INVESTIGATIVE REPORT

Expression of SOX18 in Mycosis Fungoides

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SOX18 is a transcription factor involved in the development of hair follicle, blood and lymphatic vessels, as well as regenerative processes. In addition, accumulated data indicate the role of SOX18 in tumourigenesis. So far, no studies on the role of SOX18 expression in mycosis fungoides (MF), the most common primary cutaneous T-cell lymphoma, have been performed. Therefore, we evaluated SOX18 expression in MF at the mRNA and protein level. SOX18 expression was observed predominantly on the blood and lymphatic vessels, in the intratumoural and peritumoural microenvironment of MF. The intratumoural, but not peritumoural, expression of SOX18 correlated positively with the advancement of the disease, cutaneous involvement and extracutaneous metastases at the protein level (p < 0.001, p < 0.001, p = 0.004, respectively). Significantly lower SOX18 mRNA expression was correlated with lymph node involvement (p = 0.01). In conclusion, we hypothesize that SOX18, as a marker of neovascularization, may be involved in the progression of MF.

Key words: mycosis fungoides; SOX18; angiogenesis; lymphangiogenesis; proliferation markers; Ki-67.

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Mycosis fungoides (MF) is the most common subtype of primary cutaneous T-cell lymphomas (CTCL), defined by skin-only involvement without extracutaneous spread at the time of presentation. MF is characterized by a long-term disease course, slow progression and favourable prognosis, in which it resembles indolent nodal non-Hodgkin’s lymphomas. Clinically, in the early stages, MF manifests with itchy patches and plaques that strongly assimilate benign dermatoses, i.e. eczema, lichen planus or psoriasis vulgaris, and therefore represents a diagnostic challenge for dermatologists and pathologists (1, 2). The skin lesions tend to withdraw and relapse, and the disease can proceed in this way for many years. In some cases, however, MF may rapidly progress, by skin tumour development and systemic dissemination, which significantly worsens the prognosis and survival. Despite intensive research over past decades, mechanisms impacting MF development and spread, as well as factors affecting the disease course have not been fully disclosed. Male sex, advanced age, advanced clinical staging, increased serum lactate dehydrogenase level and eosinophilia have been identified as unfavourable prognostic factors; however, novel diagnostic and prognostic tools in the MF evaluation are needed (3–6). Recent studies have also focused on the role of neovascularization in MF growth and dissemination (7).

SRY-related HMG-box 18 (SOX18) belongs to the SOX family of genes encoding transcription factors, structurally characterized by a high-mobility group (HMG) domain, which specifically binds to the 5’-(A/T) (A/T)CAA(A/T)G-3’ DNA sequence motif (8, 9). Based on their amino acid homology, members of the SOX family have been divided into 10 groups (A–J). Along with SOX7 and SOX17, SOX18 belongs to the group F, and plays an important role in the vascular development and lymphatic fate specification (10). Mutation of SOX18 has been associated with aberrant blood and lymphatic system development, resulting in hypotrichosis-lymphoedema-telangiectasia syndrome, the rare developmental condition, affecting hair follicles, blood as well as lymphatic vasculature (11). In addition, a SOX18 de novo mutation has been associated with congenital disorders, including aortic dilation, hypotrichosis and telangiectasia (12). Since pathological vascularization is likely to repeat genetic programmes and signalling pathways during embryonic vessel development, it is not surprising that the markers of physiological angiogenesis and lymphangiogenesis are also found in the processes of tumourigenesis. Recently, in vitro and in vivo models, as well as clinical studies have indicated the role of SOX18 in tumour growth and spread (13–15). Duong et al. (14) in the study on a mouse model of melanoma have observed that SOX18 disruption impairs neolymphangiogenesis during tumour growth and the partial loss of SOX18 reduces cancer cell metastasis. Similarly, in another study, allograft melanoma tumours in the mice heterozygous for the dominant-negative SOX18 mutation (Sox18RaOp) or null for SOX18 showed reduced growth and microvessel density (16). Increased SOX18 expression has been demonstrated in endothelial cells of blood and lymphatic vessels indicating its role in vascularization of the neoplastic tissue (17). On the other hand, recent in vitro studies have documented SOX18 expression in breast, gastric and pancreatic cancer as well as
and metastases. In this study, we focused on SOX18 expression in tissue samples and cell lines of invasive breast cancer, Pula et al. (15) have noted increased SOX18 expression in cancer cells that correlated with higher malignancy grades. In another study by Wang et al. (19) SOX18 knockdown cell lines derived from hepatocellular carcinoma (HCC) showed inhibition of the proliferation as well significantly impaired migration and invasion.

