Histopathologic–molecular Correlation in Early Mycosis Fungoides Using T-cell Receptor γ Gene Rearrangement by Polymerase Chain Reaction with Laser Capture Microdissection

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Background/Purpose: Early mycosis fungoides (MF) is difficult to distinguish from other benign inflammatory dermatoses. We evaluated clonal T-cell receptor (TCR) γ gene rearrangement by polymerase chain reaction (PCR) as a surrogate to histologic diagnosis in early MF.

Methods: Twenty paraffin-embedded skin biopsies from nine patients diagnosed with MF were included. Two multiplex PCR encompassing various Vγ and Jγ regions were used to detect TCRγ gene rearrangements. Histologic diagnoses were categorized as “diagnostic”, “consistent”, “suggestive”, or “nondiagnostic”. We compared TCRγ PCR results with histologic parameters to determine the differences between PCR-positive and PCR-negative groups.

Results: TCRγ PCR was positive in 53% (8/15) of the patch stage, in 100% (2/2) of the plaque stage, and in 100% (3/3) of the tumor stage. TCRγ PCR was positive in 50% (4/8) of the specimens in both the diagnostic and consistent of MF groups, 71% (5/7) in the suggestive of MF group. We found that inflammation was more severe in PCR-negative specimens. Papillary dermal fibrosis was common, and differed significantly between PCR-positive and PCR-negative groups (p < 0.01). T-cell monoclonality was detected in one nondiagnostic lesion in a patient with psoriasis and MF.

Conclusion: TCRγ PCR allows the diagnosis of MF in patients with lymphocyte-poor lesions, suggestive of MF pathologically. TCRγ PCR is more likely to be negative with moderate to severe inflammation, particularly with papillary dermal fibrosis. We suggest that the ratio of malignant clonal to reactive T-cells is critical for MF diagnosis. [J Formos Med Assoc 2007;106(4):265–272]

Key Words: laser capture microdissection, mycosis fungoides, polymerase chain reaction, T-cell receptor γ gene rearrangement

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma; however its incidence is much lower in eastern than in western countries. Only one clinicopathologic study of MF has been reported in Taiwan.1 The lesions of MF can be divided into patch, plaque, and tumor stages as disease progresses. Various histologic criteria have been proposed for the diagnosis of MF.

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Received: May 17, 2006
Revised: October 19, 2006
Accepted: December 5, 2006
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the patch stage of MF; however, these features appear more common in the later plaque lesions. The histopathologic features in the early patch stage of MF are often nonspecific and nondiagnostic, and can assume nearly every histopathologic pattern of inflammatory skin diseases. The diagnosis of early stage MF is controversial and difficult for both pathologists and dermatologists.

MF is a disease of chronic antigen stimulation in which an initial inflammatory response in the epidermis leads to T-cell proliferation and, finally, to the emergency of a malignant T-cell clone. The detection of monoclonal T-cell proliferation in MF by T-cell receptor (TCR) gene rearrangement studies has enhanced diagnostic sensitivity. A dominant monoclonal T-cell population can be detected in 76–100% of patients with advanced-stage MF. In patients with an earlier stage MF, however, the detection rate is lower despite the use of various highly sensitive molecular techniques. TCR gene rearrangement studies are of relatively less importance when the histopathologic diagnosis of MF is conclusive. However, in the early stage of the disease, in which the histopathologic diagnosis is uncertain, molecular studies are particularly important as an adjunct to diagnosis.

The purpose of this study was to evaluate TCRy gene rearrangement by polymerase chain reaction (PCR) as an adjunct to the histologic diagnosis of MF, especially in early-stage MF. The results of TCRy PCR were compared with histologic parameters in an attempt to find histologic differences between the PCR-positive and PCR-negative groups. One of the aims of this study was to further define the subgroup of early-stage MF in which T-cell monoclonality is more easily detected.

Materials and Methods

Skin biopsy specimens from patients diagnosed with MF were retrospectively collected from the Department of Dermatology of National Taiwan University Hospital from January 1996 to December 2003. After excluding uninterpretable and poor quality slides, 20 formalin-fixed, paraffin-embedded specimens from nine patients with confirmed diagnosis of MF were included in the study. Demographic data, histopathologic findings, and treatment results were collected.

Positive control samples included lymph node tissue biopsies from a patient diagnosed with T-cell lymphoma, and a Jurkat T-cell line. A tissue biopsy of a reactive lymphoid hyperplasia served as a negative control throughout the assay.

