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Value of TOX Immunoexpression in the Diagnosis of Early Mycosis Fungoides

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Abstract

Background: The lack of a specific marker to differentiate malignant from reactive T-cells makes a definite diagnosis difficult in patients suspected of having mycosis fungoides. This study has evaluated value of thymocyte selection-associated high mobility group box factor expression in the differentiation of early mycosis fungoides from a benign inflammatory dermatosis.

Methods: We selected 22 mycosis fungoides cases, 18 cases suspicious for mycosis fungoides, and 12 cases of eczematous dermatitis from patients who attended Dermatology Clinic, Shiraz University of Medical Sciences (2008-2015). The obtained skin biopsies were immunostained for thymocyte selection-associated high mobility group box factor antigen in addition to pan T cell markers. The slides were evaluated for the percentage of tumor cells and intensity of immunoreactivity.

Results: From 22 cases of mycosis fungoides, 40.9% showed massive infiltration of thymocyte selection-associated high mobility group box factor-positive lymphocytes (>30%) with high (3+) intensity in the epidermis; there was no case negative for thymocyte selection-associated high mobility group box factor expression. Only one eczematous dermatitis case had expression of thymocyte selection-associated high mobility group box factor-positive lymphocytes (>30%) with high intensity and 4 cases were negative for thymocyte selection-associated high mobility group box factor expression. The frequency of thymocyte selection-associated high mobility group box factor expressing lymphocytes was higher in biopsies from mycosis fungoides compared to eczematous dermatitis ($P<0.05$). CD7- cases expressed more thymocyte selection-associated high mobility group box factor-positive lymphocytes in the dermis and epidermis, which were significantly correlated ($P=0.013$).

Conclusion: Thymocyte selection-associated high mobility group box factor, as a positive marker, in combination with pan T cell markers (especially CD7-) can be useful to detect mycosis fungoides malignant lymphoid cells.

Keywords: MF, TOX Immunoexpression, T cell markers, CD7-

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Introduction

Although mycosis fungoides (MF), the most common primary lymphoma of the skin, is predominantly a disease of adults, any age group may be involved.[1] Early MF (eMF) is one of the most difficult diagnostic challenges due to its clinical and histological resemblance to inflammatory processes such as eczema or psoriasis.[2-4] Antigen deficiencies of pan T cell markers such as CD2, CD3, CD5, and CD7 are used to diagnose cutaneous T-cell lymphomas.[5,6] CD7 deficiency may be beneficial in evaluating the nature of dermal infiltration. However, different investigations have shown the presence of CD7 negative cells in benign T-cell infiltrate.[5] T-cell receptor gene rearrangement assay aids in histologic evaluation, although it is not entirely specific or sensitive for MF.[2,7] Although there is clonal expansion of T-cells in MF, non-neoplastic diseases can also show T-cell clonality. Therefore, the diagnosis of MF is based on a combination of clinical and histopathologic features that apply molecular techniques.[1] There is no specific marker that differentiates malignant T-cells from reactive T-cells, which makes it difficult to discern between benign inflammatory dermatitis and eMF.[8] Thymocyte selection-associated high mobility group box factor (TOX) is a DNA-binding factor that is switched off before the exit of CD4+ T-cells from the thymus. It is never expressed in mature cells.[9] Thymocyte selection-associated high mobility group box factor demonstrates a strong, specific ability to label MF cells in eMF skin biopsies by immunohistochemical (IHC) staining, and could be considered a positive marker of eMF.[8] In the current study, we evaluated eMF cases for TOX immunoexpression in addition to pan T-cell markers, which are used in cases suspicious for eMF. We have compared these cases with eczematous dermatitis, as a group of benign inflammatory dermatosis.