The aim of our study was to evaluate the diagnostic and prognostic value of SOX18 in the most common CTCL, mycosis fungoides. Utilizing immunohistochemical (IHC) and molecular methods, we assessed the expression of SOX18 in relation to clinicopathological data, mostly the progression of the disease. In addition, based on the observations linking SOX18 expression with aggressive behaviour of the malignancy, we also correlated its expression with the proliferation markers Ki-67 and mini-chromosome maintenance proteins 3 and 7 (MCM-3, MCM-7). To the best of our knowledge, no studies analysing the role of SOX18 expression in CTCL, including MF, have been published until now.

METHODS

Patients

The study was conducted on archival paraffin-embedded samples collected during diagnostic procedures from 80 patients with MF (29 women, 51 men, mean age: 59.2 ± 14.2 years, median: 62.5 years, range: 19–91 years), treated between the years 1994 and 2015 in the Department of Dermatology, Venereology and Allergology (Wroclaw Medical University, Poland). The diagnosis of the disease was established or re-evaluated based on clinical, histopathological and IHC examinations, according to the World Health Organization (WHO) classification (2008) (20). The staging was assessed according to TNMB (Tumour, Nodes, Metastases, Blood) system (ISCL/EORTC revision (Tables S1 and SII)) (21). Forty-seven patients were classified as the early stage (IA–IIA) and 33 were in the advanced stage (IIB–IVB). Healthy skin and chronic dermatitis samples in order to elucidate the differences between benign and malignant skin conditions. From 26 patients (13 women, 13 men; 16 in the early and 10 in the advanced stage, mean age 55.3 ± 15.7 years) and 7 controls skin biopsies were obtained. Forty-seven patients were classified as the early stage (IA–IIA) and 33 were in the advanced stage (IIB–IVB). Healthy skin and chronic dermatitis samples in order to elucidate the differences between benign and malignant skin conditions. From 26 patients (13 women, 13 men; 16 in the early and 10 in the advanced stage, mean age 55.3 ± 15.7 years) and 7 controls skin biopsies were obtained. Forty-seven patients were classified as the early stage (IA–IIA) and 33 were in the advanced stage (IIB–IVB). Healthy skin and chronic dermatitis samples in order to elucidate the differences between benign and malignant skin conditions. From 26 patients (13 women, 13 men; 16 in the early and 10 in the advanced stage, mean age 55.3 ± 15.7 years) and 7 controls skin biopsies were obtained.
Western blot

Frozen samples were thawed in CellLytic MT Cell Lysis Solution (Sigma Aldrich, Munich, Germany) with the addition of protease inhibitors, Benzonase – 50 U/ul (Merck; Millipore, Bedford, MA, USA) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations of whole cell lysates were determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Equal amounts of total protein (30 µg) were mixed with sample buffer and dithiothreitol (DTT) and resolved by SDS-PAGE. After the completion of the electrophoresis, the samples were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA) and incubated in a 4% BSA solution in Tris-buffered saline (TBS) with the addition of 0.1% Tween-20. Later on, the membranes were incubated with mouse anti-human SOX18 antibody – 1:100 (sc-166025; Santa Cruz Biotechnology Inc.) for a night at 4ºC. At the end the membranes were treated with the peroxidase-conjugated donkey anti-mouse secondary antibody – 1:3,000 (715-035-150; Jacksons Immunoresearch, Suffolk, UK) for 1 h, rinsed, and incubated with the Immun-Star-HRP Chemiluminescent Substrate (Bio-Rad, Hercules, CA, USA). Protein quantifications were based on the total protein normalization.

Statistical analysis

All data were analysed with Statistica12.0 software (Statsoft, Krakow, Poland). Patients and controls were compared using χ² test, unpaired Student’s t-test, Mann-Whitney U test and analysis of variance with Scheffé post hoc test. Relationships between quantitative data were verified with Spearman rank correlation test. Kaplan-Meier curves overall survival were calculated from the date of the start of therapy until the latest follow-up. The differences between the curves were assessed by log-rank test. p-values < 0.05 were considered as statistically significant.