DNA extraction and PCR

Four to five 8 μm sections were cut from each paraffin-embedded block and placed into an Eppendorf tube. The tissue sections were deparaffinized and digested with proteinase K overnight at 56°C. The DNA was then extracted using a QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA) following the manufacturer’s instructions. As an internal control for monitoring successful DNA extraction, β-globin was amplified by using primers GH21 and PC03 to yield a 250-bp product.

The two multiplex PCR reactions were performed using a method described previously. The primers used in Mix 1 PCR included a consensus primer Vγ1 cons 5′-CTG-GTA-CC-TACCA-GGA-GGG-GAA-3′ (covering Vγ1–Vγ8) and a mixture of three joining primers (Vγ2S2 [5′-CCT-GTG-ACA-ACG-GAT-TGC-3′], JP [5′-TG-GTC-CGG-GAG-GAA-ATC-CC-A], and JP1/2 [5′-CCA-GGT-GAA-GTG-CTT-ATG-AG-3′]). The primers used in Mix 2 PCR included three variable region family primers (Vγ9 [5′-GAA-AGG-ATC-TCC-GTC-TGC-3′], Vγ10 [5′-GCA-GCA-TGG-GTA-AGA-GAA-GC-3′], and Vγ11 [5′-GAT-TGC-1CA-GGT-GGG-AAG-AC-3′]) and the same three joining primers. Each DNA sample underwent two PCR reactions separately.

The PCR conditions were the same for both Mix 1 and Mix 2. In brief, after initial heating at 93°C for 10 minutes, 35 cycles of PCR were carried out (93°C denaturation for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute), followed by a 10-minute extension at 72°C. For the second round of PCR, 1% of the
first-round PCR product was used, and the same conditions were repeated for another 35 cycles. All of the reactions were performed on a Perkin-
Elmer thermocycler (Gene Amp PCR system 9600). The amplified products were verified on 8% polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light.

Histopathologic findings and statistical analysis
H&E-stained sections from each MF case were reviewed by three physicians (P.F.H., C.H.H., T.E.T.). The histologic parameters for the diagnosis of MF were chosen based on previous studies and are listed in Table 1. The histologic parameters were graded from 0, 1+, 2+ to 3+. Dermal fibrosis was designated as presence or absence. The degree of each histologic parameter was classified into two groups (0/1+ and 2+/3+; absence and presence), and Fisher’s exact test was used to analyze if there was any difference between the PCR-positive and PCR-negative groups. A p value of less than 0.05 was considered statistically significant.

When there was a clear-cut Pautrier’s microabscess, or a number of medium to large-sized hyperconvoluted lymphocytes, the lesion was considered as “diagnostic of MF”. If most criteria were met, but were not sufficient to pronounce a diagnosis of MF, the lesion was referred to as “consistent with MF”. If only one or two of the criteria were met, the lesion was considered to be “suggestive of MF”. When none of the above criteria were met, the lesion was interpreted as “nondiagnostic”. In this group, two specimens from one patient with both psoriasis and MF were histopathologically diagnosed as psoriasis.

Laser capture microdissection (LCM)
One 7 μm section cut from the paraffin-embedded tissue block was stained with HistoGene staining solution (Arcturus). LCM was performed with PixCell II LCM System (Arcturus Engineering Inc., CA, USA). Epidermotropic lymphocytes (Pautrier’s microabscess, basilar lymphocytes) and dermal lymphocytes (perivascular lymphocytic infiltration) were microdissected on different slides, and underwent two multiplex TCR γ PCR as described above.

Results
The clinical manifestations and histopathologic findings of all specimens in each patient are shown in Table 2. The clinical morphology of lesions as well as the frequency of clonal TCR γ gene rearrangement detected in each group is shown in Table 3. There was 50% clonal TCR γ gene rearrangement in the “diagnostic of MF” and “consistent with MF” groups (eight lesions with four PCR-positive rearrangements) (Table 4). The “suggestive of MF” group had 71% of TCR γ gene arrangements (five of seven lesions yielding PCR-positive results) (Table 4). Three tumor lesions were excluded from the histopathologic analysis as they did not fit into the diagnostic criteria used for early MF.
Clear bands around 230 bp and 125 bp were produced in Mix 1 and Mix 2 PCR, respectively (Figure 1). T-cell monoclonality detected in either Mix 1 or Mix 2 PCR was interpreted as positive. Most of the specimens (11/13, 85%) were positive on both Mix 1 and Mix 2 PCR. Only two specimens were positive on Mix 1 PCR alone.

