

## Original Article

## Evaluation of the molecular marker TOX protein in diagnosis of early stages of mycosis fungoides

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### ABSTRACT

**Background:** Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma (CTCL). Differentiation of MF especially early stages (eMF) from its benign mimickers is important to ensure proper management. TOX is a critical regulator of early T-cell development in the thymus that is considered as a useful marker for MF diagnosis and prognosis.

**Objective:** The aim of the current study was to evaluate the ability of molecular marker TOX protein in diagnosis of eMF, and its ability to differentiate eMF from similar benign inflammatory skin diseases (BIDs).

**Methodology:** This is a case control study and was carried out on 60 subjects; 20 patients as eMF, 20 patients as (BIDs) and 20 normal skin specimens as control cases. The diagnosis was established after clinicopathological correlation. Immunohistochemistry (IHC) was done for MF, BIDs and normal skin cases for TOX and CD4 IHC stains.

**Results:** TOX expression showed a significant positive expression in MF cases compared to BIDs and control groups, with 100% sensitivity and 95% specificity. The pattern of TOX IHC stain in MF was diffuse, while in BIDs was focal.

**Conclusion:** TOX might be considered a diagnostic marker for eMF that can differentiate eMF from BIDs mimickers.

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**Keywords:** Cutaneous T cell lymphoma, mycosis fungoides, early mycosis fungoide, benign inflammatory skin diseases, Thymocytes selection associated HMG-box

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### INTRODUCTION

Mycosis fungoides (MF) is the most common type of Cutaneous T-cell lymphoma (CTCL), which is characterized by localization of neoplastic T lymphocytes to the skin. MF represents about 50% of all primary cutaneous lymphomas<sup>[1]</sup>. In MF, there is a clonal expansion of atypical CD4+ T helper skin-homing lymphocytes expressing a memory cell phenotype. The clinical course of MF is characterized by being indolent and prolonged course lasting over years or sometimes decades, that progress from patches to more infiltrated plaques and finally tumors<sup>[2]</sup>. In early mycosis fungoides (eMF), the lesions are mostly limited to skin as flat erythematous skin patches, but in late stages of MF the lesions progress

gradually to plaques and tumors. Also, there is extra cutaneous dissemination of malignant lymphocytes to lymph nodes, peripheral blood and visceral organs. There is positive relation between the stage of the disease and the survival rate of MF patients<sup>[3]</sup>.

Differentiation of true MF from dermatological conditions that mimic MF clinically and histopathologically such as psoriasis and chronic eczema is important to ensure proper management<sup>[4]</sup>. An integrated algorithm of clinical and histological criteria for diagnosis of MF has been established by International Society of Cutaneous Lymphomas (ISCL)<sup>[2]</sup>. But, in eMF (patch and early thin plaque

MF), the diagnosis is difficult even for experienced dermatologists because of the morphological and histological similarities of MF to BIDs or discordance between clinical and pathological findings<sup>[5]</sup>.

The lack of specific cellular or molecular markers that can reliably differentiate the malignant T cells from the abundant reactive T cells that are present not only in BIDs mimickers of eMF but also in the eMF lesions themselves gives another difficulty in diagnosis of eMF<sup>[6]</sup>. 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 as it can be observed in MF with non-classic presentation such as hypopigmented type<sup>[7]</sup>. In the early phases of the disease, there is an observed loss of CD7 expression. However, some BIDs show also loss of CD7, consequently the isolated negativity of CD7 is not a sufficient criterion for diagnosis of eMF<sup>[2,8]</sup>. Therefore, investigations were carried out to establish specific markers for MF diagnosis by comparing eMF lesions with normal skin and BIDs<sup>[9]</sup>. One of these markers, a gene called Thymocyte Selection - Associated High Mobility Group Box gene (TOX), which is a critical regulator of early T-cell development, and strictly regulated in thymocyte differentiation<sup>[10]</sup>. After completion of T-cell development, TOX is firmly suppressed, consequently normal mature CD4 T-cells do not have significant expression of TOX<sup>[11]</sup>. It has been reported that TOX showed significant expression in eMF lesions versus biopsies from BIDs<sup>[9]</sup>.

