TCRγ gene rearrangement analysis in skin samples and peripheral blood of mycosis fungoides patients

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ABSTRACT

Background: Diagnosing *mycosis fungoides* (MF) can be challenging in the early stage of the disease because histopathological features may simulate a variety of benign inflammatory skin diseases. Assessment of T-cell clonality was found to be useful in diagnosis and follow-up of patients.
Objective: In this study, PCR-based TCRγ gene rearrangement analysis was performed in skin and peripheral blood samples of patients with MF treated at the two largest referral centers in Serbia, and the results obtained were correlated with clinical and follow-up data.
Methods: Skin and peripheral blood samples were obtained with informed consent from 37 patients treated at the Department of Dermatology of the Military Medical Academy and the Medical Center of Serbia from 2001 to 2006. The median time of follow-up was 4 years. Multiplex PCR was used for TCRγ gene rearrangement analysis in skin and peripheral blood samples. Clonality results were correlated with the clinical data and disease course data.
Results: Monoclonality was detected in skin samples of 30/37 patients (81%), in 2/5 patients with large-plaque parapsoriasis (LPP), in 28/32 (88%) patients with histologically proven MF, and in 1/16 (6%) patients with benign inflammatory dermatoses. A monoclonal pattern in both skin and peripheral blood was detected in 7/16 (44%) patients in the late stage of the disease, and in 1/7 (14%) patients in the early stage of the disease. A dominant clone was found in both skin and peripheral blood in 1/4 patients in remission, 2/5 with a stable disease, and 4/9 (44%) with disease progression.
Conclusion: TCR-γ gene rearrangement analysis can be regarded as a useful adjunct to diagnosis of epidermotropic lymphoproliferative disorders. The presence of a dominant clone in both the skin and peripheral blood was more frequently detected in late stages and in patients with disease progression, confirming the usefulness of clonality detection by TCR-γ gene rearrangement analysis in follow-up of patients with primary cutaneous T-cell lymphomas.

KEY WORDS

T-cell clonality, TCRγ rearrangement, mycosis fungoides, diagnosis

Introduction

Mycosis fungoides (MF) is a primary cutaneous epidermotropic lymphoma of small- and medium-sized T-cells with cerebriform nuclei, which is characterized by indolent evolution and slow progression of the dis-

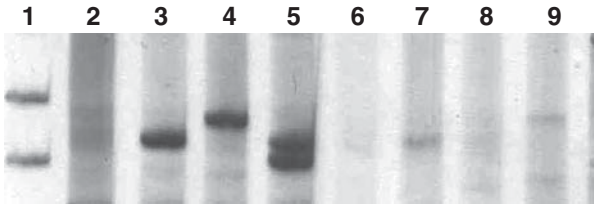

Figure 1. T-cell clonality analysis on 10% PAGE (silver staining). Samples 3, 4, 7, and 9 indicates monoclonality; sample 5 indicates biclonality; samples 2, 6, and 8 indicate polyclonal pattern and column one indicates DNA molecular weight marker.

ease for years and even decades through four stages: patch, plaque, tumor, and visceral involvement (1). It begins with a lightly erythematous macular eruption usually located on the trunk (the patch stage), and evolves into well-demarcated plaques (the plaque stage). Later on, tumors develop on the preexisting plaques or on uninvolved skin and, in certain patients, the disease progresses to erythroderma. Lymph node, peripheral blood, and visceral involvement can be detected in late stages, which is characterized by resistance to therapy and poor prognosis.

The diagnosis of the disease relies on correlation of clinical manifestations and histopathological examination. In early stages, histopathological features may simulate a variety of benign inflammatory skin diseases. This is why diagnosing MF at this stage can pose a challenging problem. A frequent diagnosis at this stage is large-plaque parapsoriasis, which is now recognized as part of the spectrum of primary cutaneous epidermotropic T-lymphoproliferative diseases because 10 to 30% of patients can develop overt MF during follow-up (2). Only in 50% of patients are biopsies diagnostic from the beginning, and in the other half of patients repeated biopsies are needed to establish the diagnosis (3, 4). Because the clinical and histological features can often mimic benign inflammatory skin diseases, the median time to diagnosis of the disease in various studies is around 5 years (2).