Analysis of each histologic parameter as a discriminator between the PCR-positive and PCR-negative groups was performed using Fisher’s exact test. Differences in histologic parameters between PCR-positive and PCR-negative were not statistically significant, with the exception of the dermal fibrosis which differed significantly between

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**Table 2.** Clinical presentation and histopathologic findings of all specimens in nine patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Clinical findings</th>
<th>Histologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Morphology</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>F</td>
<td>Many reddish patches for 15 yr</td>
<td>Patch</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>M</td>
<td>Many reddish patches for 10 yr</td>
<td>Patch</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>F</td>
<td>Many brownish scaly plaques</td>
<td>Plaque</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>F</td>
<td>Many hypopigmented patches for 6 yr</td>
<td>Patch</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
<td>F</td>
<td>Many reddish macules and plaques</td>
<td>Plaque</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>M</td>
<td>Psoriasis diagnosed 10 yr ago, one tumor on right axilla, many scaly patches</td>
<td>Tumor</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>M</td>
<td>Hypopigmented macules and patches</td>
<td>Patch</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>M</td>
<td>Many reddish macules and patches</td>
<td>Patch</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
<td>M</td>
<td>Many plaques and tumor</td>
<td>Tumor</td>
</tr>
</tbody>
</table>

A = exocytosis; B = Pautrier’s microabscess; C = single basal lymphocytes; D = haloed lymphocytes; E = hyperconvoluted lymphocytes; F = dermal inflammation; G = fibrosis.

**Table 3.** Frequency of clonal T-cell receptor \(\gamma\) gene rearrangement among patch, plaque, and tumor lesions

<table>
<thead>
<tr>
<th>Clinical morphology</th>
<th>No. of lesions</th>
<th>Polymerase chain reaction (+)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patch</td>
<td>15</td>
<td>8</td>
<td>53</td>
</tr>
<tr>
<td>Plaque</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Tumor</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>13</td>
<td>65</td>
</tr>
</tbody>
</table>

**Table 4.** Frequency of clonal T-cell receptor \(\gamma\) gene rearrangement among histologically diagnostic, consistent, suggestive and nondiagnostic groups

<table>
<thead>
<tr>
<th>Histologic diagnosis</th>
<th>No. of lesions</th>
<th>Polymerase chain reaction (+)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Consistent</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Suggestive</td>
<td>7</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>Nondiagnostic</td>
<td>2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>17*</td>
<td>10</td>
<td>59</td>
</tr>
</tbody>
</table>

*Excluded three tumor lesions from analysis.
Table 5. Relationship between polymerase chain reaction (PCR) results and fibrosis or dermal inflammation

<table>
<thead>
<tr>
<th>PCR</th>
<th>Fibrosis</th>
<th>Dermal inflammation</th>
<th>No. of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>3+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1+</td>
<td>1</td>
</tr>
<tr>
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<td>+</td>
<td>0+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>3+</td>
<td>2</td>
</tr>
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<td></td>
<td>–</td>
<td>2+</td>
<td>4</td>
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<tr>
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<td>–</td>
<td>1+</td>
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<tr>
<td></td>
<td>–</td>
<td>0+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>17*</td>
</tr>
</tbody>
</table>

*Excluded three tumor lesions from analysis.

Figure 1. Detection of TCRγ gene rearrangement by Mix 1 PCR amplification (A) or Mix 2 PCR amplification (B) using 8% polyacrylamide gel electrophoresis. Lane (+): positive control (T-cell lymphoma). Lane (−): negative control (reactive lymph node). Lanes 1–7: skin biopsies showing clonal banding patterns. Lanes 5 and 6: skin biopsies with a polyclonal smear. Lane M: molecular weight markers (Bioman).

Figure 2. Detection of TCRγ gene rearrangement on laser capture microdissected specimens by Mix 1 PCR amplification using 8% polyacrylamide gel electrophoresis. Lane M: molecular weight markers (Bioman). Lane 1: microdissected intraepidermal lymphocytes. Lane 2: microdissected dermal lymphocytes. Lane (+): positive control (T-cell lymphoma). Lane (−): negative control (reactive lymph node).

One PCR-negative specimen with severe dermal inflammation from the “diagnostic” group was chosen for LCM. The microdissected intraepidermal lymphocytes were PCR-positive, but the microdissected dermal lymphocytes were PCR-negative (Figure 2).

