ABSTRACT

Background: Mycosis fungoides (MF) is the most common type of primary cutaneous peripheral T-cell lymphoma. The diagnosis of early-stage MF is challenging due to its clinical and histological resemblance to a number of benign inflammatory dermatoses. Often an immunohistochemical phenotyping is required for the diagnosis.

Purpose: To assess the diagnostic value of a panel of seven lymphocytic immunohistochemical markers (CD3; CD20; CD4; CD8; CD45RO; CD30; Bcl2) in the early diagnosis of MF.

Material/Methods: Biopsies from 30 patients with confirmed early-stage MF were stained with lymphocytic markers: CD3; CD20; CD4; CD8; CD45RO; CD30; Bcl2. The epidermal and dermal expression in this group was compared with a control group of 30 cases of benign inflammatory dermatoses, which clinically resemble MF.

Results: A statistically significant difference was found in the expression of CD3, CD4, CD8, CD45RO and Bcl2 in the epidermis between the two groups and the expression of CD3, CD4, CD8, CD45RO and CD30 in the dermal infiltrate. There was no statistically significant difference in the expression of CD20 and Bcl2 in the dermal infiltrate between the two groups.

Conclusions: There is no single marker that has enough sensitivity and specificity to be used independently for the diagnosis of MF. High epidermal expression of CD3, CD4, and CD45RO by infiltrating lymphocytes is diagnostic for MF. The most sensitive marker for MF is the CD4/CD8 ratio in the dermal infiltrate; it takes into account the statistically different expression of CD4 and CD8 in both groups.

Keywords: cutaneous T-cell lymphoma, mycosis fungoides, immunohistochemistry,
from patients with early MF among the slides available at the Histology and immunohistochemistry laboratory at the Military Medical Academy, Sofia.

The control group (CG) included 30 randomly selected paraffin-embedded biopsy specimens, who were examined in the same laboratory setting with initial clinical suspicion of MF, but the diagnosis of MF has not been confirmed histologically. The final histological report in all selected cases has been consistent with a benign inflammatory dermatosis.

Biopsies from both MF group and control group (60 specimens in total) were stained with a panel of seven immunohistochemical lymphocytic markers: CD3; CD20; CD4; CD8; CD45RO; CD30 and Bcl2. In total, 420 immunohistochemical staining have been performed.

II. Methods
• Immunohistochemical (IHC) staining technique

IHC staining technique is based on the affinity of standardized monoclonal antibodies to respective receptor molecules. The link between the antibody and the receptor is visualized under a light microscope with a specific colour in the area of the bond.

The IHC technique used in this study followed the instructions of the manufacturer (DAKO, Denmark). Formalin-fixed paraffin-embedded tissue samples were used. Histological slides were incubated in a micro-wave to 100°C for 10-15 mins for deparaffination. After that, the samples were cooled and rinsed in Tris-BSA buffer (Tris Buffered Saline with 1%BSA, pH 8.0, Sigma - Aldrich Chemie Dmbh, Germany) for 10 mins. The primary autoantibody (CD3; CD20; CD4; CD8; CD45RO; CD30; Bcl2) was incubated for 60 mins. DAKO ChemMate APAAP (Alkaline Phosphatase-Antialkaline Phosphatase) Kit with red chromogen – immune complex with the inclusion of the primary and secondary autoantibody in the reaction was used for antibody retrieval.

Specifications of the studied lymphocytic receptors and antibodies are listed in table 1.

