THE VALUE OF TOX AS A DIAGNOSTIC MARKER FOR MYCOSIS FUNGOIDES (MF)

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ABSTRACT

Background/Aim: Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma (CTCL). Differentiation of MF from its benign mimickers is important to ensure proper management. TOX is a critical regulator of early T-cell development that is considered as a useful marker for MF diagnosis and prognosis. The current study aimed to test the role of TOX immunohistochemistry (IHC) as a diagnostic marker of MF differentiating it from its benign mimickers.

Materials and Methods: This work was carried out on 103 skin biopsies, 51 MF cases and 52 inflammatory mimickers. The diagnoses were established after clinicopathologic correlation. IHC was done in MF cases for TOX, CD4, CD8 and CD7 (in TOX negative cases), while the other mimickers stained only by TOX.

Results: TOX expression showed a significant positive expression in MF cases compared to inflammatory dermatoses, with 62% sensitivity and 100% specificity. There was positive correlation between TOX staining and CD4/CD8 ratio in MF cases. In addition, TOX positivity in plaque MF was stronger than in patch MF. Also, TOX negative MF cases were negative for CD7.

Conclusions: Positive TOX expression favors MF, but its negativity doesn't exclude the diagnosis of MF. Combining TOX with CD4/CD8 ratio and CD7 may be useful in diagnosis of MF beside the clinical and histopathological diagnosis. The stronger TOX positivity in plaque more than patch MF may correlate with the progression of the disease.

Key Words: TOX, mycosis fungoides, cutaneous T cell lymphoma, marker.

INTRODUCTION

Mycosis fungoides (MF) is the most common cutaneous T-cell lymphoma (CTCL), accounting for nearly 50% of all primary cutaneous lymphomas according to World Health Organization - European Organization for the Research and Treatment of Cancer (WHO- EORTC) 2005 classification of cutaneous lymphomas **[1]**.

It is a chronic and often indolent primary CTCL in which a malignant clone of CD4+T helper cells expressing a memory cell phenotype infiltrates the skin **[2]**. Clinically, early stages of MF present as flat erythematous skin patches whereas in the later stages MF cells gradually form plaques or tumors **[3]**.

Diagnostic histopathology of MF depends on identification of epidermotropism of cytologically atypical T-cells both singly and in small collections (Pautrier microabscesses) with little spongiosis and/or a band-like dermal infiltrate of cytologically atypical T-cells **[4]**. (Olsen et al., 2007).

Diagnosis of MF depends on correlating clinical criteria with routine histopathology, according to International Society for Cutaneous Lymphoma (ISCL) Algorithm **[4]**. Differentiation of true MF from dermatologic conditions mimicking MF clinically and histopathologically such as psoriasis and chronic eczema is important to ensure proper management **[5]**.

Elevated CD4/CD8 ratio certainly favors MF, mainly when this ratio is greater than> 2 and has considered being a valuable method for diagnosis of MF cases. However lowered CD4/CD8 ratio doesn't exclude the diagnosis of MF and can be observed in MF with non-classic presentation such as hypopigmented type **[6, 7, 8]**.

The loss of CD7 expression can be observed even in the early phases of the disease. However, isolated negativity for CD7 is not a sufficient criterion for diagnosis, as it can be shown in some inflammatory dermatoses **[4, 9]**.

Many investigations were carried out to characterize positive identification markers for MF by comparing MF lesions with inflammatory dermatitis. Zhang et al **[10]**, reported that thymocyte selection-associated high mobility group box factor (TOX) showed significant expression in early MF lesions versus biopsies from dermatitis.

TOX is a critical regulator of early T-cell development, strictly regulated in thymocyte differentiation specifically during the

transition from CD4CD8 precursors to CD4+ T cells. However, upon completion of this process, it is firmly suppressed, so that normal mature CD4 cells do not have significant expression of this protein. It encodes a nuclear protein of the high-mobility group (HMG) family that act as transcription factor and is highly but transiently expressed in thymic tissue **[11]**.

