Improved Sensitivity of T-Cell Clonality Detection in Mycosis Fungoides by Hand Microdissection and Heteroduplex Analysis

Olivier Dereure, MD, PhD; Edi Levi, MD; Eric C. Vonderheid, MD; Marshall E. Kadin, MD

Background: The presence of a dominant T-cell clone is an important diagnostic criterion in cutaneous T-cell lymphomas (CTCLs) and in atypical T-cell cutaneous infiltrates. Procedures based on polymerase chain reaction (PCR) are the most sensitive to detect clonality, but heteroduplex analysis is less sensitive than other methods such as denaturing gradient gel electrophoresis.

Objective: To assess whether a gross hand microdissection of the superficial (papillary) portion of the dermal infiltrate may improve the sensitivity of T-cell clonality detection by PCR-heteroduplex analysis in CTCL.

Setting: Regional university hospital (secondary or tertiary referral center).

Patients: A total of 29 patients with a definite diagnosis of mycosis fungoides based on typical histologic and immunophenotypic features were selected with patch (16) or plaque (13) stages.

Main Outcome Measures: Assessment of T-cell clonality by PCR amplification of the T-cell receptor \(\gamma\) chain followed by heteroduplex analysis using DNA extracted from the entire biopsy specimen and after gross microdissection of the subepidermal bandlike dermal infiltrate.

Results: T-cell clonality was demonstrated in 24 of 29 cases when hand microdissection was used compared with 16 of 29 cases with DNA analysis from entire biopsy specimens; thus, hand microdissection resulted in a sensitivity improvement of approximatively 50%.

Conclusions: Hand microdissection substantially improves the detection of a T-cell clone in CTCL when using a PCR-heteroduplex analysis and could be used routinely in the clinical evaluation of T-cell infiltrates. Importantly, the microdissection method was found to be more useful for the detection of T-cell clones in early patch stages of CTCL than in plaque-stage disease.

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Mycosis fungoides (MF), the most common cutaneous T-cell lymphoma (CTCL), is a proteiform entity in which a definite clinical and pathologic diagnosis may be difficult to establish in the initial stages. Molecular methods searching for a dominant T-cell clone in the infiltrate have been advocated, but a clear correlation between initial molecular data, final diagnosis, and patient outcome could not be established in ambiguous cutaneous lymphoid infiltrates. Conversely, in definite MF cases, the presence of a detectable dominant T-cell clone seems to be an independent risk factor for an unfavorable outcome and resistance to therapy. Another difficulty lies in the sensitivity of molecular methods, with the possibility of overlooking an authentic dominant T-cell clone when the percentage of neoplastic clonal cells falls below the threshold of the method’s ability to detect them. Southern blot, the sensitivity of which is about 5% at best, has been superseded by more sensitive methods based on polymerase chain reaction (PCR) capable of detecting as few as 0.1% of clonal T cells in a mixed lymphocytic infiltrate.

Heteroduplex analysis is probably one of the simplest of PCR-based methods and has already been clearly validated in CTCL diagnosis, especially by using products of T-cell receptor (TCR) \(\gamma\) chain gene amplification. Although easy and fast, this method is generally considered less sensitive than more complex procedures such as single-strand conformation polymorphism, temperature or denaturing gradient gel electrophoresis, ribonuclease protection analysis, and fragment analysis procedures, but it may detect between 1% and 5% of clonal T cells within a polyclonal background.
Microdissection applied to lymphoma has been already shown to improve the resolution of tumor-specific PCR products by reducing amplification of background polyclonal sequences in nodal and ocular B-cell lymphomas where neoplastic cells are admixed with reactive cells within foci of chronic inflammation to prove the presence of 2 genetically different B-cell clones or to demonstrate the presence of key gene mutations restricted to the neoplastic cells. With respect to cutaneous lymphoid infiltrates, recent reports indicate the utility of laser-beam–based microdissection and seem to establish that previous microdissection might improve the detection of a dominant T-cell clone in cutaneous T-cell lymphoma when epidermis alone is used rather than the entire specimen with dermis and epidermis. Another report using laser-assisted single-cell microdissection of MF lesions on a limited number of patients found that all lymphoid cells present in the epidermis were clonal, whereas most, but not all, dermal T cells were reactive cells. We hereby investigate the hypothesis that the detection of a dominant T-cell clone by PCR-heteroduplex analysis within cutaneous infiltrates of definite cases of CTCL can be improved by a simple hand microdissection of tissue samples, a procedure designed to offer a feasible method that can be used routinely in pathology laboratories.

