YALE JOURNAL OF BIOLOGY AND MEDICINE 72 (1999), pp. 365-375.

ORIGINAL CONTRIBUTION

Evaluation of T-cell Receptor Gene Rearrangements in Patients with Recurrent Patch/Plaque (T2) CTCL (Mycosis Fungoides)

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Cutaneous T-cell lymphoma is typically a clonal neoplasm of epidermotropic CD4+ T-lymphocytes that includes the entity mycosis fungoides (MF). After identification of patients with recurrent MF treated with total skin electron beam therapy (TSEBT) at the Yale University School of Medicine, this study attempted to compare T-cell receptor (TCR) γ gene rearrangements via polymerase chain reaction (PCR) in both original and recurrent skin biopsies from these patients. Between 1974 and 1996, a total of 95 T2 MF patients were treated with TSEB, and four of these were identified for the study. Slides and tissue samples of both primary and recurrent skin biopsies for each patient were confirmed as being consistent with MF. DNA for PCR was isolated from paraffin-embedded tissue samples. Using consensus primers that hybridize with conserved regions of the TCR gene, these regions of the genome were amplified. The PCR products were then analyzed by acrylamide gel electrophoresis. Of the primary and recurrent samples from four patients with a median disease-free interval (DFI) of 1222 days, only two showed evidence of a dominant TCR clone. A number of factors, including lack of sequence homology between the primers and the gene segments, the existence of multiple neoplastic cell lines, DNA degradation in the archival samples, and the presence of reactive as well as malignant lymphocytes, may have prevented the detection of dominant TCR rearranged clones in the samples. Despite the results of this study, TCR analysis via PCR and gel electrophoresis continues to be of utility in the evaluation of patients with MF when used in conjunction with other diagnostic modalities and in cases with nonspecific clinical, histopathological, and immunophenotyping findings.

INTRODUCTION

Cutaneous T-cell lymphoma (CTCL)[e] is most commonly a clonal malignancy of epidermotropic T-lymphocytes, usually of the CD4+ phenotype, which presents with a spectrum of clinical manifestations including mycosis fungoides (MF) and the Sézary syndrome [1]. CTCL also encompasses the less common clinical entities such as pagetoid reticulosis (Woringer-

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[e] Abbreviations: C, constant; CCR, complete clinical response; CLA, cutaneous lymphoid antigen; CTCL, cutaneous T-cell lymphoma; D, diversity; DFS, disease-free survival; HTLV-1, human lymphotropic virus type-1; J, joining; MF, mycosis fungoides; PCR, polymerase chain reaction; TCR, T-cell receptor; TNM, tumor-node-metastasis; TSEBT, total skin electron beam therapy; V, variable; PUVA, psoralen with ultraviolet A light.
Received: November 17, 1999; Accepted: April 1, 2000.

Kollop disease), suppressor (CD8+) T-cell lymphoma, granulomatous slack skin, peripheral T-cell lymphoma, adult T-cell leukemia/lymphoma, CD30+ large cell lymphoma, and lymphomatoid granulomatosis [2]. Evidence from immunophenotyping and T-cell receptor gene rearrangement studies supports the classification of these dermatoses as forms of CTCL [3].

A relatively rare disease, CTCL has an incidence rate of four per 1,000,000. However, the incidence appears to have increased by a factor of at least 3.2 between the years 1973 and 1984 [4]. Several registries indicate that African-Americans have the highest incidence of CTCL, almost twice that of whites. Males are also twice as likely to be affected as females. Although risk increases with age, with most cases being diagnosed during the fifth and sixth decades of life, one-eighth of all cases are diagnosed in patients below the age of 40, with documented cases in childhood [5, 6]. Approximately 1,000 new cases of CTCL are diagnosed annually in the United States [2]. A recent population-based analysis of nine cancer registries also shows that median survival for MF patients in the study was 9.7 years, with five- and ten-year survival rates of 66 percent and 49 percent, respectively [7].

No clear etiology for CTCL has been identified. Early investigations postulated that chronic antigenic stimulation of lymphocytes in the form of exposure to environmental agents such as industrial chemicals, metals, pesticides, and tobacco predisposed to the development of CTCL by promoting the malignant transformation of affected lymphocytes [8]. These theories, however, have not been supported by recent case-control studies [9, 10]. The possibility of a retroviral etiology also has been considered, due to the association between human lymphotropic virus type-1 (HTLV-1) and T-cell leukemias. Although most patients with CTCL have negative serologies for HTLV-1, viral particles have been isolated from the peripheral blood of some CTCL patients; thus, the role of HTLV-1 in CTCL is still uncertain [11]. An association between CTCL and Epstein-Barr virus has also been reported [12]. Additionally, mutations of the tal-1 and NFKB2/lyt-10 encoded transcription factors have been found in a subset of patients with aggressive disease [3].