The aberrant TOX expression in MF was subsequently confirmed in early as well as more advanced disease of MF without significant expression in chronic dermatitis by other studies. These studies suggested that TOX could be used as a marker for MF diagnosis and prognosis **[12, 13]**. Zhang et al **[10]** and Morimura et al **[13]** reported a marked increase of TOX staining in CD4+ T cells in MF samples in both dermis and epidermis, including the MF cells in the Pautrier's microabscess. However, they stated that TOX antibody did not label MF with CD4-CD8+ immunophenoype.

The aim of the current study is to test the role of TOX as a diagnostic marker of MF and also as a differentiating marker of MF and its benign mimickers.

MATERIALS AND METHODS

Collection and classification of cases:

This work was done on skin biopsies from 103 patients. Fifty one of them were diagnosed as MF and others 52 were diagnosed as inflammatory dermatoses (benign mimickers). The specimens were collected from the surgical pathology laboratory archive files of the Pathology and Dermatology Departments of Mansoura University, Egypt, through the period of December 2012 to November 2014. For each of these cases, the pathology reports were revised to obtain demographic and clinical data including age, sex and clinicopathological diagnosis of the patients. The original H&E sections were retrieved and reassessed to confirm and verify the histological diagnosis.

The MF cases were diagnosed with clinicopathological correlation according to International Society for Cutaneous Lymphoma (ISCL) Algorithm **[4]**. The MF cases included 18 patches, 17 plaques, 11 hypopigmented, 3 poikelodermic, one folliculotropic and one erythrodermic MF. The inflammatory dermatoses included 23 psoriasis, 16 chronic eczema, 8 lichen planus and 5 pitryasis rosea cases.

Immunohistochemistry:

Methodology. All archival specimens had been fixed in neutral-buffered formalin and embedded in paraffin by routine methods. Paraffin blocks of MF cases were yield to immunohistochemical staining for TOX, CD4, CD8 and CD7 (in TOX negative cases), while, the others inflammatory dermatoses stained only by TOX.

For the study, the specimens were cut in 4µ thick sections on adhesive-coated glass slides. Deparaffinization with xylene and hydration through graded alcohol series were performed. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxidase in methanol for 5 min.

Epitope retrieval was carried out by boiling in a pressure cooker with Citrate Buffer pH 6.0 for TOX and with EDTA Buffer pH 8.0 for CD4, CD8 and CD7. Sections were then incubated with proteinase K 0.04% for 5 min. After washing with phosphate-buffered saline, the sections were incubated with the anti-human monoclonal antibodies.

The used Monoclonal antibodies in this study were: polyclonal rabbit anti-human TOX antibody (NOVUS BIOLOGICALS, code No NBP2-20628, conc 1:400),monoclonal mouse anti CD4 (Clone 4B12, DAKO, code No IR649, Conc 1:50), monoclonal mouse anti CD8 (Clone C8/144B, DAKO, code No IR623, Conc 1:50) and monoclonal mouse anti CD7(Clone MEM-186, NOVUS BIOLOGICALS, code No NB500-326 Conc 1:150).The primary antibodies were detected with the streptavidin-biotin for 30 min at room temperature. Diaminobenzidine was used as the chromogen, and incubation was carried out for 5 min at room temperature. Sections were counterstained in hematoxylin.

For the positive control, a thymic tissue was used in TOX, a tonsillar tissue was used in CD4, CD8 and CD7 and, for the negative control; the primary antibody was omitted, and phosphate-buffered saline was used in each experiment.

Interpretation of immunohistochemical results.

1-TOX expression

The TOX expression was estimated by counting the number of cells with brown nuclear staining comparable to the total number of infiltrating lymphocytes. The staining is semi-quantitatively graded as (negative<10%, weak positive 10-30% and strong positive >30% of total infiltrating lymphocytes) **[13]**.

2-CD4, CD8 and CD7 expression

CD4 and CD8 positivity were determined by membranous staining (brown) in the infiltrating lymphocytes and were counted in the epidermis in a minimum of ten microscopic HPF and expressed as a CD4/CD8 ratio. This ratio is considered to be valuable in diagnosis of MF when, CD4:CD8> 2 in epidermotropic cells **[6,7]**.CD7 expression was determined by membranous staining (brown) of the infiltrating lymphocytes. It is considered to be a valuable method in diagnosis of MF when CD7 is expressed in lesser than 10% of epidermotropic cells(CD7 negativity) **[4]**.