Materials and Methods

Patient selection

The study included 22 cases of eMF, 18 cases suspicious for MF, and 12 cases of eczematous dermatitis who referred to the Dermatology Clinic at Shiraz University of Medical Sciences (2008-2015). The diagnosis of eMF was made by considering histologic features of the hematoxylin and eosin (H&E) stained slides. The features included the presence of atypical lymphocytes with hyperchromatic nuclei larger than dermal lymphocyte nuclei that had an irregular nuclear border surrounded by a clear halo and were limited to the basal layer or in both the stratum basale and stratum spinosum layers as single cells (Figure 1) or Pautrier microabscess formation.[10] Dermal lymphocytes appeared to have mostly mature characteristics. Suspicious cases did not fulfill the clinical and histological criteria for eMF. Histopathologic findings of suspicious cases included the presence of mature as well as a few atypical lymphocytes with minimal spongiosis. The clinical impression of those cases included parapsoriasis, eMF, and eczema. All 22 cases of eMF were in the patch stage 1. Clinical findings that included impressions by dermatologists and IHC findings (pan T-cell markers) were also considered. Skin biopsies from 12 patients with eczematous dermatitis were selected as inflammatory dermatosis (non-neoplastic group) for comparison. We performed immunostaining with TOX antigen in all 52 cases of eMf and the non-neoplastic group.

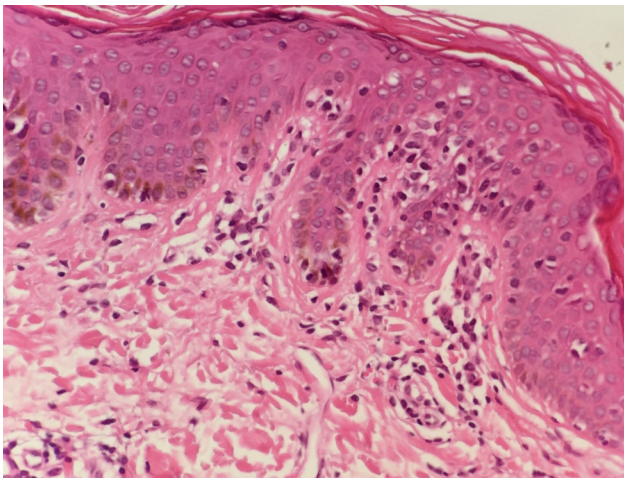

Figure 1. Early mycosis fungoides (eMF) showed epidermotropism of atypical lymphocytes in the basal and spinous layers of epidermis. [Hematoxylin and eosin (H&E), 200×].

Table 1. Frequency and percentage of thymocyte selection-associated high mobility group box factor (TOX) positivity as evaluated by two pathologists.

	Pathologist	<10% n (%)	10%-30% n (%)	>30% n (%)
Epidermis	1	8 (15.4)	6 (11.5)	38 (73.1)
	2	8 (15.4)	11 (21.2)	33 (63.4)
Dermis	1	8 (15.4)	8 (15.4)	36 (69.2)
	2	6 (11.5)	11 (21.2)	35 (67.3)

IHC study

Analysis for TOX was performed on 5-µm-thick tissue sections from formalin-fixed (neutral buffered 10% formalin), paraffin embedded blocks. Unstained sections were collected onto poly-L-lysine coated slides for IHC staining. Unstained slides were incubated in an oven at 60˚C for 30 min, cooled, deparaffinized in xylene (3 times, each for 10 min). The slides were gradually rehydrated in an ethanol series (100%, 96%, 70%, each for 20 s), followed by washing with phosphate-buffered saline for 5 min. The slides were incubated in 3% H_2O_2 for 20 min until there were no visible bubbles on the surface. After the slides were washed in phosphate-buffered saline, we performed the antigen retrieval step in TRIS buffer (pH 9) for 10 min. Next, we used Dako pen and added goat serum diluted in phosphate-buffered saline to 10% concentration for 20 min. In this phase, The slides were incubated with rabbit polyclonal anti-TOX antibody, as the optimal primary antibody, at a dilution of 1:150 (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and washed with phosphate-buffered saline (2 times, each for 5 min). Next, we used the Envision (Dako) kit and washed the slides with phosphate-buffered saline (2 times, each for 5 min), then added 3,3´ diaminobenzidine (DAB) chromogen for 5 min, followed by washing with phosphate-buffered saline for 5 min. Finally, the slides were counterstained with hematoxylin, rinsed in running water for a number of minutes, dehydrated in a graded ethanol solution, cleared with xylene, and mounted.