The microRNA -223(miR-223), which downregulates TOX expression is significantly reduced in MF. Therefore, miR-223 regulation of TOX should have significant effects for MF growth and pathogenesis<sup>[12]</sup>. Results from many experiments demonstrated that, GATA3 regulates TOX expression as TOX expression was decreased after GATA3 knockdown, indicating that GATA3 may be able to drive the increased expression of TOX that is seen in MF pathogenesis and progression<sup>[13]</sup>. A strong and specific ability of TOX IHC to stain MF cells in eMF skin biopsies by IF and IHC has been demonstrated. Consequently TOX could be used as useful marker for early diagnosis and prognosis of MF<sup>[14, 15]</sup>. The aim of the current study was to evaluate the ability of molecular marker TOX protein in diagnosis of eMF, and its ability to differentiate eMF from similar BIDs.

## PATIENTS AND METHODS

The current study is case-control, it included three groups; 1st group is twenty patients diagnosed clinically and histopathologically as eMF according to Pimpinelli et al<sup>[5]</sup> collected from about 60 patients presented clinically as MF, in the form of [ patches (15 cases), thin plaques (4cases) and one case showed mixed patches and thin plaques], 2<sup>nd</sup> group is twenty

patients diagnosed clinically and histopathologically as BIDs in the form of [8psoriasis, 8chronic eczema, 2 Lichen planus and 2 Pityriasis rosea] and 3<sup>rd</sup> group is 20 healthy volunteers similar in ages and sex served as control groups. The patients were collected from the outpatient clinic of dermatology and venereology department of Al Zahra, Al-Hussein and Zagazig Universities Hospitals, during the period from October 2015 to May 2017. Data regarding age, sex, occupation, past history, medical history and clinicopathological diagnosis of the patients were recorded. The study was approved by the research ethical committee of the faculty of medicine for girls Al Azhar University and all participants gave an informed consent.

### Inclusion criteria

1. Males and females were included.
2. Age ranged from 18-70 y.
3. Patients didn't receive any treatment for MF at all or at least for 6 months prior to study.
4. Classic MF, including early stages (patch and thin plaque).

### Exclusion criteria

1. Patients with any chronic debilitating diseases such as chronic renal failure, hepatic failure and malignant diseases.
2. Patients should be free from any chronic immunological diseases.
3. Patients on phototherapy (PUVA or UV-B), irradiation therapy, cytotoxic chemotherapy and topical chemotherapy (nitrogen mustard or carmustine therapy).

### Clinical evaluation of the patients:

Our mycosis fungoides patients were collected clinically as eMF according to Pimpinelli et al<sup>[5]</sup>

1. Patch stage: persistent and progressive nonspecific patches on non-sun exposed areas (lower trunk and buttocks).
2. Plaque stage: persistent and progressive thin plaques on non-sun exposed areas (lower trunk and buttocks).
3. The number of lesions is multiple, size of lesions is variable.
4. Poikiloderma.

The intensity of itching was evaluated according to visual analogue scale (VAS)<sup>[16]</sup>.

### Immunohistochemistry:

#### ➤ Methodology:

The histopathological diagnosis was reassessed and confirmed by the original H&E sections. All specimens were fixed in neutral-buffered formalin and embedded in paraffin by routine methods. Paraffin blocks of MF, BIDs and control groups were yield to immunohistochemical IHC staining for TOX and CD4. The DAKO automatic stainer was used for Staining, by using the envision method (Dako Cytomation, Denmark). Sections of 3–5 µm were cut

and mounted on positively charged slides from the formalin-fixed-paraffin-embedded blocks. Deparaffinization with xylene and hydration through graded alcohol series were performed. The activity of endogenous peroxidase 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. All sections had been incubated with proteinase K 0.04% for 5 min. The sections were incubated with the anti-human monoclonal antibodies after washing with phosphate-buffered saline. Rabbit monoclonal TOX (Sigma-Aldrich, HPA018322; St. Louis, MO, U.S.A., 1:200) and mouse monoclonal CD4 antibodies (Clone 4B12, DAKO, code No IR649, Conc 1:50) were used.