Statistical analysis

Data entry and statistical analyses were performed using SPSS (statistical package of social sciences) version 16.0 (SPSS Inc., Chicago, IL, USA). Parametric data were expressed in mean ± standard deviation. Non parametric data were expressed in median, minimum and maximum. Normality of data was first tested by one sample K-S test.

In addition, independent t test was used to compare means for continuous parametric variables of each two different groups. Also, Mann-Whitney U test (z) was used to compare non parametric continuous variables in two different groups. Pearson Chi-square tests were used to compare the categorical variables between groups. Roc curve was used to estimate area under the curve (AUC), sensitivity and specificity and cutoff values indicating diagnostic accuracy of TOX expression gene in diagnosis of MF. Calculation for degree of agreement between two tests was done according to equation (Addition of product of the diagonal /total %). P value < 0.05 was considered as statistically significant.

RESULTS

Clinical characteristics of the studied cases:

The age and male gender in MF cases were significantly higher than inflammatory dermatoses group (table 1).

Table 1: Age and gender among MF and inflammatory dermatoses group

	MF (n=51)	Inflammatory dermatoses (n=52)	P value
Age (years)	46.09 ±18.5	37.9 ± 16.7	0.02*
Sex			
Male	34 (66.7%)	24 (46.2%)	0.03*
Female	17 (33.3%)	28 (53.8%)	

Immunohistochemical results:

I Immunohistochemical staining of TOX

TOX expression in our study revealed significant positive expression by IHC in MF cases in comparison with inflammatory dermatoses at both weak and strong positivity(p value =0.02, =0.03 respectively) (table 2) (Figure 1).

Table 2: Role of TOX in diagnosis of MF in comparison with inflammatory dermatoses

TOX expression(scoring)	Clinicopathological diagnosis		
	Inflammatory dermatoses	MF	P value
	n=52	n=51	
Negative	52	18	1
	100.0%	35.3%	
Weak positive	0	12	<0.001*
	.0%	23.5%	
Strong positive	0	21	<0.001*
	.0%	41.2%	

Diagnostic accuracy of TOX was determined by ROC curve test. ROC curve analysis revealed that TOX has higher specificity (100%) than sensitivity (62%) at Cut off point 17.5 (table 3).

Table 3: ROC curve shows the diagnostic accuracy of TOX

Cut off point	Sensitivity%	Specificity%	Area under curve	95%CI
17.5	62	100		
22.5	56	100		
27.5	52	100	0.84	0.76- 0.92

There was statistical significant increase in TOX titre in Plaque MF that showed stronger positivity more than Patch MF in our study (p=0.01) (table 4)(figure 1: photo b and c).

Table 11: Relation of TOX titre with clinicopathological MF type (patch and plaque)

TOX titre	N	Median	Minimum	Maximum	P value
Patch	18	27.50	.00	50.00	0.01*
Plaque	17	40.00	.00	70.00	

In the current study, CD4/CD8 ratio was calculated, and demonstrated in table 5.

Table 5: Results of CD4/8 ratio in diagnosis of MF group

IHC	MF group
	No (%)
CD4 / CD8 ratio	
Not valuable	16(31.4)
Valuable	35(68.6)

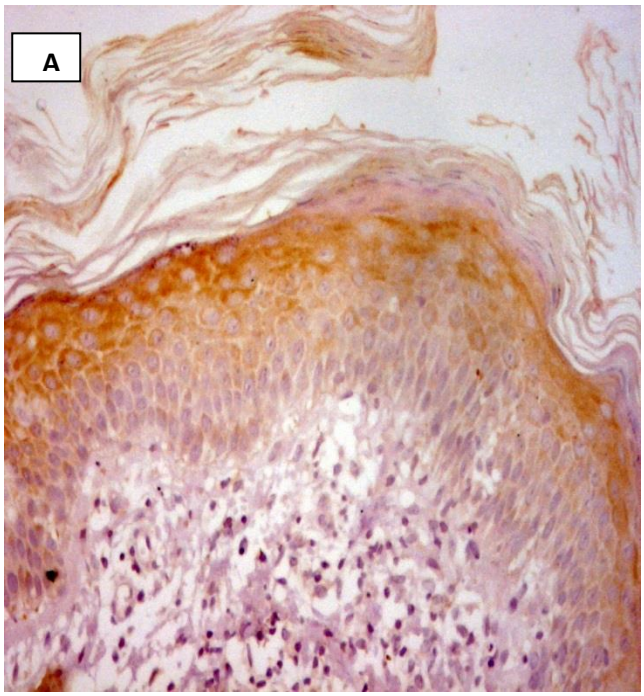
The current study revealed positive TOX staining in only CD4+ T cells in MF cases that had valuable elevated CD4/CD8 ratio(Figure 2) with strong reliability (agreement) with it and its negativity (Figure 1 photo d) in MF(mainly hypopigmented variant) with invaluable CD4/CD8 ratio (table 6).