The pathological features of CTCL typically correspond to the developmental stage of the lesions. Biopsies from the early stages of disease often show nonspecific changes. The early features include the presence of a sparse lymphocytic infiltrate in the papillary dermis with occasional intraepidermal lymphocytes. With disease progression, lymphocyte atypia becomes evident, and the infiltrate also tends to include other cell types such as eosinophils and plasma cells. Epidermotropism, or the colonization of the epidermis by cerebriform T-lymphocytes, clusters of which are known as Pautrier's microabscesses, are also seen with disease progression [8]. The predominant cell type in CTCL is the CD4+ helper T-cell, which expresses cutaneous lymphoid antigen (CLA), a P-selectin cell surface glycoprotein that binds to E-selectin on endothelial cells in the skin [12]. CLA as well as interferon elaborated by both neoplastic and normal T-cells facilitate the epidermotropism of the tumor cells. With further disease progression, epidermotropism is often lost, and tumor nodules involving the dermis and subdermal structures are observed.

Differentiating CTCL from a benign inflammatory reaction may be difficult. Phenotypically, both may show loss of CD7 and Leu-8 markers while retaining CD3 and CD4 positivity [8]. However, the expression of activation and proliferation antigens (CD25, CD30, HLA-DR or Ki-67) and the loss of pan T-cell antigens suggest neoplastic progression. Monoclonality

can be demonstrated by T-cell receptor gene rearrangement analysis, a process whereby DNA from biopsy specimens is extracted, digested with restriction enzymes, amplified by the polymerase chain reaction, and fractionated on an agarose gel [13, 14]. These techniques have proven to be extremely useful in supporting a diagnosis of CTCL in very early stages when histological changes are often nonspecific [15, 16]. The identification of a T-cell rearrangement signifies the existence of dominant clone, though not necessarily a malignant one. Clonality alone is not sufficient for the diagnosis of malignancy; certain conditions such as Waldenstrom's macroglobulinemia may be caused by a monoclonal lymphocyte population. Hence, evidence from T-cell receptor gene rearrangement studies must be interpreted within the larger context of clinical and pathological findings [8].

The most widely used staging system for CTCL emphasizes the tumor-node-metastasis (TNM) classification (Table 1) [17]. Essentially, the first three phases of disease correspond to T1 or T2 disease, while the tumor phase is T3. Erythroderma is always considered T4 disease. Table 2 shows the stage groupings of MF based on TNM status [17].

The treatment of MF includes a variety of modalities [18-27]. An evaluation of tumor burden, atypia, and immunocompetence is essential before the initiation of therapy, which consists of three broad categories: skin-directed therapy, biological response modifiers, and chemotherapy

Table 1. TNM classification of CTCL[a]

T: Skin[b]	
T0	Clinically and/or histopathologically suspicious lesions
T1	Limited plaques, papules, or eczematous patches covering <10 percent of the skin surface
T2	Generalized plaques, papules, or erythematous patches covering >10 percent of the skin surface
T3	Tumors
T4	Generalized erythroderma
N: Lymph nodes[c]	
N0	No clinically abnormal peripheral lymph nodes, pathology negative for CTCL
N1	Clinically abnormal peripheral lymph nodes, pathology negative for CTCL
N2	No clinically abnormal peripheral lymph nodes, pathology positive for CTCL
N3	Clinically abnormal peripheral lymph nodes, pathology positive for CTCL
M: Visceral organs	
M0	No visceral organ involvement
M1	Visceral involvement (must have pathology confirmation and organ involved should be specified)

[a] The complete classification contains a category in which the number of circulating atypical cells are noted. This category, however, has never been used for the clinical staging and is, therefore, not included here. It has been demonstrated that it is a useful parameter in this classification except in T4 disease [16].
[b] Pathology of T1-4 is diagnostic of CTCL. When more than one T stage exists, both are recorded, and the highest is used for staging (e.g., T4) [3].
[c] Record number of sites of abnormal nodes (e.g., cervical [left and right], etc.).

Table 2. TNM staging of mycosis fungoides.