**Table 1. Primers Used for T-Cell Receptor y Gene Amplification**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Vγ1</td>
<td>5’-TACATCCACTGGTACCTACACA-3’</td>
</tr>
<tr>
<td>Vγ9</td>
<td>5’-CTACACTGGTGGTGCCTCC-3’</td>
</tr>
<tr>
<td>Vγ10</td>
<td>5’-GGAGCAGTGGTAAGACAAGC-3’</td>
</tr>
<tr>
<td>Vγ10/11</td>
<td>5’-TCATTCACTGGTACCGGCAGAAACAA-3’</td>
</tr>
<tr>
<td>Jγ1/2</td>
<td>5’-CCCTGCTGACACTCCTGAAATGTGTTATTC-3’</td>
</tr>
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**Methods**

**Tissues and Patients**

Twenty-nine cutaneous biopsy specimens from 29 patients were selected, including 13 paraffin-embedded and 16 snap-frozen samples. In all cases, a diagnosis of MF was made by integration of clinical, histologic, and immunohistologic data. Biopsies were carried out on patch (16 cases) or plaque (13 cases) lesions. In most cases, the dermal lymphocytic infiltrate was a bandlike moderate one involving part or all of the upper third of the dermis. In some cases the involvement was larger, including the upper two thirds of the dermis, or smaller, with only scattered cells beneath the epidermis. Epidermal involvement with single cells or small clusters of lymphocytes (Pautrier microabcesses) was occasionally seen. Immunophenotyping was performed in all but 3 cases and showed a dominant CD3+, CD4+, CD25+, CD30+ profile intermixed with some CD3+CD8+ cells, typical of MF. Peripheral blood mononuclear cells from healthy donors and paraffin-embedded tissues from 10 patients with typical lichen planus were used as negative controls, while Jurkat cells, a non-Hodgkin T-lymphoma-derived cell line, and Mac-2A cells from a cutaneous anaplastic large cell lymphoma, each with known monoclonal TCR rearrangement, were used as positive controls.

**Microdissection Procedure**

Sections 20 µm thick were cut from frozen or paraffin-embedded tissues from available samples of MF and lichen planus. The sections were transferred onto superfrosted glass slides and stained lightly with eosin either directly after spontaneous rehydration by air exposure (for frozen tissues) or after removal of wax by a standard xylene and ethanol procedure (for paraffin-embedded tissues). After slight further rehydration with distilled water, the upper part of the dermis (papillary dermis) along with the very lowest part of the epidermis were carefully removed with a 30-gauge needle under microscopic visual control (objective lens magnification ×40) (Figure 1). The retrieved material was then transferred in a 1.5-µL Eppendorf tube. Eight to 10 sections were harvested per case.

**Genomic DNA Extraction**

Sections 25 µm thick without previous microdissection (with wax removal by xylene and ethanol standard protocol for paraffin-embedded tissues) or tissue fragments resulting from gross microdissection were transferred to 1.5-µL Eppendorf tubes and digested with 0.1 mg of proteinase K overnight in a shaking water bath at 37°C. After desalination with 5M sodium chloride and centrifugation, genomic DNA in clear supernatant was precipitated with 2 volumes of absolute ethanol. Pelleted DNA was washed with 70% ethanol, dried, dissolved in 50 µL of 10mM Tris (pH 7.6); 1mM edetic acid (pH 8), and kept refrigerated at 4°C until further use. Genomic DNA was extracted from Jurkat cells using a Tissuelyte DNA Extraction Kit (Quiagen Inc, Santa Clara, Calif) according to the manufacturer’s instructions.

**PCR Amplification**

Four primer pairs were designed to amplify the V/J region of the TCRγ chain according to published reports (Table 1). Each primer pair includes the same J consensus primer specific to the J region of the TCRγ chain, and a different V primer, depending on the targeted variable V region: V1 as a consensus primer for V regions 1 to 8, V9, V10, and V11 for the V regions 9, 10, and 11, respectively. Analysis was performed on a Perkin-Elmer-Cetus N801-0150 DNA Thermal Cycler (Boston, Mass), with 100 ng of genomic DNA, 100 pmol of each primer, and Ready-To-Go PCR beads (Pharmacia Biotech, Piscataway, NJ); 200 µmol/L each of deoxadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate; 50 mM of potassium chloride; 1.5 mM of magnesium chloride; 10 mM of Tris-hydrochloric acid (pH 9.0); and 1.5 U of Taq DNA polymerase, with a final volume of 2 µL. The samples were denatured at 95°C for 5 minutes then amplified for 35 cycles consisting of...
of 30 seconds at 95°C, 30 seconds at 65°C, and 1 minute and 30 seconds at 72°C, followed by a final extension segment of 10 minutes at 72°C. The quality of the PCR was controlled by electrophoresis of a 5-µL sample on a 2% agarose gel.