Table 1. Lymphocytic receptors and primary antibodies

<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>NATURE</th>
<th>ANTIBODY</th>
<th>DELUTION AND RETRIEVAL</th>
<th>SECTIONS</th>
<th>POSITIVE EXPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>T-cell coreceptor</td>
<td>CD3 /Monoclonal Mouse anti-Human CD3, Clone F7.2.38/</td>
<td>1:25; water bath</td>
<td>paraffin-embedded</td>
<td>mature T-cells, neoplastic epidermotropic T-cells in MF</td>
</tr>
<tr>
<td>CD20</td>
<td>B-cell receptor</td>
<td>CD20/Monoclonal Mouse anti-Human CD20cy, Clone L26/</td>
<td>1:200; water bath</td>
<td>paraffin-embedded</td>
<td>normal reactive B-cells neoplastic T- and B-cells</td>
</tr>
<tr>
<td>CD4</td>
<td>surface glycoprotein</td>
<td>CD4 /Mouse Anti-Human CD4 Antigen, Clone MT310/</td>
<td>without dilution; water bath</td>
<td>paraffin-embedded</td>
<td>T-helper lymphocytes, neoplastic MF cells</td>
</tr>
<tr>
<td>CD8</td>
<td>co-receptor of the TCR</td>
<td>CD8 /Mouse Anti-Human CD8 Antigen, Clone DK25/</td>
<td>without dilution; water bath</td>
<td>paraffin-embedded</td>
<td>cytotoxic T-cells, NK cells and dendritic cells, rarely neoplastic MF cells</td>
</tr>
<tr>
<td>CD45RO</td>
<td>lymphocytic marker</td>
<td>CD45Ro / Monoclonal Mouse Anti-Human CD45RO Antigen, Clone UCHL1/</td>
<td>without dilution; water bath</td>
<td>paraffin-embedded</td>
<td>activated T-lymphocytes or T-memory cells, MF cells</td>
</tr>
<tr>
<td>CD30</td>
<td>cell membrane protein of the tumor necrosis factor receptor family and tumor marker</td>
<td>CD30 /Mouse Anti-Human CD8 Antigen, Clone DK25/</td>
<td>1:20; water bath</td>
<td>paraffin-embedded</td>
<td>virus-infected lymphocytes, neoplasms of lymphoid origin, a subset of activated T cells, MF cell in large transformation</td>
</tr>
<tr>
<td>Bcl2</td>
<td>anti-apoptotic oncoprotein</td>
<td>Bcl2 /Monoclonal Mouse Anti-Human BCL2 Oncoprotein, Clone 124/</td>
<td>without dilution; water bath</td>
<td>paraffin-embedded</td>
<td>loss of normal expression of Bcl-2 protein in melanoma, breast, prostate, chronic lymphocytic leukemia, and lung cancer</td>
</tr>
</tbody>
</table>
• Evaluation of the IHC expression

The IHC expression was evaluated under light microscopy (OLYMPUS BX51TF).

The staining with CD3, CD4, CD8, CD45RO and Bcl2 in the epidermis was evaluated as the average absolute number of positively stained cells in field x400 magnification after examination of 5 random microscopic fields.

The staining with CD3, CD20, CD4, CD8, CD45RO, CD30 and Bcl 2 in the dermis was evaluated as a percentage of the dermal infiltrate.

For documentation, a microscopic digital camera OLYMPUS DP70 has been used.

• Statistical methods

Rstudio ver. 0.97.551 software has been used for statistical evaluation of the data. The Welch Two Sample t-test has been implemented to test the hypothesis that there is no significant difference in the means in the two groups (MF group and CG) with a confidence interval (CI) of 95%. In cases, when this hypothesis has been ruled out, a conclusion has been made that there is a significant difference in the means in the two groups and respectively the marker has diagnostic value.

RESULTS

Expression in the epidermis

The results from our study showed that the average expression of CD3 in the epidermis in the material from the MF group (9.1 Ly/field x400) was significantly higher than in the CG (5.4 Ly/field x400) (p = 0.00282, 95% CI). Similar results were observed regarding the expression of CD4 in epidermotropic lymphocytes in both groups (6.4 Ly/field x400 in MF group; 1.6 Ly/field x400 in CG, p = 1.766e-07, 95% CI). On the contrary, the average number of CD8-positive lymphocytes was significantly higher in the epidermis in the CG (3.5 Ly/field x400) than in the MF group (1.7 Ly/field x400)(p = 9e-04, 95% CI). The mean calculated CD4/CD8 ratio in the MF group, and the CG was respectively: 3.7 and 0.45.

Table 2. Comparison between the IHC expression of lymphocytic markers in the epidermis in MF group and control group (presented as an average number of positive cells/field x400)

<table>
<thead>
<tr>
<th>IHC marker</th>
<th>mean value (MF group)</th>
<th>mean value (control group)</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>9.100000</td>
<td>5.433333</td>
<td>0.00282</td>
<td>1.343967</td>
</tr>
<tr>
<td>CD4</td>
<td>6.466667</td>
<td>1.666667</td>
<td>1.766e-07</td>
<td>3.261452</td>
</tr>
<tr>
<td>CD8</td>
<td>1.733333</td>
<td>3.533333</td>
<td>9e-04</td>
<td>-2.8228749</td>
</tr>
<tr>
<td>CD45RO</td>
<td>6.200000</td>
<td>2.566667</td>
<td>9e-04</td>
<td>1.904693</td>
</tr>
<tr>
<td>Bcl2</td>
<td>5.033333</td>
<td>1.500000</td>
<td>1.688e-06</td>
<td>2.264234</td>
</tr>
</tbody>
</table>