Lack of well-established histological (and immunohistochemical) criteria for diagnosing MF led to the development of molecular genetic tests used to detect T-cell clonality. MF (and other primary cutaneous lymphoproliferative disorders) are monoclonal in origin; that is, they develop from a single T-cell, which forms a malignant clone of skin-homing T-cells. The T-cell receptor is a specific marker of that clone because every T-cell bears a unique antigen receptor on its surface. This unique structure of the T-cell receptor on every normal T-cell is formed during the rearrangement of the T-cell receptor gene during lymphocyte development. During this process, various genes from V (variability), D (diversity), and J (joining) gene regions of the T-cell receptor gene are joined and only this unique combination is expressed as the final TCR protein. This final V-D-J (in α and γ chains of TCR) or V-J (in β and δ chains of TCR) structure acquires a unique nucleotide sequence, and its product, which is expressed on the surface, is a specific marker of the T-cell and its clonal progeny (5).

The T-cell receptor rearrangement analysis has been used for detection of clonality in lymphomas for the past 15 years. Southern blot analysis was initially used, but this technique is time-consuming, large skin samples are required, and it has a detection limit of 5 to 10% of clonal subpopulation of cells (5, 6). In recent years, PCR-based methods were developed for TCR gene rearrangement analysis, which is much more sensitive and easier to perform. Rearrangement of the TCRg gene is usually analyzed because it is rearranged early in T-cell development, both in $\alpha/\beta+$ (95% of circulating T cells) and $\gamma\delta+$ (the remaining 5%) T cells. Also, the structure of the TCRγ gene is simple (compared to, e.g., TCRβ), so it is possible to use fewer sets of primers to detect most probably gene rearrangements (5, 6).

In recent studies, detection of T-cell clonality in the

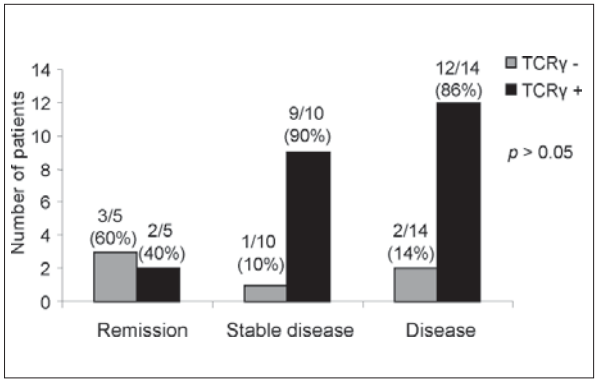

Figure 2. T-cell clonality analysis in skin samples of patients with *mycosis fungoides* and course of disease.

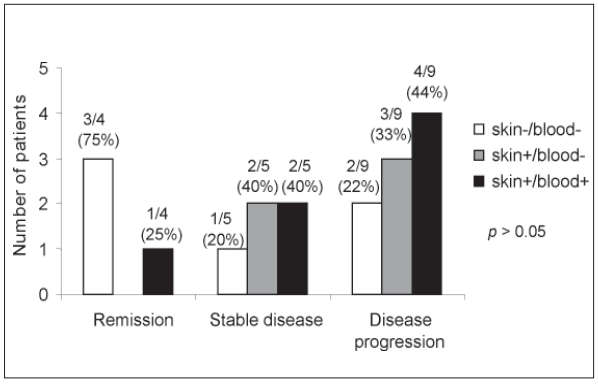

Figure 3. TCR-g gene rearrangement analysis in skin and/or peripheral blood of patients with *mycosis fungoides*.

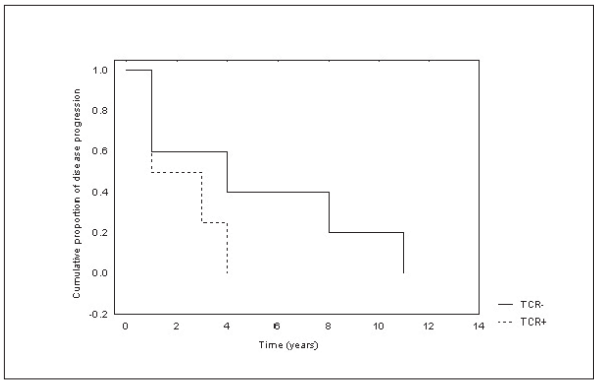

Figure 4. Cumulative proportion of disease progression over time and finding of a dominant clone in skin and/or peripheral blood.

skin was found to be very useful for early detection of MF and related disorders, and it improved classification of cutaneous T- and B-cell lymphoproliferative disorders as well (5, 6). Also, detection of clonality in skin and peripheral blood was found to be an independent prognostic factor in primary cutaneous T-cell lymphoma (7, 8).

This study used PCR-based TCRγ gene rearrangement analysis for detection of clonality of T-cells in skin infiltrates and peripheral blood of patients with primary cutaneous T-cell lymphoproliferative disorders in two dermatological referral centers in Serbia from 2001 to 2006. The data were correlated with clinical data and the course of the disease.