HETERODUPLEX ANALYSIS

Heteroduplex analysis was performed as previously described. Briefly, 5 µL of the PCR mixture was mixed with an equal volume of denaturing buffer (93% formamide, 5% 0.5M edetic acid [pH 8.1], 0.025% xylene cyanol, and 0.025% bromphenol blue), and the resulting solution was heated at 93°C for 5 minutes for denaturation of the double-strand PCR products. Heteroduplex formation was then allowed to proceed by random renaturation at 50°C for 1 hour. Samples were immediately cooled on ice for 5 minutes and loaded on a nondenaturating 12% polyacrylamide gel without glycerol. The samples were run at 4°C at 5 W for 16 to 20 hours. Finally, the gels were read by UV transillumination after conventional ethidium bromide staining for 45 minutes and washing with distilled water for 15 minutes.

RESULTS

In each case, genomic DNA was obtained from the entire specimen and from the upper (papillary) dermal lymphoid infiltrate after gross microdissection. The PCR amplification was performed in all cases with the 4 primer pairs together in a multiplex reaction, and a PCR product of the expected size, appearing as a sharp band of slightly variable width, was obtained in all cases.

Heteroduplex analysis revealed a smear without any monoclonal band for peripheral blood mononuclear cells and lichen planus samples with and without microdissection for the lichen planus specimens, a pattern consistent with a polyclonal nature. Conversely, a clear monoclonal band without background smear was present for DNA extracted from Jurkat and Mac 2A T cells, as expected. In MF cases, a monoclonal band usually associated with a faint background smear was obtained in 16 of 29 cases for entire specimens, with slight size differences between the patients. Using DNA from the upper dermal infiltrate only, the same bands were obtained in all 16 previously positive cases, and a monoclonal band was visible in an additional 8 cases. Overall, a monoclonal pattern was obtained in 55% (16/29) of MF cases without microdissection, and in 83% (24/29) with microdissection, for a gain of clonal detection of 51%.

No correlation could be established between the pattern of the band obtained on 2% agarose gel and the detection of a monoclonal band with heteroduplex analysis. On the other hand, the microdissection procedure did not influence this pattern.

The enhancement of detection of T-cell clone by the described gross microdissection procedure did not seem to depend on whether the tissues were previously frozen or formalin-fixed and paraffin-embedded. Indeed, the relative gain of detection of a clone was 44% and 55% for frozen and paraffin-embedded tissues, respectively, as detailed in Table 2. Conversely, the gain of sensitivity was higher in early-stage than in more advanced-stage diseases. More precisely, the percentage of gain of clonality detection was 70% (7/16 vs 12/16) in patch-stage and 33% (9/27 vs 12/13) in plaque-stage disease.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Without Microdissection</th>
<th>With Microdissection</th>
<th>Gain of Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>7/13 (54%)</td>
<td>11/13 (84%)</td>
<td>55%</td>
</tr>
<tr>
<td>Paraffin-embedded</td>
<td>9/16 (56%)</td>
<td>13/16 (81%)</td>
<td>44%</td>
</tr>
</tbody>
</table>

COMMENT

This study investigated the possibility that an easy-to-perform hand microdissection procedure carried out on the superficial (papillary) dermis in tissue slides from cutaneous lesions of MF could improve the rate of positive search of a dominant T-cell clone using a PCR amplification and heteroduplex analysis method applied to the TCRα chain gene. In a series of 29 histologically definitive MF cases, we demonstrated that this procedure increases the sensitivity of detection of clonal T cells by approximately 50%, in fixed, paraffin-embedded sections and in frozen samples. Conversely, the same method showed a consistent polyclonal pattern in benign lymphoid infiltrates (lichen planus).