The most important findings included: a significantly higher percentage of CD3-positive cells in the dermal infiltrate in the MF group (81%) than in the CG (65%), (p=1.647e-12, CI 95%). Similar results were observed regarding the expression of CD4 - 69% positive cells in the MF group and 39% in the CG (p= 1.879e-15, 95% CI) – and

Expression in the dermis

The results of our study regarding the expression of the IHC markers in the dermis are presented in table 3.

Table 3. Comparison between the IHC expression of lymphocytic markers in the dermis in MF group and control group (presented as a percentage of the dermal infiltrate)

<table>
<thead>
<tr>
<th>IHC marker</th>
<th>mean value (MF group)</th>
<th>mean value (control group)</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>80.83333</td>
<td>65.33333</td>
<td>1.647e-12</td>
<td>12.06896</td>
</tr>
<tr>
<td>CD20</td>
<td>3.433333</td>
<td>3.933333</td>
<td>0.2483</td>
<td>-1.360059</td>
</tr>
<tr>
<td>CD4</td>
<td>68.83333</td>
<td>39.33333</td>
<td>1.879e-15</td>
<td>24.34522</td>
</tr>
<tr>
<td>CD8</td>
<td>23.83333</td>
<td>33.66667</td>
<td>0.0002717</td>
<td>-14.905305</td>
</tr>
<tr>
<td>CD45RO</td>
<td>71.33333</td>
<td>52.50000</td>
<td>1.454e-07</td>
<td>12.98227</td>
</tr>
<tr>
<td>CD30</td>
<td>5.6</td>
<td>0.4</td>
<td>0.0005397</td>
<td>2.461484</td>
</tr>
<tr>
<td>Bcl2</td>
<td>26.50000</td>
<td>26.16667</td>
<td>0.88</td>
<td>-4.089352</td>
</tr>
</tbody>
</table>

The complete data regarding the IHC expression in the epidermis in both groups are presented in table 2.
CD45RO – 71% in the MF group and 53% in the CG (p = 1.454e-07, 95% CI 95%). The opposite tendency was observed regarding the expression of CD8, which was significantly higher in the dermal infiltrate in the CG (37%) than in the MG group (24%), (p = 0.0002717, CI 95% ). The mean calculated CD4/CD8 ratio in the MG group, and the CG was respectively: 2.9 and 1.0.

The expression of CD20 and Bcl2 did not differ significantly in both studies groups (Tabl. 3)

**DISCUSSION**

The IHC staining is the gold standard for diagnosis of early-stage MF in cases where the differential diagnosis with benign inflammatory dermatoses (psoriasis vulgaris, atopic dermatitis, ichthyosis, chronic actinic dermatitis, lichen planus, lichen sclerosus et atrophicus, sicca striatus, lichen planus-like keratosis, vitiligo, etc.) cannot be ruled out by simple histological examination. There is no single marker that is specific for MF, and usually, the use of multiple antibodies is required [3].

Immunologically, MF cells usually express a CD3+CD4+CD45RO+ phenotype and represent memory T-lymphocytes. Rarely, CD3+CD4-CD8+ cytotoxic/suppressor phenotype is observed in MF with generally no difference in the clinical course and prognosis [4].

Although, the research about a specific marker that could distinguish between MF and its benign inflammatory mimickers is rapidly progressing there is currently no sufficient data to implement a new marker in the clinical practice [5].

The IHC antibody panel used routinely for diagnosis of MF usually includes CD3, CD20, CD4, CD8, CD4/CD8 ratio, CD30. Rarely other markers such as Ki67, CD2, CD5, CD7 find a place in the clinical practice [6].

The data of our study about the expression of the investigated IHC panel showed that the presence of CD3+CD4+CD45RO+ epidermotropic lymphocytes was significantly higher in the MF group than in the CG. These could be explained by the accumulation of higher numbers of MF cells in the epidermis in the form of single cells and Pautrier microabcesses [7].