Materials and methods

Patients

Thirty-seven patients were enrolled in the study. The patients were treated at two dermatological referral centers in Serbia – the Department of Dermatology at the Military Medical Academy and the Institute of Dermatovenerology at the Medical Center of Serbia – from 2001 to 2006. Diagnosis of MF was made by correlating clinical, histopathological, and immunohistochemical data according to the WHO-EORTC classification of cutaneous lymphomas (1).

Informed consent was obtained for patients enrolled in the study. There were 11 women and 26 men included in the study, 31 to 81 years old (median 61 years). In 32 patients, diagnosis of MF was established, and 5 patients were diagnosed with large-plaque parapsoriasis. The median duration of follow-up was 4 years (from 1 to 10 years). Seven patients were lost to follow-up. The TNM classification of patients with MF enrolled in the study and classification in the early and late stages of the disease according to prognosis (9) are shown in Table 1.

Patients were treated with standard treatment procedures. In early stages of MF, skin-directed therapy (most commonly photochemotherapy) was used with or without IFN-α, retinoids, and topical corticosteroid creams. In late stages, chemotherapy and radiotherapy with or without IFN-α and photochemotherapy were usually employed. Sixteen patients with various benign inflammatory dermatoses were included in the control group: eczema nummular/generalized (n = 8), idiopathic erythroderma (n = 2), psoriasis (n = 2), lichen simplex chronicus (n = 1), lichen amyloidosus (n = 1), erythema annulare centrifugum (n = 1), and Lyme borreliosis (n = 1).

Samples

From 2001 to 2006, 37 skin and peripheral blood samples were analyzed as well as 16 skin and peripheral blood samples of patients with benign inflammatory dermatoses. The samples were usually taken at the time of diagnosis, or during the relapse of disease after therapy,

Table 1. TNM classification of the patients in the study.

Stage (prognostic)	Clinical stage	TNM classification			Patients(n)	Total
Large-plaque parapsoriasis	–		–		5	5
Early MF	IA	T1	N0	M0	1	9
	IB	T2	N0	M0	3	
	IIA	T1-2	N1	M0	5	
Late MF	IIB	T3	N0-1	M0	13	23
	IIIA	T4	N0	M0	0	
	IIIB	T4	N1	M0	6	
	IVA	T1-4	N2-3	M0	4	
	IVB	T1-4	N0-3	M1	0	
						37

Table 2. Primer sequences for amplification of the TCRγ hypervariable region.

Gene	Region	Nucleotide sequence
TCRγ	*V-J*	5'-C TTC ACT CAG ATG TCA CCT ACA ACT CCA AGG TTG-3'
		5'-C TTC CTG AAG ATG ACG CCT CCA CCG CAA GGG ATG-3'
		5'-C TTC CTG GGA ATG ACT ACC ACA CCT CCA GCG GTT-3'
		5'-C TTC CTG ATG GTA ACT CCT ACA ACT CCA GGG TTG-3'
		5'-GGNA CTG CAG GAA GGC AAT GGC GCA TTC CG-3'
		5'-GGNA AAA CAG GAA AGG AAT CTG GCA TTC CG-3'
		5'-AAG TGT TGT TCC TAC GCC TTT-3'
		5'-AGT TAC TAT TCT CCT AGT CCC-3'
		5'-TGT AAT GAT GGA CTT TGT TCC-3'

with excisional or shave biopsy. One-half of the sample was placed in 10% formaldehyde for further histopathological analysis, and the other half was frozen and stored at -20 °C for DNA extraction and further DNA analyses. The blood samples were taken by venepunction and used for DNA extraction and further analyses.

TCR-γ gene rearrangement analysis

DNA was isolated by phenol:chloroform:isoamil alcohol extraction (10). PCRs were performed in a 50 μL reaction volume containing 400 to 600 ng of gDNA (Master Mix, Applied Biosystems). For amplification of the hypervariable region of the rearranged TCRγ chain gene, 0.2 μM/L of each oligonucleotide primer was used (Table 2).

The PCR protocol included 40 repeats of the basic cycle (94 °C for 40 seconds, 56 °C for 1 minute, 72 °C for 1 minute). The uniformity of rearranged TCRg genes was analyzed on 10% polyacrylamide gel (PAGs) electrophoresis after staining with silver nitrate (11). To ensure that DNA was amplifiable, all samples were amplified with commercial primers for the *P53* exon 4. In order to avoid false-positive results, negative controls containing no template DNA were subjected to the same procedure. PCR products were considered to be monoclonal only if one discrete band within the expected size range (~ 200 bp) was observed on the gel after electrophoresis.