In prior studies, detection of T-cell clonality by PCR-based methods in cutaneous T-cell infiltrates of ambiguous nature provided variable results according to the stage of the disease and the method used. Heteroduplex analysis without microdissection has been reported to detect the presence of a T-cell clone in 40% to more than 85% of CTCLs, and our rate of 57% without microdissection is consistent with these previous data. The migration pattern of PCR products on nondenaturing polyacrylamide gels and more complex methods such as denaturing or temperature gradient gel electrophoresis, single-strand conformation polymorphism, and fragment analysis are more sensitive, with rates of detection between 66% and 90%, a variability likely to be at least partially related to inclusion of lesions from different stages of the disease.

The microdissection procedure we used, allowing the collection of the immediate subepidermal area, is based on the hypothesis that the ratio between neoplastic clonal cells and reactive normal lymphocytes was perhaps more important in this area and that the detection of a T-cell clone would be easier when using DNA originating exclusively from this zone. Indeed, reactive cells might be more numerous in the dermal infiltrate than previously thought, and they might be responsible for dilution of tumor-specific DNA below the threshold of clonal detection. These reactive cells are most likely scattered throughout the dermal infiltrate, as opposed to neoplastic cells, which are usually concentrated in the upper papillary dermis. Furthermore, the substantial background of the DNA-rich epidermis is almost totally removed by our method; this may be important because it cannot be ruled out that keratinocyte DNA, although theoretically not amplified by rearranged TCRγ gene–specific primers, may somehow interfere with subsequent enzymatic
reaction in a way that cannot be predicted. Overall, experimental findings support this working hypothesis and confirm that PCR-heteroduplex analysis may be rendered more sensitive by microdissection of the subepidermal area. It must be pointed out that some sensitivity was probably lost by using a simple consensus primer, but this did not invalidate the results because the samples were analyzed in a comparative way.

Conversely, it must be clearly emphasized that the experimental protocol we used was not designed to give further insight into the main location of the neoplastic clone in MF lesions, which is still a matter of debate especially in early stages (epidermis only vs dermis only vs both). Interestingly, however, the results do not seem to support the hypothesis that the clone may be confined to the epidermis; since removal of epidermal DNA did not prevent the detection of a clonal T-cell population. The hypothesis that neoplastic cells are present in the epidermis alone has been mainly based on indirect data (immunohistochemical techniques and analysis of the cytokine profile) or on PCR results without further analysis of the amplified fragments, which are not always reliable indicators of clonality. Furthermore, it has been recently demonstrated by a very elegant but difficult to implement and time-consuming procedure of laser-assisted single-cell microdissection of cutaneous samples of 5 MF samples that all epidermal lymphocytes were clonal, whereas the dermis actually contains neoplastic cells, although in the minority, admixed with a majority of nonclonal reactive cells.

Our results do not fully contradict these latter data, since it is conceivable that the detection of dermal clonal T cells, although not in the majority, is still improved by an initial hand microdissection even if all epidermal T cells, often scarce when present, are clonal. Moreover, our work cannot be compared with the studies by Cerroni et al and Gellrich et al, who used very elaborate methods even on a semiroutine basis. The procedure we developed is clearly not meant to replace laser microdissection but instead to offer a feasible, easy-to-perform method that can be used routinely in standard pathology laboratories. It obviously remains to investigate whether this procedure can help to better identify the nature of the infiltrate in more ambiguous cases, an investigation that will represent a second-step study requiring a higher number of adequate controls.

Interestingly, we found a tendency toward a higher gain in sensitivity of clonal detection in early patch-stage disease than in more advanced diseases, even though no statistical analysis was performed owing to the small sample size. This tendency was not unexpected because the detection of a dominant T-cell clone in skin lesions is more frequently reported in advanced diseases than in early stages, rendering the gain of sensitivity more difficult to appreciate when a microdissection procedure is used; this gain of sensitivity is more obvious in early stages of CTCL. However, this apparent tendency will have to be confirmed by further studies.

In summary, the present study reports the value of performing hand microdissection of the subepidermal area on a semiroutine basis to detect T-cell monoclonality in MF, especially in early patch-stage lesions. Moreover, it might also allow convenient access to key genes to better understand the pathogenesis of MF.

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Dr Levi is a fellow of the Lymphoma Research Foundation of America.

Corresponding author and reprints: Olivier Dereure, MD, PhD, Department of Dermatology, Hopital Saint-Eloi, University Hospital of Montpellier, 80 ave Augustin Fliche, 34295 Montpellier Cedex 05, France (e-mail: o-dereure@chu-montpellier.fr).

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