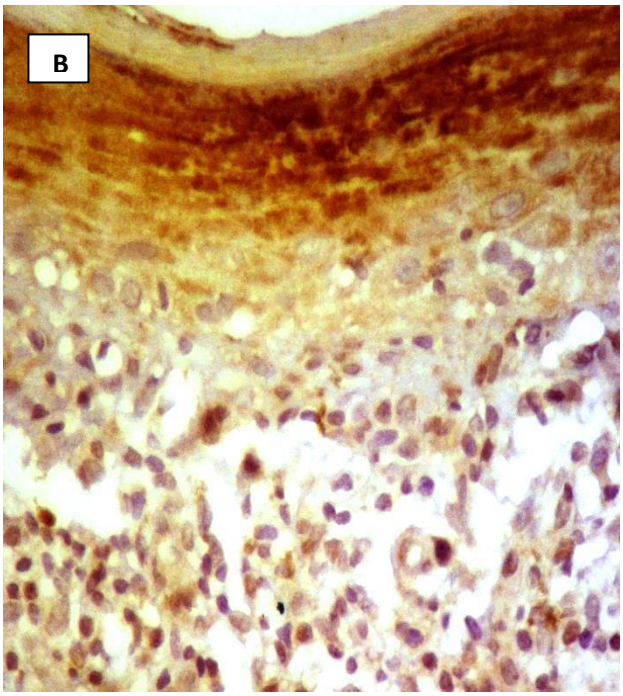
Table 6: Reliability (agreement) of TOX with CD4/CD8 ratio

		TOX expression		
CD4/CD8 ratio		Negative	Positive	Total
	not valuable	16	0	16
		88.9%	.0%	31.4
	valuable	2	33	35
		11.1%	100.0%	68.6
Total		18	33	51
		100.0%	100.0%	100.0%

Reliability =16+33/51= 0.96.

When we stained the TOX negative cases by CD7, all of them showed negative CD7 expression.