Although some cases of CD3+CD4-CD8+ MF were included in this study, generally, the CD8+ lymphocytes that infiltrate the epidermis in MF are from the reactive origin and do not represent neoplastic MF cells. In the control group, all infiltrating lymphocytes are reactive, and this explains the significantly higher expression of CD8 in the epidermis in the CG [8].

Our study shows a predominantly CD3+ cell’s infiltrate in both groups with a significantly higher percentage of CD3+ cells in the MF group. The CD3 receptor is expressed on all mature T-lymphocytes – both reactive and neoplastic. The higher expression in the MF group may be due to the relative restriction of other cells elements, such as CD20 and CD8, as a result from the tumorigenesis. This tendency has been previously reported by other authors [9].

Our results differ from the data published in an original study by Asadullah et al. which shows a higher percent-age of activated CD8+ lymphocytes and NK cells in MF than in inflammatory dermatoses (psoriasis vulgaris; atopic dermatitis) and healthy volunteers. The authors support the thesis that the CD8+ are polyclonal and play a key role in anti-neoplastic immunity [10].

Further studies are needed to understand the role of infiltrating CD8+ lymphocytes in MF.

Although both individual expressions of CD4 and CD8 differ significantly in both groups, the calculated CD4:CD8 ratio in the dermal infiltrate seems most important for the diagnosis of MF.

According to our study, the mean calculated CD4/CD8 ratio in the MF group, and the CG is respectively: 2.9 and 1.0. Our data confirm the findings of Nuckols et al. who report a CD4/CD8 ration above 2 in 65% of the cases of MF.

Some authors report even higher values of CD4/CD8 ratio ≥6 in MF. This higher value could possibly be explained by the fact that not only early diagnostic biopsies of MF were included in the study. In advanced stages, an increase in the CD4/CD8 ratio may be expected due to the lower rates of reactive infiltrating CD8+ lymphocytes [11].

CD20 is expressed on the cellular membrane of normal reactive B-cells. Rarely, some neoplastic T-cells may express CD20. The predominant expression of CD3 in the neoplastic infiltrate is suggestive for a T-cell neoplasia, while the predominance of CD20 weights towards a B-cell neoplasia. All imitators of MF represent T-cell mediated skin reactions and have low expression of CD20.

Actually, the main diagnostic value of CD20 in early MF is to distinguish between a T-cell and a B-cell neoplasia, which can be challenging in some cases [12]. CD20 has no use in differentiating between MF and benign T-cell mediated skin reactions.

CD30 may be considered a specific, but a not sensitive marker to differentiate MF from benign inflammatory dermatoses since it is rarely expressed by neoplastic cells in early MF and is related to the large cell transformation (LCT) of MF in more advanced stages. CD30 has minimal or no expression in cases of benign dermatoses [13].

Existing data suggest significant abnormalities in the antiapoptotic oncogenic molecule of Bcl2 in MF. Previous studies suggest lower expression of Bcl2 in MF and in other solid tumours [14].

However, our study does not demonstrate a statistically significant difference in the expression of Bcl2 in the MF group and in the CG, and we consider that the expression of Bcl2 in the dermal infiltrate could not be used for differentiating MF from benign inflammatory dermatoses.

**CONCLUSIONS**

Our study examined the usefulness of a panel of seven antibodies (CD3, CD20, CD4, CD8, CD45RO, CD30 and Bcl2) for the diagnosis of early-stage MF, by comparing their expression in early diagnostic biopsies of MF and a control group of benign inflammatory imitators of MF.

There is no single marker that has enough sensitivity and specificity to be used independently for the diagnosis of MF. High epidermal expression of CD3, CD4, and CD45RO by infiltrating lymphocytes is diagnostic for MF. The most
sensitive marker for MF is the CD4/CD8 ratio in the dermal infiltrate, which incorporates both the statistically different expression of CD4 and CD8 in the two groups. Further studies are needed to find the most appropriate range for this ratio as a diagnostic criterion in MF. Expression of CD20 and Bcl2 in the dermal infiltrate could not be used to differentiate MF from clinical and histological imitators.

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ABBREVIATION LIST:
PC-PTCL – primary cutaneous peripheral T-cell lymphoma
MF – mycosis fungoides
ICH – immunohistochemistry
CG – control group
Ly – lymphocytes
TCR – T-cell receptor

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