Statistical analysis

Comparisons of categorical data were done by using a χ^2 test or the Yates χ^2 test. The prognostic value of clonality in MF was assessed using survival analysis methods. The entry date of our study was the histological diagnosis of MF. Survival was calculated from the time of diagnosis to the date of death or "lost to follow-up," and disease progression from the time of diagnosis to the date of disease progression through stage. Survival and disease progression curves were calculated using the Kaplan-Meier method. Differences between survival and disease progression curves were calculated by the log-rank test. Statistical analyses were performed using Statistica, software version 6.0 for Microsoft Windows (StatSoft, Tulsa, USA).

Results

Detection of T-cell clonality in skin samples

Monoclonality was detected in the skin samples of 30/37 patients (81%). In patients with large-plaque parapsoriasis (LPP), monoclonality was detected in 2/5 patients. In patients with histologically proven MF, monoclonality was detected in 28/32 patients (88%) (Figs. 1 and 2), but the differences did not reach statistical significance due to the small number of patients with LPP. Monoclonality was detected in 1/16 (6%) skin samples of patients with benign inflammatory dermatoses.

Probably due to the small number of patients in the early stage of disease, no correlation was found between the presence of a dominant clone in the skin and T stage of disease, type of lesion, and clinical stage.

Clonality detection results were further correlated with the follow-up data (Figure 2). In 3/5 patients in remission, a polyclonal pattern was detected in the skin samples, whereas in patients with a stable disease or disease progression a polyclonal pattern was seen in 3/24 (13%) patients, without a statistically significant difference between groups.

Detection of T-cell clonality in peripheral blood samples

In 23 patients with primary cutaneous lymphoproliferative diseases and in 16 patients with benign inflammatory dermatoses TCR-g gene rearrangement analysis was performed in peripheral blood samples. In all patients with benign inflammatory dermatoses, a polyclonal pattern was detected. Based on these results,

patients were divided into three groups: Group 1 = patients with a dominant clone found in both skin and peripheral blood samples, Group 2 = patients with a dominant clone in skin, and no dominant clone found in peripheral blood, and Group 3 = patients with a polyclonal pattern found in both skin and peripheral blood.

A monoclonal pattern in both skin and peripheral blood (group 1) was detected in 7/16 (44%) patients in the late stage of the disease, and in only 1/7 patients in the early stage of the disease. In patients with parapsoriasis (n = 3), a polyclonal pattern was detected in skin and peripheral blood in all patients.

The results of TCR-γ gene rearrangement were correlated with disease course data. A polyclonal pattern in skin and peripheral blood was found in 3/4 patients in remission, in 1/5 patients with a stable disease, and in 2/9 patients with disease progression. A dominant clone was found both in skin and peripheral blood in 1/4 patients in remission, 2/5 with a stable disease, and 4/9 with disease progression (Fig. 3).

In patients with a dominant clone in the skin and peripheral blood, there was a trend toward a shorter time to progression, but the difference was not statistically significant (log-rank test, $p > 0.05$) (Fig. 4).

In relation to survival, no difference was found in the cumulative proportion of survival between patients with a dominant clone in skin and/or peripheral blood and patients with a polyclonal pattern in skin and/or peripheral blood (not shown).

Discussion

In recent years, TCR gene rearrangement analysis has been used in the diagnosis of T-cell lymphomas as well as in the diagnosis of cutaneous T-cell lymphoproliferative disorders. A dominant T-cell clone can be detected using PCR-based methods for TCR-γ gene rearrangement analysis in up to 90% of patients with MF and in up to 50% of patients with large-plaque parapsoriasis. This further indicates that these entities belong to the same disease spectrum (5, 6). A monoclonal pattern can be found in up to 100% of cases in the tumor stage of disease, in 83% of patients with erythrodermic MF, and in 52 to 75% of patients with the patch/plaque stage of disease (7, 11–13).