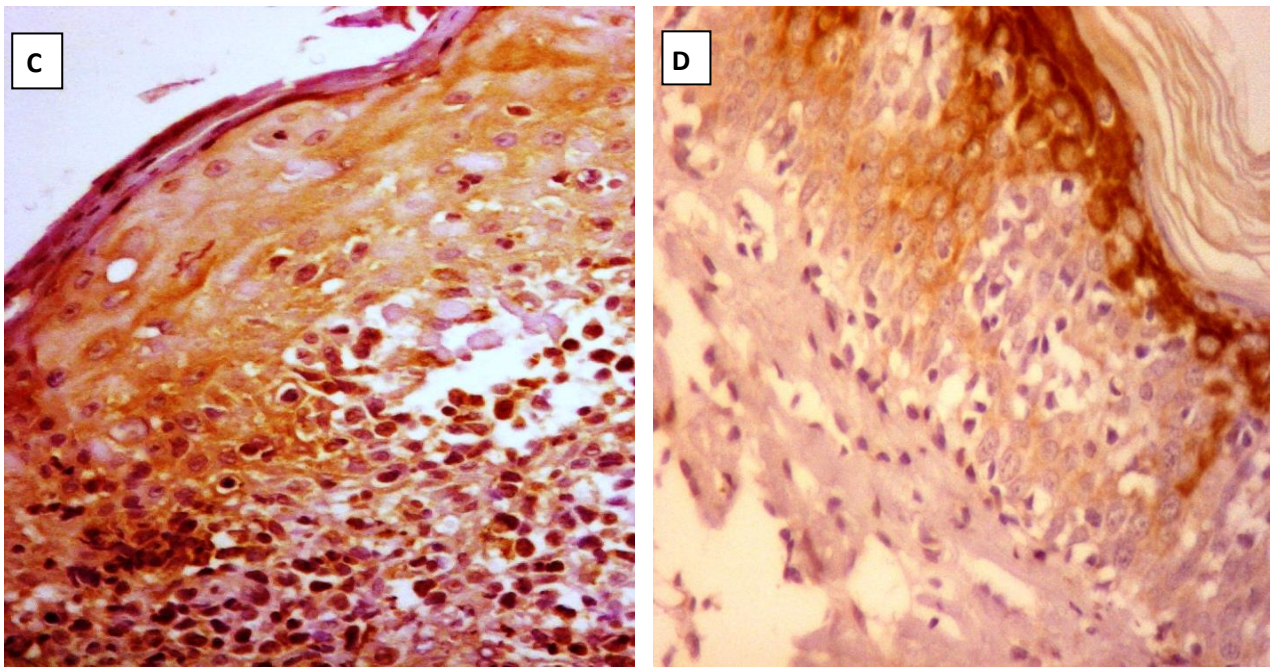


Figure 1. Different TOX expression in the studied cases (A) Photo of negative TOX in lymphocytes nuclei in an inflammatory mimicker (pitryasis rosea); IHC×200. (B) Photo of weak positive TOX in infiltrating lymphocytes nuclei in a patch MF; IHC ×400. (C) Photo of strong positive TOX in lymphocytes nuclei in a plaque MF; IHC ×200. (D) Photo of negative TOX in lymphocytes nuclei in hypopigmented MF; IHC×200.

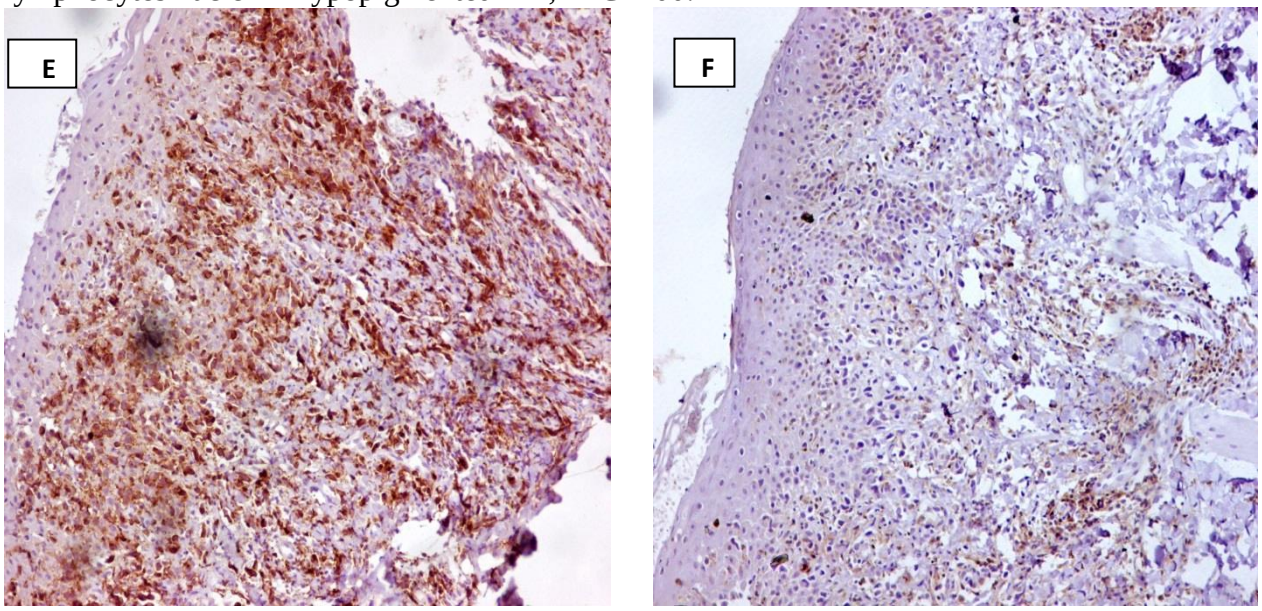


Figure 2. Elevated CD4/CD8 ratio in a case of plaque MF (E): Photo of plaque MF case with Predominance of CD4+ T cells IHC ×100. (F) Photo of the same MF case shows scattered CD8+ lymphocytes IHC ×100.