➤ **Interpretation of immunohistochemical results:**

1. **TOX expression:** The TOX stain is nuclear IHC stain, and semi-quantitatively graded as follow, negative: Nothing of immunoreactive cells is stained, weak positive <10% of immunoreactive cells are stained, moderate positive 10-30% of immunoreactive cells are stained and strong > 30% of immunoreactive cells are stained<sup>[17]</sup>.
2. **CD4 expression:** CD4 is IHC stain. It was used to detect CD4+T cells of the infiltrating lymphocytes in both MF and BIDs groups. It was evaluated as follow, stained or not (+ve or -ve).

**Statistical analysis:**

The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative percentages. Fisher exact (chi-square corrected) test was used to calculate difference between qualitative variables. Validity data were calculated using:

- Sensitivity= true positives/ (true positive + false negative),
- Specificity=true negatives/ (true negative + false positives),
- Positive predictive value (PV+)= true positive/ (true positive + false positive),
- Negative predictive value (PV-)= true negatives/ (true negatives +false negatives) and
- Accuracy = (true positive + true negative)/ Total number.

The significance level determined as p- value of <0.05 indicate significant results.

## RESULTS

### Demographic and clinical results

In our study the selected patients were eMF in the form of three cases were stage IA, While 17 cases were stage IB. Regarding the age and gender of patients in our study, the range of age in MF patients was 17-63years with a mean  $44.15 \pm 11.54$  y which is the average age of onset of MF and male to female ratio was nearly 2:1. In the current study, there was no relation between TOX expression in MF cases with sex (P=0.96). The two diseased groups didn't show any statistical significant difference in BSA affection, but there was a statistical significant increase in frequency of sever itching (P=0.002) and patches lesion (P=0.003) among MF group compared to BID group. Also there was a statistical significant increase in disease duration among MF group compared to BID group (p=0.03).

### Immunohistochemical (IHC) results of TOX and CD4:

In MF group all cases showed positive TOX stain in the form of 4cases (weak20%), 6 cases (moderate 30%) and 10cases (strong 50%) ,whereas in BIDs group only 2 cases showed weak stain and control group showed negative stain . Regarding CD4, in MF group all cases (20) showed positive staining (100%), in BID group 17 cases (85%) were positive, whereas all cases in control group showed negative stain. There were statistical significant increase in frequency of +ve cases in TOX stain among MF group compared to BIDs and control groups with P=<0.001. Also, there were statistical significant increase in frequency of +ve cases among both MF and BID group compared to control group in CD4 stain with P=0.001. There was increase in positivity of TOX stain in MF in comparison to BIDs and control groups with P=0.001. (**Table 1**). The sensitivity of TOX stain in diagnosis of MF was 100%, specificity 95%, accuracy 96.7%, positive predictive value (PVP) 90.9% and negative predictive value (PVN) 100%. There was statistical significant agreement between histopathology and TOX stain in diagnosis of MF, as all cases that were diagnosed histopathologically as eMF were TOX +ve (**Table 2**).

In our study, there was a statistically significant increase in severity of itching (p=0.02) and BSA (p=0.001), with strong TOX expression. In the current study, a statically highly significant relation between intensity of TOX IHC stain and progression of the MF (P<0.01) was detected, as intensity of TOX in stage IB was more than stage IA. Stage IA includes 3 cases with weak TOX expression, whereas stage IB includes 17 cases with 1case weak TOX expression, 6 cases moderate TOX expression and 10 cases strong TOX expression.