This study found monoclonality in 40% of patients with large-plaque parapsoriasis, and in 83% of patients with MF. In patients with an early (stage I/IIA) disease, monoclonality was found in 9/9 patients (100%), and in 19/23 (83%) of patients in late stages (IIB and more), which is comparable with the results of other studies. Although previous studies found a correlation between TCR-γ gene rearrangement analysis results and type of lesion (i.e., T stage of disease), this study found no correlation between the T stage of disease (patch/plaque vs. tumor/erythroderma) and clonality detection results. This may be due to the small number of patients in an early stage of the disease as well as the presence of false negative results in later stages of disease. False negative results are possible in cases with poor sampling; that is, taking a skin sample with a small number of malignant T-cells, and the possibility that primers used in these studies do not cover all possible TCR-γ gene rearrangements (5, 6, 14, 15). In addition, in the late stages of disease there is a possibility of TCR gene deletion during malignant transformation (14, 15). It is less possible that previous treatment is responsible for negative results in the late stages of disease. First of all, samples were taken before treatment or in disease relapse during treatment. In addition, in other studies, a dominant clone was preserved in more than 90% of skin samples in patents with clinical and histological remission of disease after treatment with standard therapy (PUVA, electron beam therapy) (12, 16).

Some previous studies have found that monoclonality in skin samples of patients with early MF is an independent factor of poor prognosis; that is, that TCR-γ gene rearrangement analysis can identify patients that have a greater risk for disease progression (7, 17). In this study, in 3/5 of patients in remission a polyclonal pattern was found, whereas, in patients with a stable disease and disease progression, polyclonality was present in only 3/24 (13%) samples studied. However, this difference was not statistically significant due to the small number of patients in remission.

A dominant clone in peripheral blood can be detected in a high percentage of patients with cutaneous T-cell lymphomas, even in the earliest disease (8, 12, 13, 18, 19). The significance of peripheral blood monoclonality is therefore not yet established because it is also found in healthy subjects over 80 years old, as well as in patients with certain autoimmune disorders (12, 13, 18). However, if the dominant clone found in peripheral blood is the same as the dominant clone found in the skin sample (and/or lymph node), it can be taken as a disease manifestation and in this case is an independent factor of poor prognosis (8, 18–20). In this study, the presence of a dominant clone in both skin and peripheral blood was detected in 7/16 (44%) of patients with a late-stage disease, whereas in patients with an early disease it was present in 1/7 patients. In 3 patients with large-plaque parapsoriasis, a polyclonal pattern was detected in both the skin and peripheral blood in all samples studied. In 3/4 of patients with disease remission, a polyclonal pattern was detected, whereas in patients with a stable disease and disease progression, a polyclonal pattern in skin and/or blood was present in only 3/14 patients (21%). These differences, however, were not significant due to the small number of patients in remission. Also, a trend toward a shorter time to progression was found in patients with a dominant clone in both the skin and peripheral blood, in comparison to patients in whom a domi-

nant clone was not found. Although these differences were not statistically significant, they can denote the prognostic importance of a dominant clone in the skin and/or peripheral blood, which was found in other studies (8, 19, 20). In relation to survival, no differences were found between patients with and without a dominant clone in the skin and/or peripheral blood.

In patients with benign inflammatory dermatoses, a polyclonal pattern was found in both skin and peripheral blood in all but one patient with erythroderma, in whom monoclonality was detected in the skin sample. Despite thorough investigation, the cause of erythroderma was not revealed. Pathohistological analysis of the skin sample was consistent with nonspecific dermatitis, and pathohistological analysis of the lymph node revealed dermopathic lymphadenopathy; peripheral blood smear and pathohistological analysis of the blood marrow were normal, with no enlarged chest, abdominal, and pelvic lymph nodes (CT scan). The patient was lost to follow-up. This result could also be a false positive, which is possible in cases in which more than 1% reactive benign lymphocytes are present in the skin infiltrate. In these cases, monoclonality is detected but is not a sign of malignancy. In other studies, monoclonality was detected in 0 to 6% of skin samples from benign inflammatory dermatoses, similar to our series (5–7, 11–13). The finding of non-specific dermatitis and monoclonal pattern found on PCR is called "clonal dermatitis."

Patients with "clonal dermatitis" require close follow-up and repeated biopsies because of a possible underlying lymphoproliferative disorder, not recognized at the start of symptoms and signs of disease (21). Alessi et al. analyzed TCR-γ gene rearrangement in patients with a clinical diagnosis of primary cutaneous lymphoproliferative disease, which was not confirmed by histopathological analysis. Of 29 patients in whom monoclonality was detected in skin samples, a diagnosis of MF was established in 15 after repeated histopathological analysis and correlation with clinical findings (22).

Based on the results of our study, TCR-γ gene rearrangement analysis can be regarded as a useful adjunct to diagnosis of lymphoproliferative disorders of the skin. Also, the presence of a dominant clone in both the skin and in peripheral blood was more frequently detected in late stages and in patients with disease progression. These findings further denote the usefulness of clonality detection by TCR-γ rearrangement analysis in diagnosis and follow-up of patients with primary cutaneous T-cell lymphomas.

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