Stage	T	N	M
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T1-2	N1	M0
IIB	T3	N0-1	M0
III	T4	N0-1	M0
IVA	T1-4	N0-2	M1

[28]. Skin-directed therapies are most effective in early disease and are the first line treatment for T1-T3 stage disease. They include steroids, topical nitrogen mustard (mechlorethamine), carmustine (BCNU), retinoids, phototherapy with ultraviolet A light (PUVA), and radiotherapy, which includes both x-ray and electron beam therapy. Skin-directed therapy may be used alone or concurrently with other therapies. Biological response modifiers target abnormal T-cell function and altered cytokine profiles observed in MF. Extracorporeal photochemotherapy is presently employed for T4 disease, and trials with other agents such as retinoids, cytokines, immunotoxins, and vaccines are under way [28]. Since biological response agents are typically used for refractory or advanced disease, they are often administered in conjunction with skin-directed therapies. Chemotherapeutic agents used in MF include methotrexate, and T-cell specific agents such as adenosine deaminase inhibitors also have been used in advanced disease [28, 29].

Recent studies by Wilson et. al., have identified a subset of T2 MF patients who fail after 2.5 or more years following total skin electron beam therapy [25, 30]. It was postulated that these patients who present with "late failures" are actually suffering from second primary tumors rather than true recurrences, which are usually seen in the patients who fail within two years of initial treatment. Using the database of patients treated at Yale-New Haven Hospital for patch/plaque MF, our study attempts to evaluate whether or not these "late failures" actually represent the persistence of original neoplastic clones or the emergence of second primary neoplastic T-cell clones. By isolating DNA from original and recurrent tumor biopsy specimens and amplifying the T-cell receptor variable regions by PCR, the clonality of the original and recurrent tumors was compared. Ideally, this information will impact upon the clinical management of these patients. If the late failures represent true recurrences, then further research upon more aggressive adjuvant and maintenance therapy would be warranted. If the failures proved to be second primaries, this information would indicate the need to develop modalities to detect them at earlier stages when they are more likely to be amenable to a complete clinical response.

METHODS

Patient population

Between 1974 and 1996, a total of 95 T2 MF patients were evaluated and treated at the Yale University School of Medicine. Of these 95 patients, eight were noted to have recurrences 2.5 or more years after the completion of total skin electron beam therapy (TSEBT). Four of these patients were excluded from the study due to either (1) the unavailability of biopsy material

from the time of diagnosis or recurrence or (2) the fact they may have received radiation therapy from an outside institution prior to initial treatment with electron beam therapy.

All patients were evaluated by members of the Yale University School of Medicine Departments of Dermatology and Therapeutic Radiology. A complete history and physical examination of the skin and lymph node sites were done prior to the initiation of therapy for each patient. Three of the four patients also had abdominal computed tomography and liver spleen scans. None of the four cases exhibited visceral involvement. All of the patients in the study were Caucasian; the median age at diagnosis was 55.8 years, and there was an equal gender distribution in the patient population.

Clinical staging

All patients were staged according to the guidelines of the TNM system for CTCL [17]. None of the patients had nodal nor visceral involvement.

Pathology

Both original and relapse histopathology specimens from each patient were reviewed by members of the Yale University Department of Pathology or the Section of Dermatopathology prior to the initiation of therapy. These specimens were re-examined and the diagnosis confirmed before the gene rearrangement studies were performed. Specimens that exhibited colonization of the epidermis with a lymphocytic infiltrate with atypical nuclei and a superficial band-like lymphoid infiltrate were considered diagnostic of MF. Specimens that exhibited two of these three criteria (epidermotropism, atypical nuclei, or band-like lymphoid infiltrate) were considered to be consistent with MF. All of the samples in the study were either diagnostic of or consistent with MF.

Therapy prior to TSEBT

Only one of the patients had received therapy, consisting of topical steroids, prior to the initiation of TSEBT. Prior treatment did not influence the TSEBT protocol.

TSEBT protocol

Each of the patients in the study were treated with TSEBT delivered by a 6 mEV linear accelerator at a distance of 7 meters to a total dose of 36 Gy over a nine to 12 week period. Patients received treatment to six fields with orthovoltage boosts to the perineum (120 kvp, 1 Gy x 20), soles of feet (120 kvp, 1 Gy x 20), and apical scalp (120 kvp, 2 Gy x 3). External and internal eye shields were used throughout therapy, and the hands and feet were shielded for 50 percent of the course. Therapy was administered and supervised by a member of the Department of Therapeutic Radiology (LW, BK). The most common sequelae of therapy included erythema, pruritis, lower extremity edema, and alopecia.