**Table (1): Pattern of TOX & CD4 IHC stains results among the three studied groups**

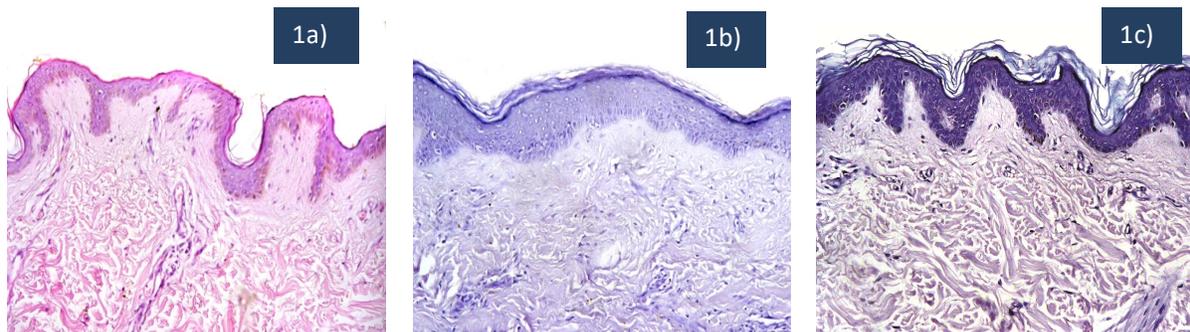
Variables	Group I (MF) (n=20)		Group II (BID) (n=20)		Group III (Control) (n=20)		Sig. test	P
	No	%	No	%	No	%		
<b>TOX:</b>								
- (Negative)	0	0	18	90	20	100	55.16	0.001*
+ (Weak)	4	20	2	10	0	0		
++(Moderate)	6	30	0	0	0	0		
+++ (strong)	10	50	0	0	0	0		
<b>CD4:</b>								
-	0	0	3	15	20	100	49.2	0.001*
+	20	100	17	85	0	0		

\*Significant P-value, MF: mycosis fungoides, BID: Benign inflammatory skin disease, IHC: Immunohistochemical stains

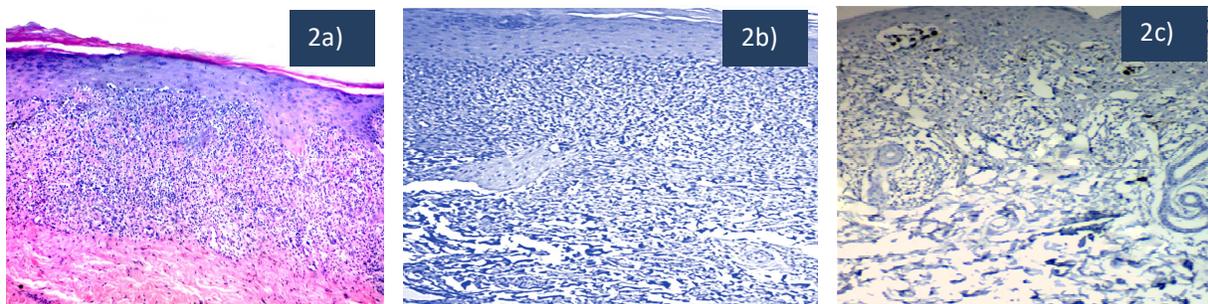
**Table (2): Validity of TOX IHC stain as a marker in diagnosis of mycosis fungoides**

TOX	Histopathology		Total	P
	MF	BID		
<b>Positive</b>	20	2	22	0.001*
<b>Negative</b>	0	18	18	
<b>Total</b>	20	20	40	
<b>Validity</b>	Sensitivity: 100% PPV: 90.9%		Specificity: 95% NPV: 100 %	
<b>Accuracy</b>	96.7 %			

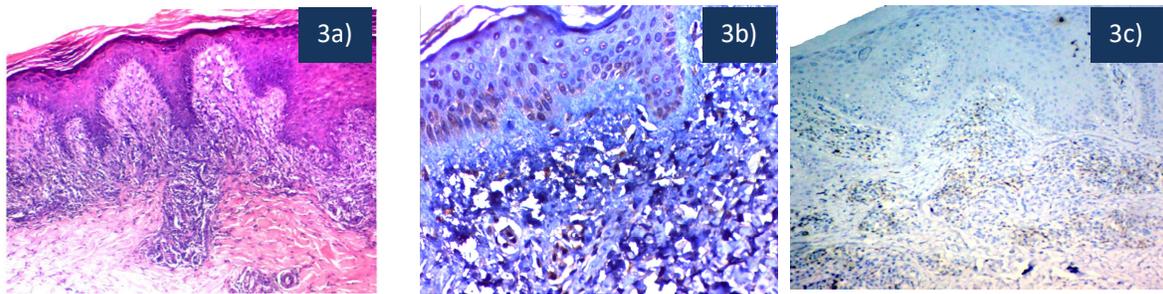
\* Significant p-value, MF: mycosis fungoides, BID: Benign inflammatory skin disease, PPV: Positive predictive value, NPV: Negative predictive value, IHC: Immunohistochemical stains.