The slides were separately evaluated by a dermatopathologist and a molecular pathologist for both tumor cell percentage and intensity in the epidermis and dermis. The tumor cell percentage showed the frequency of TOX+ lymphocytes in comparison with CD3+ infiltrating T lymphocytes at a high power field magnification (400×) and was determined to be <10%, 10%-30%, and >30%.[5] The intensity was scored as 0 (negative), w+ (very weak), 1+ (weak), 2+ (moderate), and 3+ (strong). Of note, the pan T-cell markers CD3, CD5, CD7, CD4, CD8, and CD30 as well as B cell markers CD20 and CD43 were previously used to assess the cases suspicious for eMF. The same markers were used for eczematous dermatitis. Finally, we compared the IHC findings with morphological features and clinical data such as differential clinical diagnoses, patient's age, biopsy site, and number of biopsies.

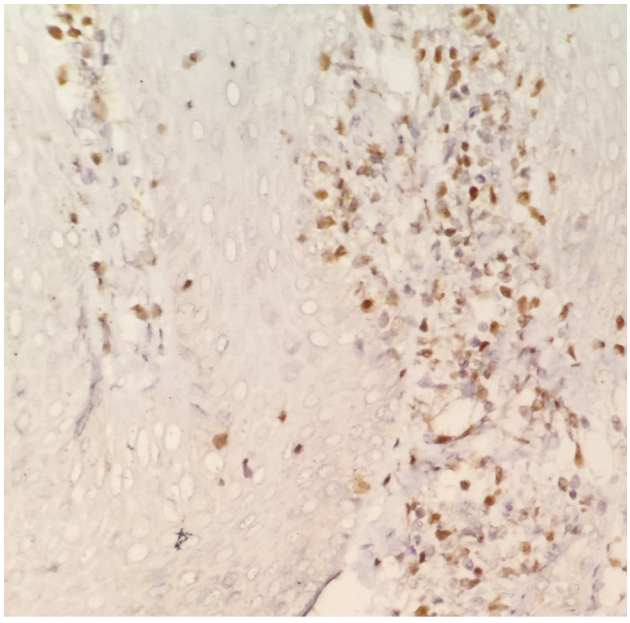

Figure 2. Early mycosis fungoides (eMF), thymocyte selection-associated high mobility group box factor TOX (3+) immunoexpression of lymphocytes in the epidermal and dermal layers (200×).

Table 2. Frequency of thymocyte selection-associated high mobility group box factor (TOX) intensity as evaluated by two pathologists.

	Pathologist	0	W+	1+	2+	3+
		n (%)	n (%)	n (%)	n (%)	n (%)
Epidermis	1	6 (11.5)	0 (0.0)	6 (11.5)	26 (50.0)	14(27.0)
	2	6 (11.5)	1 (1.9)	6 (11.5)	22 (42.3)	17 (32.7)
Dermis	1	6 (11.5)	0 (0.0)	6 (11.5)	25 (48.2)	15 (28.8)
	2	4 (7.7)	3 (5.8)	6 (11.5)	21 (40.4)	8 (34.6)

0: Negative; W+: Very weak; 1+ Weak; 2+ Moderate; 3+: Strong

Statistical analysis

We used SPSS version 17 for data analyses. We applied the t-test, chi-square and Fisher`s exact tests to determine the correlation between different parameters. The diagnostic values (sensitivity, specificity, positive predictive value, and negative predictive value) of TOX immunoreactivity were also calculated.

Results

We categorized the patients into 3 groups: eMF (n=22), suspicious for MF (n=18), and eczematous dermatitis (control, n=12). There was a wide age range of patients in the eMF group (6-64 years) with a mean of 33.4 years. Most patients were 15-45 years old.

Skin biopsies from eMF and suspicious cases varied in number and anatomical site. The number of biopsies (1 to 3) and the most common anatomical biopsy site in the eMF and suspicious groups was the trunk (19.2%). In the eczema group, it was the leg (18.1%). There was no significant correlation between the number of biopsies and final diagnosis of MF (P>0.05). Papillary dermal fibrosis (chicken wire) was seen in 17 (77.2%) cases from the eMF group, but only 4 (22.2%) of the suspicious group. There was a significant correlation between the presence of this feature and the diagnosis of MF (P=0.001).