RESULTS

Expression of SOX18 in regard to patients’ clinical and pathological data

The expression of SOX18 was observed in 77 out of 80 MF samples and 11 out of 18 controls (p<0.001). The studied marker was expressed predominantly in the nuclei of endothelial cells of blood and lymphatic vessels. The expression was noted in the peritumoural area as well as in the intratumoural microenvironment (Fig. 1C–D). In some cases nuclear expression of SOX18 was found in neoplastic cells, but reactivity was weak, therefore, it was not taken into further consideration. Among the tested samples, the intratumoural expression of SOX18 was significantly higher in MF compared with chronic benign dermatoses (mean ± standard deviation (SD): 5.9 ± 3.0 and 4.2 ± 2.0, respectively, p=0.03). To determine the correlation between SOX18 expression and the advancement of the disease, its expression was analysed with respect to the MF staging. SOX18 intratumoural expression was significantly higher in advanced stages compared with early ones (p<0.001) (Table I). We also found increased expression of intratumoural SOX18 in more infiltrated skin lesions (tumours, T3) compared with the less infiltrated ones (patches, plaques (T1, T2) (p<0.001) and erythroderma (T4) (p=0.03) (Table I). Regarding metastases, the increased expression of intratumoural SOX18 was linked with the disease dissemination to the lymph nodes (p=0.04). Analysing the peritumoural SOX18 expression, no significant differences were observed between MF and benign dermatoses, as well as between various MF groups divided based on its advancement.

To support the results obtained by IHC reaction we also studied SOX18 expression in frozen samples of MF and control group (19 and 6 subjects, respectively) using the Western blot technique. The analysis revealed slightly higher expression of SOX18...
in the MF group (0.07 ± 0.06 vs. 0.06 ± 0.04, \( p = 0.79 \)), albeit not reaching statistical significance.

Real-time PCR analysis of \( SOX18 \) mRNA expression was performed in 26 MF samples and 7 control cases. Significantly lower mean \( SOX18 \) mRNA levels were observed in MF group than in chronic benign dermatoses (RQ 0.6 ± 0.3 and 1.4 ± 0.9, respectively, \( p < 0.001 \)). In addition, higher \( SOX18 \) mRNA expression was associated with less advanced lymph node involvement (stage N0: 0.68 ± 0.37 vs. N1: 0.48 ± 0.17 vs. N2: 0.18 ± 0.03, Scheffé post hoc test: N2 vs. N0: \( p < 0.001 \), N2 vs. N1: \( p = 0.01 \)), but with more infiltrative nature of MF (stage b vs. stage a: 0.6 ± 0.2 vs. 1.0 ± 0.4, \( p = 0.03 \)).

Correlation of \( SOX18 \) expression with proliferation markers: Ki-67, MCM-3 and MCM-7

In the present study we found a statistically significant correlation between intratumoural and peritumoural \( SOX18 \) expression (\( r = 0.37; p < 0.001 \)) (Fig. 2A). We also focused on the expression of the proliferation markers: Ki-67, MCM-3 and MCM-7 in relation to \( SOX18 \) and we noted a statistically significant correlation between intratumoural \( SOX18 \) and Ki-67, MCM-3 and MCM-7 expressions (Fig. 2B–D). In contrast, statistical analysis did not reveal any significant correlation between peritumoural \( SOX18 \) and Ki-67, MCM-3 and MCM-7 expression.