**Figure 1 (a, b, c): A case of normal skin with negative TOX expression**  
 a)-Normal skin with H&E, b)- Normal skin with –ve TOX, c)- Normal skin with–ve CD4

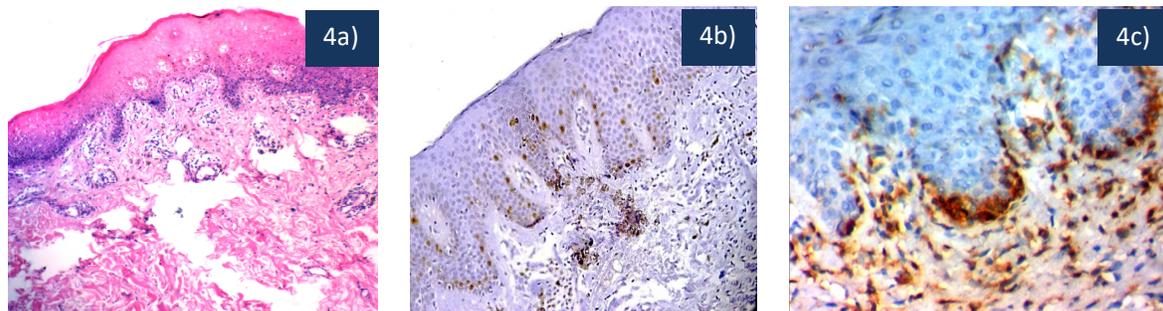


**Figure 2 (a, b ,c): Benign cutaneous inflammatory dermatoses with negative TOX expression:**  
 a) A case of lichen planus showing orthokeratosis, focal hypogranulosis, irregular acanthosis and hydropic degeneration of the basal layer. The dermis showing heavy band-like inflammatory lymphocytic infiltrate (x200, H&E).  
 b) A case of lichen planus showing negative TOX- expression in the epidermis and upper dermis (IHC TOX X 400).  
 c) A case of lichen planus showing few positively stained CD4+ T –cells mainly in the upper dermis (IHC CD4X 400).



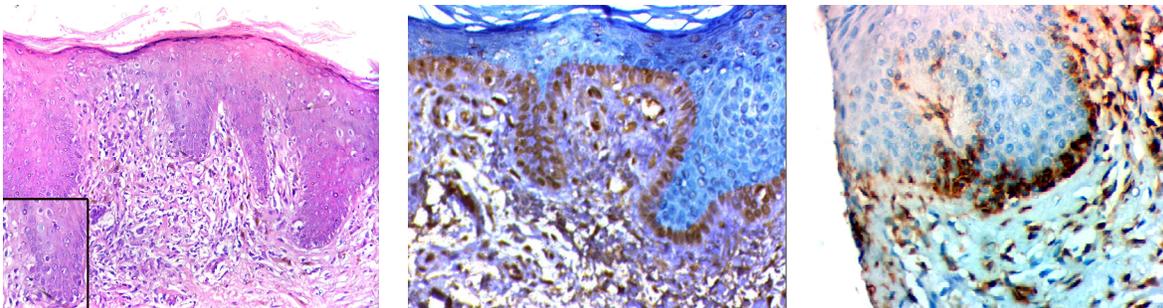
**Figure 3 (a, b, c): Benign cutaneous inflammatory dermatoses with weak TOX expression:**

- a) A case of chronic dermatitis showing hyperkeratosis, acanthosis, exocytosis and peri - vascular inflammatory infiltrate in the dermis (x200, H&E).
- b) A case of chronic dermatitis showing scores 1(weak) TOX expression with few aggregates of positive TOX-T-cells in epidermis and dermis (IHC TOX HP X 400).
- c) A case of chronic dermatitis showing positive CD4 expression with numerous CD4+ T -cells mainly in the dermis and some cells in epidermis (IHC CD4X200).