DISCUSSION

Considering the difficulty in differentiation between MF and its benign mimickers, our study was conducted to highlight the role of thymocyte selection-associated high mobility group box factor (TOX) IHC in this aspect. However, the studies in this aspect were limited, only three studies **[10, 12, 13]**.

Regarding the age and gender of patients in our study the mean age of MF presentation was 46.09 ± 18.5 ranged between 6-78 years and male to female ratio was 2:1. The age and male gender in MF cases were significantly higher than inflammatory diseases.

In agreement with our results, Mokhtar et al, **[14]** in Egypt, reported an average age of 47.5 years and a male to female ratio 1.8:1 in MF.

TOX expression in our study revealed significant positive expression by IHC in MF cases in comparison with inflammatory dermatoses. This was demonstrated by AUC value of 0.84 (95% CI =0.76- 0.92, P < 0. 001). When cutoff was set at 17.5, TOX had 62% sensitivity and 100% specificity for MF.

In agreement with our results, Zhang et al **[10]** and Morimura et al (2014) reported similar results as regard specificity and sensitivity. However, lower specificity (75%) and higher sensitivity (90.3%) was reported by Huang et al. [**12]** as they worked on larger number of cases with different methodology at genetic level and lower cutoff value.

Our results that used Morimura et al **[13]** score revealed stronger TOX positivity in plaque more than patch MF. In agreement with our results, Huang et al **[12]** and Morimura et al **[13]** furthermore reported that thicker skin lesions such as plaques and tumors expressed higher TOX levels than patches and correlated the higher TOX levels with the increase risks of disease progression and disease-specific mortality .So clinical staging and good follow up of the patients with stronger TOX expression are needed.

Zhang et al **[10]** and Morimura et al **[13]** reported a marked increase of TOX staining in CD4+ T cells in MF samples in both dermis and epidermis, including the MF cells in the Pautrier's microabscess. However, they stated that TOX antibody did not label MF with CD4-CD8+ immunophenoype.

In agreement of them, the current study revealed positive TOX staining in only CD4+ T cells in MF that had elevated valuable CD4/CD8 ratio with strong reliability to it and its negativity in MF with CD4-CD8+ immunophenoype and invaluable ratio.

This can explain the negativity of TOX expression in about 1/3 MF cases with predominance of negativity in hypopigmented variant that had CD8+ immunophenotype which was common in Egypt as reported by Hassab-El-Naby and El-Khalawany **[15]**.

However, in our study we had 2 MF cases that showed elevated CD4/CD8 ratio and were negative for TOX. So, collectively, the MF cases that were negative for TOX which were 18 in number, only 2 of them had valuable CD4/CD8 ratio, however the remaining 16 cases had invaluable CD4/CD8 ratio.

When we stained these TOX negative cases by CD7, all of them showed CD7 negativity. These results, confirming the diagnosis of MF beside the clinical and histopathological diagnosis as reported by **[4]**.However, isolated negativity for CD7 is not a sufficient criterion for diagnosis, as it has lower specificity and can be shown in some inflammatory dermatoses **[9]**.So, we need a panel of IHC in diagnosis of MF. As, TOX seems to be good positive marker for MF diagnosis, however, its negativity doesn't exclude the possibility of MF diagnosis.

Huang et al **[12]** stated that TOX may behave as a novel therapeutic target for MF in the future .So, only cases that were positive for TOX can get benefit from therapy in future. Further studies are needed to evaluate TOX expression in MF by different methods including PCR, IF ,IHC-F that need fresh frozen tissue for more accurate results as reported by Zhang et al **[10]**.

In conclusion, our results have suggested that positive TOX expression favors MF however negative TOX doesn't exclude the diagnosis of MF. Combining TOX with CD4/CD8 ratio and CD7 may be useful in diagnosis of MF beside the clinical and histopathological diagnosis. The stronger TOX positivity in plaque more than patch MF may correlate with the progression of the disease.

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