Discussion

In the present study, TCRγ PCR was positive in 71% of the specimens in the “suggestive of MF” group, 50% of the specimens in the “diagnostic” and “consistent” groups. There is a variability of detection rates in the “suggestive of MF” group in different studies. Several factors accounting for the variability include the different sensitivity of PCR detection methods, the different stage and duration of early lesions, the inclusion of non-specific lesions, and the subjectivity of interpretation by different pathologists. Tok et al.14 also included patients with cutaneous T-cell lymphoma and reported a high detection rate (71%) in the “suggestive” group. However, similar detection rates were also found in both the “diagnostic” (74%) and “nondiagnostic” groups (73%). As the authors suggested, a significant variation of interpretation between the three dermatopathologists may be a confounding factor in this study. Other studies included a more heterogeneous group of patients,15,26 most of whom did not develop MF, which partly explained the low PCR-positive rate (14–16%) in the group not histologically typical.
of MF. A recent large study by Massone et al also collected early lesions from patients with confirmed diagnosis of MF and reported a 53% detection of a monoclonal population of T lymphocytes by PCR. However, no histopathologic subgroup analysis was performed in this study. Although the number of cases in this study was limited, all the patients were diagnosed and treated as MF later in their course, and the follow-up period of these patients was from 1 to 6 years (mean, 3.7 years). Specimens from confirmed MF patients were utilized in the present study and served to strengthen interpretations in spite of the small number of patients. Results of the present study reported a 20% difference between the "suggestive of MF" and the "diagnostic" groups. We further explored the histopathologic findings from the specimens to search for a possible explanation of the results.

After analyzing seven histologic parameters, we found that, although the number of atypical lymphocytes (Pautrier’s microabscess, single basal lymphocytes, haloed lymphocytes, and hyperconvoluted lymphocytes) in the PCR-negative group was higher than that in the PCR-positive group, this does not represent the main difference between the two groups. Instead, in the PCR-negative group, only one specimen displayed a mild degree of dermal inflammation (1+) while the rest of the specimens showed severe dermal inflammation (2+/3+). The importance of dermal inflammation has rarely been addressed. We infer that inflammation leads to elevated levels of reactive lymphocytes, which may influence the sensitivity of the PCR detection by lowering the PCR signals. Two of the three PCR-negative specimens in the "diagnostic" group had severe dermal inflammation (2+/3+), which may partially explain the relatively low rate of PCR-positives in this group. In early, lymphocyte-poor cases of MF, which pose a major diagnostic difficulty, PCR-based techniques for detection of TCR gene rearrangement were more helpful in making a diagnosis since there was less dilution of clonal T-cells. A similar hypothesis was made by Liebman et al, although no further morphologic features were noted in their report to support this theory. The histopathologic analysis performed in the present study supports this theory.

To confirm the above hypothesis, we microdissected one PCR-negative specimen with severe dermal inflammation from the "diagnostic" group using LCM. TCRγ PCR was positive in the microdissected Pautrier’s microabscess from the epidermis, and negative in the microdissected dermal inflammatory cells around the vessels. In the previous study in which LCM was performed, microdissected lymphocytes from dermis were chosen instead of the intraepidermal lymphocytes. Simple laser-beam microdissection to separate the epidermis from the dermis also found a higher detection of clonal T cells in epidermal samples than in dermal samples. Our report was the first to demonstrate this difference in detection with more specific use of LCM. We believe that the ratio of neoplastic to reactive lymphocytes plays a crucial role in the detection of the T-cell monoclonality, as clarified in determining the sensitivity of various PCR-based techniques. In this respect, molecular studies are not very useful when the histopathologic findings show a more severe inflammation, because inflammation dilutes the malignant T-cell clones. Papillary dermal fibrosis is the only histologic parameter, which differs significantly between the PCR-positive and PCR-negative groups (p = 0.01). Dermal fibrosis does not directly influence the detection of clonal T cells, but indirectly reflects the degree of dermal inflammation and disease chronicity.

In a recent study by Ponti et al, the multivariate logistic regression showed that the density and extent of the infiltrate, the degree of epithilomatropism, and the presence of cytologic atypia share an independent predictive value for clonality in early cutaneous T-cell lymphoma. Among these histologic parameters, the density of the cell infiltrate showed a higher odds ratio. In our study, we only included patients with T1 to T2 stage in the analysis and further divided these patients into different pathologic subgroups using histopathologic criteria for MF. Although our results seemed contradictory to this report, we
want to remind that in a subset of early MF patch lesions, especially when clonal T cells are minimal, the dilution of clonal T cells by reactive lymphocytes should be considered when the lesions yield negative PCR results. More similar lesions need to be included for further study in the future.

Acknowledgments

This study was supported by a grant NSC92-2314-B-002-339 from the National Science Council of Taiwan.

References

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