Assessment of response

Patients were seen in follow-up four to eight weeks after the completion of electron beam therapy. They were scored as having either a complete clinical response (CCR) or a partial response based upon the presence of cutaneous lesions. Each of the four patients in the study had a complete clinical response to therapy by eight weeks after the completion of TSEBT.

Adjuvant therapy

Adjuvant therapy was defined as treatment administered to patients after a CCR. Two of the four patients had adjuvant therapy consisting of six cycles of cyclophosphamide with intravenous doxorubicin on day one of therapy [31]. One patient received PUVA therapy, consisting of the oral administration of 8-methoxypsoralen followed by the exposure of the cutaneous

Table 3. PCR cycling conditions for T-cell receptor gene rearrangement study.

Temperature	Time	No. of cycles
98.4°C	10 minutes	1
94°C 55°C 72°C	0.5 minutes 0.75 minutes 0.75 minutes	34
72°C	10 minutes	1
30°C	1 minute	1

surface to UVA light (320-400 nm) [23, 32, 33]. Treatments were initially two to four times weekly and then tapered to once monthly with the maintenance of a CCR. One patient received adjuvant extracorporeal photopheresis as described by Edelson et. al., in which the patient received 8-methoxypsoralen followed by the removal of blood, pheresis of leukocytes, treatment of leukocytes with ultraviolet light at 25 C, and reinfusion [34]. The interval between ECP treatments was extended as the patient maintained a CCR for greater than six months.

DNA extraction and T-cell receptor gene rearrangement studies

After histopathological confirmation of a diagnosis of MF, DNA was isolated from each of the paraffin-embedded samples using the procedure and reagents of the Easy DNA Kit for Genomic DNA Isolation from Invitrogen™. After microdissection, the tissue samples were washed with xylene, hydrated, and then rinsed again with ethanol. The tissue was then pelleted by microcentrifugation, dried overnight, and resuspended in TE buffer with pH 8.0. The quality and quantity of extracted DNA were assessed by spectrophotometry and gel electrophoresis. Consensus primers that hybridize with conserved sequences in most of the variable and joining regions of the germline genome allow for the determination of clonality. Numerous studies have identified the optimal conditions and the specific primer sequences for this assay. Specifically, the following T-cell receptor primers were used: (1) Beta Chain – Consensus J region 5′AGCAC(GCT)GTG AGCC(GT)GGTG CC3′ Consensus D region 5′CAAAGCTGTAACATTGTG GGGAC3′ (2) Gamma chain – Consensus J region – 5′CGTCGACAACAAGTG TTGTTCCAC3′ Consensus V region – 5′AGGGTGTGTTGGAATCAGG3′ [13, 35]. The presence of amplifiable DNA in the reactions was confirmed by simultaneous amplification of a segment of the beta-globin gene or the HLA gene. The PCR reactions are performed in 30 µl aliquots with 300 ng of sample DNA in 3 µl and 27 µl of reaction mix, which is composed of 0.12 µl of Amplitaq polymerase-gold (Perkin-Elmer) and 26.88 µl of reaction mix. The PCR cycling conditions are listed in Table 3. Two µl of each of the PCR products from the β-globin, immunoglobulin heavy chain, and T-cell receptor β chain reactions were mixed with 4 µl of the loading dye solution and then loaded into the wells of the gel. One µl of the PCR product of T-cell receptor gamma chain reaction was first denatured in 3 µl of stop solution composed of 950 µl formamide, 2 µl 5M NaOH, 25 µl 1 percent Bromophenol blue, 25 µl 1 percent xylene

cyanole. Electrophoresis was performed using a 15 percent acrylamide gel for 75 minutes at 220v. Appropriate molecular weight markers were run in one lane in every gel (AT Biochem Biomarkers EXT Plus). The gels were bathed in distilled water and ethidium bromide (10μg/ml/100ml H_2O) for three minutes. Afterwards, the gels were viewed on an UV light table and recorded using a digital imager system from Alpha Innitech Corporation [13].