Clinical findings and impression by the dermatologists were considered as diagnostic criteria for eMF. From 40 cases of the eMF and suspicious groups, 6 (15%) cases were diagnosed with eMF as the exclusive clinical diagnosis and 28 (70%) with ≥2 differential diagnoses, of which MF was either the first, second, or third differential diagnosis. These 34 (85%) cases were positive for clinical criteria. There was no definite association between the number of differential diagnoses and final diagnosis of MF (Fisher's exact test = 0.565). There was no correlation between the histologic diagnosis of MF and the number of clinical differential diagnoses (Fisher's exact test = 0.08). The existence of MF as the first differential diagnosis also did not affect the results.

Immunohistochemical findings were another diagnostic criterion for eMF. From 22 cases of eMF, 90.9% were CD7- CD5+; one (4.55%) case was negative for both CD7 and CD5; one (4.55%) was positive for both markers. From 18 cases suspicious for eMF, 66.7% were CD7- CD5+; one (5.5%) was CD7- CD5-; and 5 (27.8%) were positive for both antigens. In the eczematous dermatitis group, only one (8.3%) out of 12 cases was CD7- CD5+.

CD4 and CD8 markers had different frequencies between the cases. In the eMF group, 22.7% were CD4+ CD8- in both the dermis and epidermis and 4.5% were CD4- CD8+ in the epidermis. The average CD4/CD8 lymphocyte

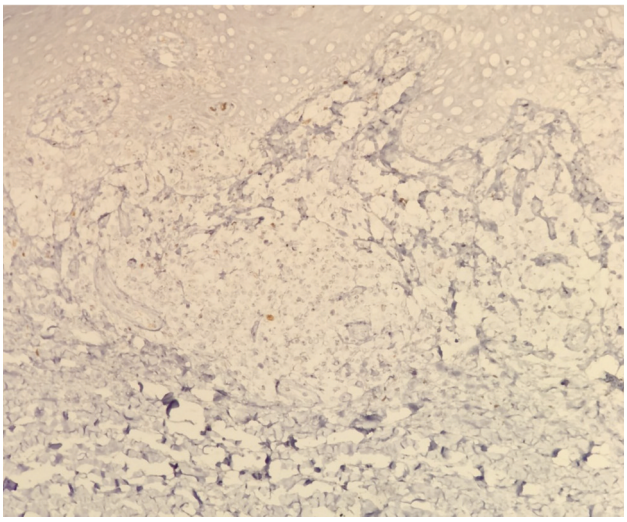

Figure 3. Eczematous dermatitis, negative for thymocyte selection-associated high mobility group box factor (TOX) immunoexpression in the dermal and epidermal layers (200×).

Table 3. The t-test for equality of means for thymocyte selection-associated high mobility group box factor (TOX) expression.

Histological diagnosis	Pathologist 1				Pathologist 2			
	Dermis		Epidermis		Dermis		Epidermis	
	Mean (%)	*P*- value	Mean (%)	*P*- value	Mean (%)	*P*- value	Mean (%)	*P*- value
eMF* or suspicious for MF	58.1	0.004	61.3	0.01	55.1	0.037	55.7	0.012
Not MF	31.9		35.7		35.3		30.7	

*eMF: Early mycosis fungoides

ratio in the epidermis was 1.5 in this group. In the suspicious group, 50% were CD4+ CD8-; 11.1% were CD4- CD8-; and 5.5% were CD4- CD8+ in the epidermis. The average CD4/CD8 lymphocytes ratio in epidermis was 1.7. In the eczematous group this ratio was 1.4 in the epidermis.

None of the groups showed any correlation between the ratio of CD4/CD8 lymphocytes in the epidermis and TOX positivity in the epidermis. The eMF and suspicious groups had a *P*-value = 0.49 and the eczema group had a *P*-value=0.71. In the eMF group, 4 (22.2%) cases were positive for CD30, 14 (77.7%) were negative, and 4 cases had no report for this marker.

IHC expression of TOX

We assessed the percentage and intensity of TOX+ lymphocytes in comparison with CD3+ infiltrating T-lymphocytes at high power field magnification. TOX+ lymphocytes showed nuclear staining. The results are shown in tables 1 and 2.