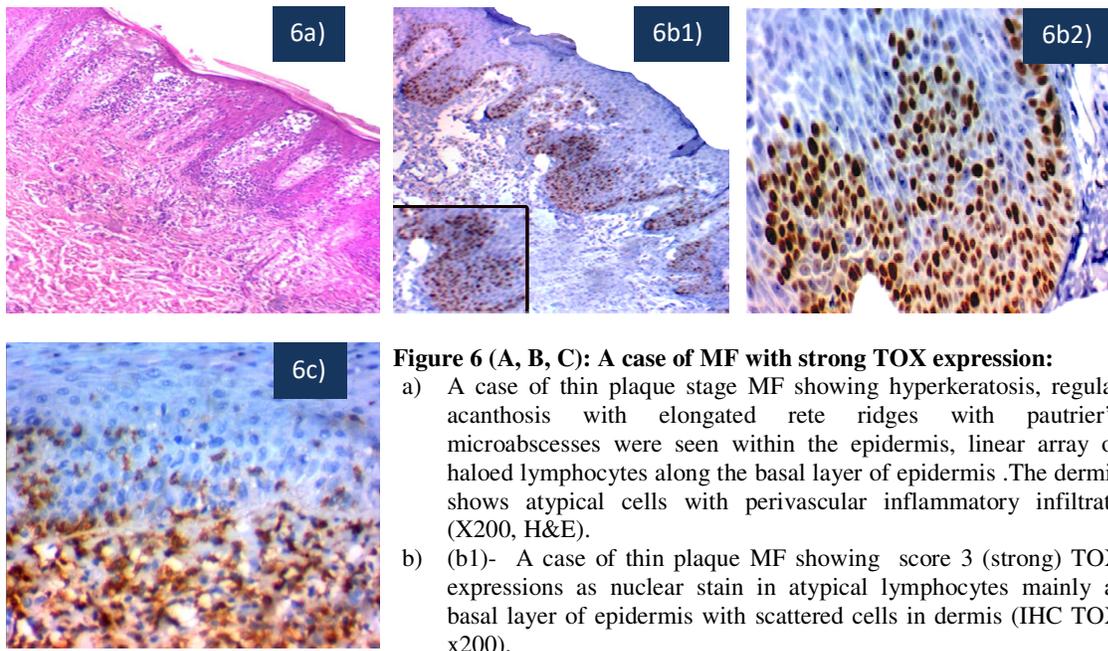
**Figure 4(a, b, c): A case of MF with weak TOX expression:**

- a) A case of patch stage MF showing acanthosis with Pautrier's microabscesses were seen within the epidermis and linear array of atypical cells along the basal layer of epidermis. The dermis shows atypical cells with perivascular inflammatory infiltrate (x200, H&E).
- b) A case of patch stage MF showing score 1(weak) TOX expression as nuclear stain in malignant lymphocytes mainly along basal layer of epidermis and scattered cells in dermis (IHC TOX x200).
- c) A case of patch stage MF showing positive expression of CD4 with clusters of CD4+T-lymphocytes in the dermis and linear array of CD4+T-lymphocytes along the basal layer of the epidermis (IHC CD4 HPx400).



**Figure 5 (A,B,C): A case of MF with moderate TOX expression:**

- a) A case of patch stage MF showing acanthosis & thickened rete ridges with atypical cells surrounded by clear halo along the basal cell layer of epidermis. The dermis shows atypical cells with perivascular inflammatory infiltrate (X200 H&E).
- b) A case of patch MF shows score 2 (moderate) TOX expressions as nuclear stain in the malignant lymphocytes mainly in epidermis especially at basal layer with scattered cells in dermis (IHC TOX X HP 400).
- c) A case of patch MF shows positive CD4 expression with numerous CD4+T-lymphocytes arranged in aggregate mainly in the upper dermis and positive cells along the basal layer of epidermis (IHC CD4x HP400).