RESULTS

Clinical results

Disease-free survival (DFS) was calculated from the end of TSEBT until the time of relapse, which, for each of the patients in the study, was biopsy-documented after examination by the radiation oncologist or the dermatologist. As Table 4 indicates, the median follow-up from the completion of TSEBT for the patients in the study was 3593 days, with a median disease-free interval from the completion of TSEBT was 1222 days. After relapse, two patients received PUVA therapy, one received an additional course of TSEBT, and one received ECP and limited electron beam therapy. Three of the four patients achieved a second CR, while one achieved a partial response. During the course of follow-up, one patient was noted to have a basal cell carcinoma and a squamous cell carcinoma. Another developed a melanoma *in situ* and a basal cell carcinoma. At the time of analysis, the patients had an overall survival of 100 percent.

TCR gene rearrangement study results

The results of the TCR gamma gene rearrangements for both original and recurrent biopsy specimens along with DNA markers are shown (Figure 1). This technique allows for the identification of dominant clones of malignant cells by the presence of distinct bands upon the gel. The presence of a broad smear of bands as opposed to distinct bands indicates the presence of multiple clones. PCR products from original and recurrent biopsy specimens for each patient were run adjacent to each other for comparison of TCR gene rearrangements. Multiple lanes for either original or recurrent biopsies indicate multiple biopsy sites. PCR products in all lanes except for 6 and 7 show a broad smear of multiple bands, indicating the presence of multiple clones. Lane 6 represents the recurrent biopsy for Patient 2, and lane 7 represents the original biopsy of Patient 3. Both of these lanes show a range of polyclonal bands as well as a range of more distinct dominant clonal bands. Thus, the presence of dominant rearranged clones of the TCR gamma chain is only observed in lanes 6 and 7. Both original and recurrent biopsies for patients 1 and 4, the original biopsy for patient 2, and the recurrent biopsy for patient 4 do not exhibit the presence of

Table 4. Patient profile.

Patient No.	Race	Diagnosis	Age at Stage	Sex	DFI (days)	F/U (days)
1	Caucasian	51	T2N0M0	M	1310	3204
2	Caucasian	70	T2N0M0	F	1419	2173
3	Caucasian	69	T2N0M0	F	1027	4134
4	Caucasian	33	T2N0M0	M	1130	4859

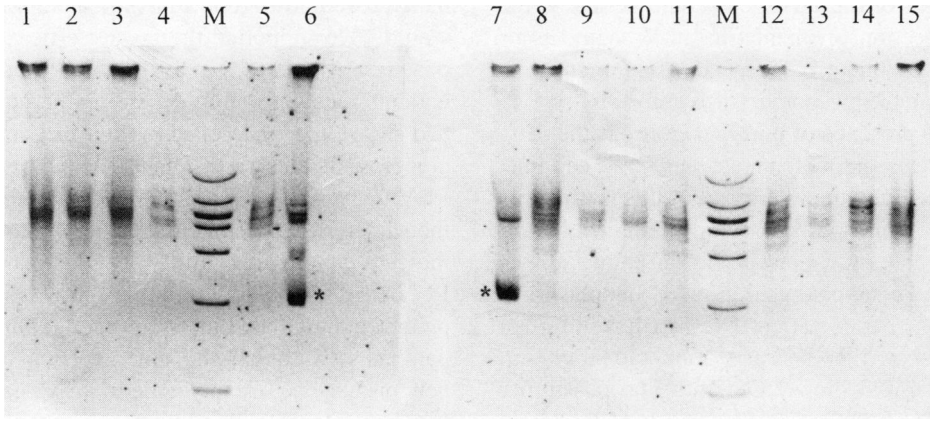
dominant clones of malignant cells. The presence of the reannealed bands identified by the asterisks also indicate the presence of dominant clones in lanes 6 and 7.

DISCUSSION

Molecular analysis of CTCL, specifically MF, has been extremely valuable in its diagnosis, particularly in its ability to distinguish MF from benign lymphoproliferative processes, particularly in the early stages of disease when histopathological findings can be nonspecific. Immunophenotyping, essential to the identification of T-cell lymphomas, may pose problems in the diagnosis of cases with an absence of cell surface markers such as certain immature T-cell lymphomas and in neoplastic infiltrates, which also include significant populations of benign T-cells with phenotypic features of malignant cells. Evaluation of TCR gene rearrangements and the identification of dominant T-cell clonal populations from biopsy specimens using Southern blot analysis have been well documented [36, 37]. The limitations of this technique include the fact that it is very labor intensive, time-consuming, and requires 10^5 to 10^6 cells for a single analysis, a quantity not always available from biopsy specimens [15].