Both pathologists confirmed TOX expression in the epidermis (kappa index=0.685) and dermis (kappa index=0.642).

There was a significant mean difference for TOX expression in the dermis and epidermis between cases of eMF or suspicious for MF and eczematous dermatitis. A significant correlation existed between histologic diagnosis and TOX expression (Table 3).

TOX expression in the eMF group showed massive infiltration (>30%) with high (3+) intensity of TOX+ lymphocytes in 9 (40.9%) cases in the epidermis as reported by both pathologists (Figure 2). There was no case negative for TOX expression, while in the suspicious group 4 cases (22.2%) were negative for TOX expression. Only one (8.3%) out of 12 cases with eczematous dermatitis expressed TOX+ lymphocytes (>30%) with high (3+) intensity and there were 4 cases that did not express TOX (Figure 3). These findings showed that the frequency of TOX expressing lymphocytes was higher in the eMF group compared to the eczematous dermatitis group ($P<0.05$). Expression of TOX in the epidermis had a sensitivity of 72.5% and specificity of 82.5%, whereas the sensitivity in the dermis was 83.3% with a specificity of 75%. The positive predictive value for TOX expression in the epidermis was 93.5% with a negative predictive value of 91.7%. Positive predictive value for TOX expression in the dermis was 47.6% and the negative predictive value was 56.3%.

Due to complete agreement between the two pathologists, we performed statistical analysis on the results reported by one of the pathologists. The most common negative antigen in the MF group was CD7. We evaluated the correlation between CD7 (negative marker) and TOX (positive marker) expression (Table 4).

The cases without CD7 expression showed more TOX+ lymphocytes in the dermis and epidermis. There was a significant correlation between these two markers ($P=0.013$).

Discussion

Early MF cannot be diagnosed with certainty due to its resemblance to benign inflammatory dermatitis and lack of a specific molecular marker.[11] This causes difficulty in the management of patients suspected to have MF.[8] Although a

Table 4. Correlation between CD7 and thymocyte selection-associated high mobility group box factor (TOX) expression in the dermis and epidermis.

CD7	TOX+ lymphocytes in the dermis and epidermis		Total
	<30% n (%)	≥30% n (%)	
Positive	10 (19.6)	8 (15.7)	18 (35.3)
Negative	7 (13.7)	26 (51.0)	33 (64.7)

scoring approach is available to evaluate eMF, it greatly depends on the pathologist's experience.[2,8] Massone and colleagues reported the presence of a Pautrier microabscess in 19% of MF cases and atypical lymphocytes in 9% in a large histology study. Epidermotropism, a pathognomonic phenomenon in MF, was completely missed in 4% of the cases.[8,12] Thus, finding a specific marker would be of tremendous benefit for the diagnosis of MF, particularly during the early stages of this disease when there are few numbers of malignant cells.[8] Recently, TOX was reported to be a useful molecular marker for histological diagnosis of eMF.[8,11,13] This DNA-binding protein plays an important regulatory role in CD4+ T-cell development and is aberrantly overexpressed in the majority of MF patients.[11]

In the current study, we observed that both eMF and suspicious biopsy samples had a higher frequency of TOX-expressing atypical T-cells compared to eczematous samples. Most showed massive infiltration of TOX+ cells (>30% of infiltrating CD3+ lymphocytes) and were highly (3+) immunoreactive. The results of our study agreed with recent studies. An earlier study by Zhang et al. on 21 biopsies from eMF patients, 15 biopsies from benign inflammatory disease, and 21 samples from normal skin showed a marked increase of cells with TOX staining as well as bright diffuse nuclear staining in the eMF biopsies.[8] Morimura et al. performed TOX IHC staining on 56 cases of various cutaneous lymphomas, including MF, along with 4 cases of atopic dermatitis and 10 cases with normal skin. They found high specific nuclear staining of TOX in MF, Sezary syndrome, and peripheral T-cell lymphoma tissues compared to normal skin. In this study, CD4+ atypical lymphocytes in the epidermis were positive for TOX.[9]