### Table I. Expression intensities of intratumoural and peritumoural \( SOX18 \) in mycosis fungoides

<table>
<thead>
<tr>
<th>Stage</th>
<th>Intratumoural ( SOX18 )</th>
<th>Peritumoural ( SOX18 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median (range)</td>
</tr>
<tr>
<td>T1</td>
<td>4.8 ± 2.1</td>
<td>5.3 (0–9.3)</td>
</tr>
<tr>
<td>T2</td>
<td>4.9 ± 2.5</td>
<td>5.0 (0–8.7)</td>
</tr>
<tr>
<td>T3</td>
<td>9.0 ± 3.8</td>
<td>8.0 (4.0–16.0)</td>
</tr>
<tr>
<td>T4</td>
<td>6.2 ± 1.8</td>
<td>6.3 (3.3–10.0)</td>
</tr>
<tr>
<td>Stadium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>4.9 ± 2.2</td>
<td>5.3 (0–8.7)</td>
</tr>
<tr>
<td>b</td>
<td>5.2 ± 2.5</td>
<td>5.2 (0–9.3)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>5.4 ± 2.9</td>
<td>5.3 (0–14.0)</td>
</tr>
<tr>
<td>N1–3</td>
<td>6.7 ± 3.0</td>
<td>6.3 (3.3–16.0)</td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0</td>
<td>5.9 ± 3.0</td>
<td>5.7 (0–16.0)</td>
</tr>
<tr>
<td>B1</td>
<td>5.7 ± 1.8</td>
<td>5.7 (3.3–8.0)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>4.9 ± 2.3</td>
<td>5.3 (0–9.3)</td>
</tr>
<tr>
<td>Advanced</td>
<td>7.4 ± 3.2</td>
<td>6.7 (3.3–16.0)</td>
</tr>
<tr>
<td>Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>5.5 ± 2.9</td>
<td>5.3 (0–16.0)</td>
</tr>
<tr>
<td>Dead</td>
<td>6.2 ± 2.7</td>
<td>6.0 (0–14.0)</td>
</tr>
</tbody>
</table>

Analysis of variance with post hoc test Scheffe and Student’s t-test. Significant values are shown in bold.

Fig. 2. Spearman-rank correlation test revealed moderate, albeit significant, positive associations between intratumoural expression of \( SOX18 \) and peritumoural \( SOX18 \) (A), Ki-67 (B), MCM-3 (C) and MCM-7 (D) in patients with mycosis fungoides.
tumoural SOX18 expression and studied proliferation markers. Detailed IHC analysis of the expression of proliferation markers Ki-67, MCM-3 and MCM-7 in CTCL was published previously (22).

The detailed analysis of patients’ survival in regard with advancement of the disease and the epidemiological data were shown in our previous report (22). In the present study we focused on the patients’ survival in regard to the SOX18 expression and we did not find any significant relationship between patient’s survival and intratumoural, as well as peritumoural SOX expression. In addition, no other associations between SOX18 immunoreactivity and sex, age and disease duration were noted (data not shown).

**DISCUSSION**

Accumulated data indicate the role of SOX18 in tumourigenesis. Recent studies have revealed the increased SOX18 expression in tumour cells as well as in endothelial cells of blood and lymphatic vessels in intratumoural and peritumoural areas, which may indicate a double role of SOX18 in the growth and spread of neoplasms (15, 19, 23).

In our study, for the first time in the literature, we have documented the expression of SOX18 on both mRNA and protein level, and its correlation with the progression of MF. In other recent studies focusing on the SOX18 expression in lung, breast and ovarian tumours, the expression of the analysed transcription factor was observed in both cancer and endothelial cells of blood and lymphatic vessels (15, 23, 24). On the contrary, in MF, the significant expression of SOX18 was found almost exclusively in the blood and lymphatic endothelium of intratumoural and peritumoural areas. Therefore, we assumed that the role of this factor in the development and spread of MF might be associated with neovascularization of the malignancy. Vascularization of the tumour is essential for its growth and dissemination. It is also one of the key markers of disease outcome. Numerous studies published over the past decades showed a close relationship between the vascularization of the tumour, its histological grade and clinical progression and poor prognosis in non-Hodgkin’s lymphomas (NHL) (25–28). In contrast, relatively little attention was paid to primary CTCLs in the aspect of angiogenesis versus lymphangiogenesis. In our study, comparing malignant and non-malignant skin samples, we observed a higher SOX18 reaction intensity in the vessels of MF than in vessels from lichen planus and disseminated eczema samples, representing benign inflammatory dermatoses. The SOX18 overexpression in MF tissues suggests the role of this factor in tumour growth and angiogenesis rather than in the inflammatory processes. Similar results were obtained by Vacca et al. (29) who evaluated angiogenesis, measured by the MVD assessment in the skin biopsies obtained from MF patients and noticed significantly higher MVD in the skin lesions compared with the unchanged clinically patients’ skin samples. We also noted the correlation between SOX18 reaction intensity and advancement of the disease. The endothelial expression of SOX18 was significantly higher in advanced stages compared with the early ones, which may reflect the increase in vascularity associated with disease progression. These results are consistent with our previous observations concerning angiogenesis in MF, which demonstrated increased angiogenesis measured by CD34 expression in advanced vs. early stages (30). Similarly, in Sézary syndrome (SzS), an aggressive leukaemic counterpart of MF, blood and lymphatic vessel density measured by CD31 and podoplanin expression, respectively, increased in parallel with progression of the disease (7). Even though MF represents a different type of malignant disease compared to solid tumours, MF data are also supported by the study of Pula et al. (24) who reported the correlation between higher SOX18 expression and advanced disease stage in ovarian cancer.

