sequencing to study amplified immunoglobulin and the TCRG gene. Others have analyzed RNA transcripts by reverse transcriptase PCR, followed by conformational polymorphism in an assay somewhat similar to ours. Each of these approaches uses methods that would present a challenge in routine clinical laboratories. Our goal in this study was the validation of a new method for the detection of T-cell clones that would be sensitive, rapid, and as simple as possible to perform.

The PCR-SSCP analysis differs from the other methods in the analysis of the PCR product. The amplicons are denatured by heat before the electrophoresis step (Figure 3); the single-stranded DNA fragments acquire tridimensional conformation dependent on the nucleotide sequence of the PCR product. This conformation determines how the fragments will migrate through a polyacrylamide gel. Basically, interpretation of the results involves assessing whether distinct bands are present. In a clone with a monoallelic rearrangement, 2 bands are generally seen, each representing the conformation of 1 of the 2 DNA strands. Sometimes 4 clonal bands are detected, indicating that both alleles are rearranged. Occasionally, specimens show an odd number of bands, resulting from a DNA fragment that is stable in more than a single conformation. Polyclonal populations, for which amplicons contain several different N regions, will yield a smear. The use of a semiautomated electrophoresis and staining apparatus makes this approach especially rapid, reproducible, and amenable to use in clinical laboratories.

The sensitivity (73%) of our test in the present study was lower than that of an initial evaluation of various T-cell lymphoproliferative conditions. This is probably because the specimens were mostly from patients with early-stage MF with small malignant clones. The sensitivity of other PCR methods has been reported in the literature to range from 59% to 90%. The variation may be partly due to differences in the patient populations. In 3 of the patients with CTCL with only a mildly dense lymphoid infiltrate, the clonal bands were present but faint. This observation is in accordance with the gradual progression of the malignant T-cell clones in MF, as demonstrated by Bachelez et al using semiquantitative PCR techniques. An unambiguous interpretation in such patients may be difficult.

Our test failed to detect 7 (27%) of the 26 patients with clinicopathological features of CTCL. Although TCRG is generally rearranged in T-cell lymphoproliferative disorders, patients may occasionally lack rearrangement of this gene. Alternatively, the consensus primers used for PCR amplification may have failed to anneal to the segments present in the rearranged TCRG gene. Only 8 rearranging V segments and 5 J segments, of which 2 most frequently rearrange, have been described. The variable primer used anneals to all 5 of the 9 active vari-
able regions of group I of the TCRG gene; clonal populations using the remaining 3 active but less commonly used variable regions (II, III, and IV) may be missed. Three of the patients with CTCL with nondetectable TCRG gene rearrangement by PCR had multiple antigen deletions, and their diagnosis was easily confirmed by immunohistochemistry.

The 2 false-positive results in our series occurred in patients who may actually have a clonal T-cell population (ie, a history of CTCL), which may indicate a higher sensitivity of the clonality test than our standard CPC. Therefore, the specificity of the PCR-SSCP test could be higher than stated.

Immunophenotyping provides a different angle in the evaluation of the lymphoid population. Several studies have shown a different expression of surface markers in malignant T cells, compared with lymphocytes from inflammatory conditions. Whether such changes represent an aberrant antigen deletion or, rather, a distinct, yet normal, lymphoid population is debatable. A subset of T cells with cutaneous affinity and a lack of CD7 expression and a lack of CD7 expression has been reported in the peripheral blood of healthy persons and in cutaneous inflammatory infiltrates. These CD4+, CD7− T-cell populations have affinity to the skin and may undergo clonal proliferation in CTCL. Nevertheless, a lack of CD7 expression in more than 50% of the cells is exceedingly rare in benign dermatoses. We define our criteria for abnormal antigen expression cautiously. In our “abnormal phenotype,” less than a third of the mononuclear cells will express a pan−T-cell marker. Using similar criteria, Wood et al found a lack of CD7 expression in 59% of patients with MF vs in only 3% of persons with benign dermatoses. Although the detection of an abnormal phenotype is not a highly sensitive test, combining the immunophenotype with PCR-SSCP analysis allowed us to detect at least 1 abnormal test result in more than 90% of patients with CTCL.

CONCLUSIONS

The PCR-SSCP test is a rapid and reproducible semiautomated method for the detection of T-cell clonality in skin biopsy specimens, with an overall low cost. The rapid turnaround time and the simplicity of the test are major advantages over other methods for detecting T-cell clonality. The new PCR-SSCP−based method is especially useful for the diagnosis of CTCL when interpretation is combined with immunohistochemistry results and integrated into the overall clinicopathological assessment.

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