McGirt et al. performed quantitative real-time PCR and IHC studies on bisected biopsies from 28 MF patients, 6 cases of benign inflammatory disease, and 7 normal skin biopsies (control group). They found that inhibition of miR-23 (small non-coding RNA) in MF led to overexpressed TOX protein as a target of this RNA.[13] Huang et al., in a multi-center study, examined TOX mRNA expression in 113 MF biopsies. They observed that MF biopsies had higher expressions of TOX mRNA than the controls in both cohorts. Thus, TOX overexpression differentiated MF from the controls. Finally, they reported that TOX might be a useful marker in MF cases for better diagnosis and prognosis.[11]

The CD4+/CD8+ ratio of intraepidermal T-cells was 1.5 in the MF group, 1.7 in the group suspicious for MF, and 1.4 in the eczematous group. However, this ratio was more than 4 in the study conducted by Furmanczyk et al. They reported a low sensitivity and high specificity for this ratio. However, the cut-off value varied between different studies.[2,14] Another study compared the CD4:CD8 ratio in patch-stage MF versus inflammatory mimics. They selected 20 cases each of eMF and inflammatory dermatoses. The average CD4:CD8 ratio was 4.2 (range: 1-16.8) in the MF cases and 0.9 (range: 0.43-5) in inflammatory diseases. They concluded that an elevated CD4:CD8 ratio favored MF. However, an overlap existed in the lower range with pityriasis lichenoides chronica.[15]

Florell et al. reported a ratio of 3.5 in MF and 1-1.6 in benign/suspicious infiltrates. They concluded that high ratios were specific for MF, but showed poor sensitivity.[14]

According to the diagnostic algorithm by Pimpinelli et al. and a study by Furmanczyk et al.,

the loss of at least one T-cell antigen could be considered one point for IHC criteria.[2,7] Classically, the atypical lymphocytes in MF are CD3/CD4 + with loss of CD7 and less frequent loss of CD5.[16] In our study, we have observed that one (4.7%) out of 22 MF cases was negative for both CD7 and CD5, 19(90.4%) were (CD7-CD5+), and one (4.7%) was double positive for these antigens. CD7 loss was more frequent in the MF group (90.9%) compared to the eczematous group (8.3%). These results agreed with those reported by Cotta et al. who evaluated 31 cases of MF diagnosed with clinicopathologic correlation and follow-up and 11 cases with benign dermatoses. The MF group had significantly lower immunolabeling of CD7.[17] Furmanczyk et al. found that the loss of CD7 in epidermal lymphocytes varied in degree of MF compared to inflammatory dermatoses.[2] Another study showed CD7 expression in approximately 90% of CD4+ T-cells, but was often deficient in malignant T-cells. Thus, CD7 could be used to evaluate the nature of dermal lymphoid infiltrates.[18] Previously, over a 30-month period, we studied 15 eMF cases, 12 cases suspicious for MF, and 15 patients with benign inflammatory dermatosis. All patients in the eMF group lacked CD7 expression in epidermotropic mycosis cells. Low CD7 expression (CD7 expression in less than 10% of lymphocytes) had a sensitivity of 75%, positive predictive value of 100%, specificity of 100%, and negative predictive value of 83.3% for the diagnosis of the patch stage of MF. The age range of eMF cases was 6-64 years with a mean age of 33.4 years in this study, which was lower than other studies. Participants in a previous study in our center had an age range of 22-75 years.[19] A retrospective study on Iranian MF patients in the Department of Dermatology, University Hospital of Isfahan, Iran between 2003 and 2013 included 86 patients with clinical and histologic diagnoses of MF. There was a male: female ratio of 1:1.2. Patients were between 7 and 84 years of age (median: 41 years). They showed major differences in epidemiologic characteristics of MF in Iran with a lack of male predominance and lower age of patients at the time of diagnosis.[20] The study limitation was the low number of MF cases.

Conclusion

Our study has shown that TOX immunostaining is a useful technique to detect MF cells in skin biopsies in combination with pan T-cell markers as diagnostic criteria to differentiate eMF from eczematous dermatitis. It could be helpful to make a conclusive diagnosis of MF as soon as possible followed by timely and proper medical approaches. We suggest a larger study with more cases and follow-up examinations for better evaluation and determination of diagnostic and prognostic value of this marker in MF and cases suspicious for MF.

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Conflict of Interest

None declared.

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