**Figure 6 (A, B, C): A case of MF with strong TOX expression:**

- a) A case of thin plaque stage MF showing hyperkeratosis, regular acanthosis with elongated rete ridges with Pautrier's microabscesses were seen within the epidermis, linear array of haloed lymphocytes along the basal layer of epidermis. The dermis shows atypical cells with perivascular inflammatory infiltrate (X200, H&E).
- b) (b1)- A case of thin plaque MF showing score 3 (strong) TOX expressions as nuclear stain in atypical lymphocytes mainly at basal layer of epidermis with scattered cells in dermis (IHC TOX x200).  
(b2)- closer view of the same case of thin plaque MF showing Score 3 (strong) TOX expression as nuclear stain in malignant lymphocytes with large and atypical hyperchromatic nuclei (IHC TOX HP x 400).
- c) (C)- A case of thin plaque MF showing positive CD4 expression with large aggregate of CD4+T-lymphocytes mainly in the upper dermis and along the basal layer of the epidermis (IHC CD4 HP X 400).

## DISCUSSION

As a result of high similarity between MF and its mimic BIDs at both clinical and histopathological levels, the early diagnosis of MF is difficult and can be easily missed [18]. Considering this difficulty in differentiation between eMF and its BIDs, our study was conducted to estimate the role of TOX as immunohistochemical marker in diagnosis of eMF.

There are many immunophenotypic variants of MF, such as CD4+ /CD8+ ,CD4- /CD8+ ,CD4- /CD8- , CD30+ , CD20+ and CD56+ [19]. Rarely eMF represented by abnormal phenotypes such as CD4+ /CD8+ , CD4- /CD8- [20].

In current study, there was a statistically significant increase in frequency of severe itching and BSA with strong TOX in comparison to intensity of TOX stain. In present study, there was a statistically highly significant increase in the intensity of TOX stain with progression of the disease, as intensity TOX IHC stain in stage IB was more than stage IA. In agreement with our results Morimura et al. [17] and Haung et al. [21] reported that, there is positive correlation between the higher TOX levels and increase risk of progression of the disease and mortality rates specific to the disease.

In our study, expression of TOX was observed in all MF specimens as uniformly positive diffuse nuclear

immunostaining with various degree of severity in dermis and epidermis especially epidermotropic lymphocytes mainly at basal layers. However, TOX expression was observed only in two cases of BIDs,

they showed weak expression and focal staining pattern, while no TOX expression was observed

at all in normal control skin. The sensitivity of TOX stain in diagnosis of MF was 100%, specificity 95%, accuracy 96.7%, positive

predictive value (PVP) 90.9% and negative predictive value (PVN) 100%. There was statistical significant agreement between histopathology and TOX stain in diagnosis of MF, as all cases that were diagnosed histopathologically as eMF were TOX +ve.

In present study, abnormal TOX expression was subsequently confirmed in CD4+ T cells in eMF samples without significant expression in BIDs. In agreement with these results Zhang et al. [9] and Morimura et al [17] reported a marked increase of TOX stain in CD4+T-cells in samples of MF lesions in both dermis and epidermis especially in Pautrier microabscess. Moreover, they stated that TOX did not or at low level label CD4+ T cells in BIDs patients. Most previous studies reported that, in BIDs there were few or no TOX CD4 T-cells found on

histopathological examination<sup>[9], [10]</sup>. Likely our study, expressed TOX staining only in 2 cases (10%) which were generally weak and focal. Long term follow up of these cases clinically and histopathologically is mandatory because if they remain stable and respond well to treatment, it can be assumed that TOX was expressed at low levels by some reactive T-cell, but if they were not stable and didn't respond to treatment, these cases might progress into MF. Therefore studies are required to follow them up in order to establish their association with TOX expression. A study was conducted by **Schrader et al.** [22], evaluated that various types of CTCL can express TOX at different levels, and therefore is not specific for MF only. Also CTCL with CD4-/ CD8+ and CD4-/ CD8- phenotypes can express TOX, and not restricted to CTCL with a CD4+ / CD8- phenotype only. In BIDs, considerable amounts of reactive T cells expressed TOX, although less strongly and in lower percentages than in MF. Expression of TOX alone is insufficient for MF diagnosis, but it may have an adjunctive diagnostic role in conjunction with other clinical and histological criteria.