The rationale for evaluating T-cell receptor gene rearrangements in patients with MF rests upon the principle that germline T-lymphocytes undergo rearrangement of their antigen receptor genes, which are composed of variable [V], diversity [D], joining [J], and constant [C] segments, which allows for the great diversity of T-cell receptors. MF is typically characterized by a clonal proliferation of T-cells, indicating that the malignant cells have identical TCR gene rearrangements. Although the process of V, D, and J segment recombination involves the deletion and insertion of certain sequences, particularly at the junctions between segments,

Figure 1. T-cell receptor gamma gene rearrangements of T2 CTCL patients. Identification of lanes.



Patient	Original biopsy	Recurrent biopsy
1	Lanes 1, 2	Lanes 3, 4
2	Lane 5	Lane 6
3	Lanes 7, 8	Lanes 9-11
4	Lanes 12-14	Lane 15

portions of the TCR are highly conserved as well. By using primers for these highly conserved regions of the rearranged segments, PCR allows for the amplification of this portion of the TCR gene. A clonal population of cells should result in DNA fragments that are identical in size, which will appear as distinct bands after gel electrophoresis [38]. A polyclonal population will have multiple TCR gene rearrangements, and, therefore, when the PCR products are run on a polyacrylamide gel, a smear of bands representing DNA fragments of different sizes will appear. This method of evaluation of TCR gene rearrangements has its downfalls as well.

Ideally, the presence of a dominant clone should have been observed in each of the PCR products. A number of factors may have contributed to the fact that all of the lanes other than 6 and 7 demonstrated polyclonal products. The consensus primers designed for this assay have a sensitivity of approximately 90 percent [13]. Thus, there is a small possibility that the assay was not able to detect the TCR rearrangement in selected samples. This lack of sequence homology between the consensus primers and the conserved portions of the TCR gene could be due to the selection of unanticipated V segments or mutation in response to antigen stimulation [35]. Another factor contributing to the presence of multiple clones is the existence of more than one neoplastic cell line. Although the likelihood of this is rare, this phenomenon has been documented. Additionally the presence of multiple clones could indicate a high-grade neoplasm. In the case of patient No. 3, whose original biopsy demonstrates monoclonality and recurrent biopsy does not, the possibility of transformation as a result of treatment must also be considered. The most likely explanation for the inability to demonstrate monoclonality in the PCR products, however, rests on the quality and age of the samples themselves. Seventy-five percent of the original biopsy specimens were greater than 10 years old at the time of analysis. Problems associated with difficulty amplifying archival biopsy material greater than five years old due to DNA degradation has been documented [16]. Furthermore, the presence of reactive lymphocytes, which are far greater in number than the clonal malignant cells, may have prevented the detection of a single neoplastic cell line [39].

If the TCR analysis had indeed been able to establish a dominant clone in each of the samples, a number of conclusions potentially could have been drawn from the data. If, for example, both original and recurrent biopsy samples had resulted in PCR products with identical dominant clones, the analysis would have established that the later tumors represented true recurrences, indicating the persistence of the original neoplastic clone. Although the sample size of the study is quite small due to the stringent inclusion criteria, this information would have supported the need to utilize more effective adjuvant and maintenance therapies in this subset of patients. Additionally, any significant differences observed between patients who had received different adjuvant therapies would have supported the greater efficacy of one treatment. For example, if both patients receiving adjuvant chemotherapy had shown the emergence of their original clones while the two patients who received PUVA and ECP had different clones for their later biopsies, this information could indicate the greater utility of ECP and PUVA as adjuvant therapies for this patient population. Finally, if each of the patients had shown the emergence of a new primary neoplastic clone in his or her recurrent biopsies, this information would have indicated that the therapy received was adequate and could be repeated. More effective surveillance to detect these lesions at early stages when they are most amenable to treatment would also be use-

ful for this patient population.

Despite the results of this study, TCR analysis via PCR and gel electrophoresis continues to be of utility in the evaluation of patients with MF when used in conjunction with other diagnostic methods. Given the ambiguity of the clinical presentation of this disease and the difficulty associated with making a definitive diagnosis, the use of this diagnostic modality may be of particular use in cases with nonspecific clinical, histopathological, and immunophenotyping findings. In this subset of patients who may present with clinical features of MF but with no histopathological nor molecular evidence of disease, TCR gene rearrangement studies via PCR could identify those patients who will need more stringent surveillance and follow-up, since they may be harboring occult neoplasms. Furthermore, in patients with biopsy documentation of recurrent MF, this diagnostic tool, given its cost and indeterminate impact upon therapy, should not be routinely used.

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