In our study, we documented the differential SOX18 expression regarding skin and lymph node involvement in MF patients. More infiltrated cutaneous lesions were characterized by a higher intratumoural SOX18 expression, whereas no correlation between peritumoural SOX18 expression and tumour staging was found. Similarly, in the studies of Mazur et al. (31) and Jankowska-Konsur et al. (30), progression of the skin lesions correlated with higher MVD in MF. We also observed that the higher intratumoural, but not peritumoural SOX18 reaction intensity was also associated with the dissemination of malignant cells to the lymph nodes. Our results are in concordance with other studies focusing on the impact of SOX18 on cancer development and spread, e.g. the experiments on melanoma allografts in mice models have documented the metastatic potential of SOX18 tumoural expression (14). In addition, the putative impact of SOX18 on cancer dissemination was also demonstrated by Wang et al. (19) who have observed the impaired migration and invasion of SOX18 knock-down hepatocellular cancer cells in the transwell migration assay. Interestingly, although our results strongly suggest the role of SOX18 in the disease progression, its expression did not affect patient survival. On the contrary, in solid tumours (breast, ovarian and non-small cell lung cancer) higher SOX18 expression correlated with poor prognosis (15, 23, 24). Insignificant correlations between survival and intratumoural SOX18 expression might be associated with small sample size. However, these differences may also be partially explained by the different nature of MF, and further research determining the impact of SOX18 expression on the clinical outcome in MF is needed.

In the present study, we obtained divergent results concerning the levels of SOX18 mRNA and protein expression levels in MF and chronic benign dermatoses. Significantly lower SOX18 mRNA expression was...
observed in MF compared with the control group. Conversely, the **SOX18** protein expression level was higher in MF group. Recent *in vitro* and *in vivo* studies on the lung cancer have documented similar discrepancy of **SOX18** mRNA and protein level (23, 32, 33). According to the authors the reason for this divergence could be the hypermethylation, frequently observed in this type of malignancy. Accumulated data over the last decade indicates that gene expression may be regulated at the transcriptional and posttranscriptional level by a number of epigenetic mechanisms and epigenetic-related microRNA (miRNA) activity. In tumours these phenomena may be of particular importance, especially in the context of genetic instability, characteristic feature of malignancies (34, 35).

Uncontrolled proliferation is one of the hallmarks of a malignancy, indicating a tumour’s aggressiveness potential. Over 2 decades proliferation rate has been routinely evaluated using Ki-67 antigen, a non-histone nuclear protein; however, new proliferation markers, i.e. MCM, have been implemented in cancer assessment recently. Accumulated data have pointed to the correlation between proliferation rate and aggressiveness of CTCL, disease advancement and poor clinical outcome (30, 32, 37, 38). Therefore, in the current study, we checked the association between the expression of **SOX18** and the proliferation markers Ki-67, MCM-3 and MCM-7 as indicators of the malignancy aggressiveness. The significant correlations between all analysed markers of proliferation and **SOX18** found in our study suggest that both, high proliferation rate and increased vascularization of the tumour, are determinants of the aggressive course of the malignant process. Our results are consistent with the study of Jethon et al. (23), who found a positive correlation between **SOX18** and Ki-67 expression in non-small lung cancer.

This report for the first time documents the expression pattern of **SOX18** in MF, as well as its impact on the clinical progression of the disease. We found that intratumoural **SOX18** expression in the endothelial cells of the tumour vessels correlates with advanced stage, cutaneous involvement and metastatic status. Interestingly, although our results clearly indicate the role of **SOX18** in the disease progression, its expression did not affect patient survival. In our opinion, **SOX18** could be considered as a new marker of the disease advancement, as well as the subject of research on new therapeutic strategies; however, further studies are needed.

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