We had used CD4 stain as a confirmatory IHC stain to detect presence of CD4+ T-cells in both MF and BIDs in comparison to control group, and to prove if TOX is CD4+ T marker or not as evaluated by **Zhang et al.** [9] who 1<sup>st</sup> suggested that, TOX could be used as diagnostic marker for eMF. In the current study, the sensitivity of CD4 IHC stain was 92.5%, specificity

100%, positive predictive value (PVP) 100 %, negative predictive value (PVN) 85% and accuracy 96.25. In addition, we observed that TOX+ lymphocytes were a subset of CD4+ lymphocytes in the MF specimens that were atypical-appearing lymphocytes, with large and atypical hyperchromatic nuclei. This finding suggests that MF tumor cells were CD4+TOX+ lymphocytes. The hypothesis of continuous antigenic -stimulation suggests that, MF is caused by antigen persistence of T cells, which start with initial chronic inflammation (polyclonal reactive T-cell clone) and in turn leads to the development of a malignant monoclonal T-cell clone and MF formation [23]. The limitation of our study was the low number of the patients.

## CONCLUSION

We can conclude that TOX might be considered a diagnostic marker that can differentiate the neoplastic T lymphocytes from the inflammatory (reactive) T lymphocytes that are present not only in BIDs but also in MF itself especially in the setting of strongly positive staining patterns to differentiate MF especially eMF from BIDs. So we recommend that, the possible use of TOX marker as routine investigation for MF mainly eMF.

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## الملخص العربي

### المحدد الجزئي توكس بروتين في تشخيص المراحل الأولية من مرض الفطر الفطرائي

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#### ملخص البحث

**الخلفية:** مرض الفطر الفطرائي هو النوع الأكثر شيوعا من  $\square$  رطان الغدد الليمفاوية للخلايا التائية. يتشابهه مرض الفطر الفطرائي خاصة في مرحله الأولى مع الكثير من الأمراض الجلدية الإلتهابية الحميدة ولذلك يجب التفريقه بينهم لضمان التشخيص والعلاج السليم للفطر الفطرائي. التوكس بروتين هو عامل مهم لنمو وتطور الخلايا التائية في الغدة الصعترية والذي يعتبر عنصرا مهما لتشخيص وتقييم مرض الفطر الفطرائي .

**الهدف:** الهدف من الدراسة الحالية هو تقييم قدرة المحدد الجزئي توكس بروتين في تشخيص المراحل الاولية من مرض الفطر الفطرائي، وقدرته على تمييز المراحل الاولية من مرض الفطر الفطرائي عن الأمراض الجلدية الإلتهابية الحميدة المماثلة.

**الطرق:** أجريت هذه الدراسة على 60 مريضا : 20 مريضا بالمراحل الاولية من مرض الفطر الفطرائي ، 20 مريضا من الامراض الجلدية الإلتهابية الحميدة و 20 عينة جلد طبيعية كمرضى ضابطه. تم تشخيص المرضى بعد التقييم الإكلينيكي و الباثولوجي. تم صبغ العينات بالتوكس بروتين وصبغة المجموعة الاختلافية 4 للخلايا التائية.

**النتائج:** أظهرت النتائج تعبيرًا إيجابيًا كبيرًا في مرضى الفطر الفطرائي مقارنةً بالأمراض الجلدية الإلتهابية الحميدة والمجموعة الضابطه بحساسية 100% وخصوصية 95% , كما كان هناك ارتباط إيجابي بين التوكس بروتين والمجموعة الاختلافية 4 للخلايا التائية في مرضى الفطر الفطرائي .

**الاستنتاجات:** من الممكن اعتبار التوكس بروتين عامل تشخيصي قوى للمراحل الاولية من مرض الفطر الفطرائي , كما يمكنه التمييز بين المراحل الاولية من مرض الفطر عن الأمراض الجلدية الإلتهابية الحميدة المماثلة.

**الكلمات المفتاحية:**  $\square$  رطان الغدد الليمفاوية للخلايا التائية المراحل الأولية من مرض الفطريات الفطر الفطرائي

الباحث الرئيسي :

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