MicroRNAs modulate the expression of the *SOX18* transcript in lung squamous cell carcinoma

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Abstract. Recent statistics show that lung cancer is the second most common malignant tumor in the world (14% of all cancers in the USA), both in terms of morbidity and mortality. The mortality of this type of tumor shows an increasing trend (28% for men and 26% for women). Lung squamous cell carcinoma (LSCC) is the second-largest histological subtype of non-small cell lung cancers (NSCLCs) after adenocarcinoma. SRY-related HMG-box 18 (SOX18) protein is an important transcription factor involved in the development of the cardiovascular system and the lymphatic ducts. In addition, it was observed that SOX18 functions in wound healing processes and the development of atherosclerosis. Likewise, an increased level of this protein was found in melanomas and malignant pancreatic, stomach and breast tumors. Furthermore, high expression of SOX18 in gastric cancer stromal cells was found to be associated with a poor patient prognosis. In the present study, we analyzed the expression of the SOX18 protein and the mRNA level in postoperative samples of LSCC and non-malignant lung tissues (NMLTs), and a disparity in both levels was observed. Because of the fact that microRNAs (miRNAs) play important roles in the initiation and progression of lung cancer, the main aim of this study was to identify the miRNAs that interact with the *SOX18* transcript in NSCLC cases. *SOX18* mRNA expression level was significantly lower in the LSCC tissues than that noted in the NMLTs ($p<0.01$). However, protein levels were higher in the LSCC cases compared to these levels in the NMLTs ($p<0.0001$). We showed that miR-7a and miR-24-3p were expressed more highly in the NMLTs than levels in the LSCC samples, and that they could be switched off in lung cancer tissue. Additionally, correlations between RQ-values of SOX18 in NMLTs and LSCC samples ($r=0.43$, $p=0.019$), and between miR-7a and miR24-3p in NMLT cases ($r=0.4$, $p=0.057$) as well as in the LSCC samples ($r=0.51$, $p=0.012$) were noted. In conclusion, miRNAs interact with the mRNA of the *SOX18* gene, but the mechanism by which they could be inhibited in cancer cells requires further examination.

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Introduction

The molecular pathogenesis of the development of non-small cell lung cancer (NSCLC) is very complex (1). Understanding the molecular basis of the development of this malignant tumor, especially lung squamous cell carcinoma (LSCC), may enable the use of targeted therapy, which may result in a greater efficiency in the treatment of these patients. It is therefore important to search for new and more effective therapeutic strategies as well as new proteins that may be used as potential targets (1,2). In this sense, the SOX protein family appears to be an auspicious element in anticancer therapy (3).

SRY-related HMG-box (SOX) family genes were isolated in mammals in 1990 on the basis of the presence of the conservative high mobility group (HMG) box protein domain, primarily occurring in the sex-determining region Y (SRY) (4). Varying expression levels of SOX proteins have been attested depending on the type of cancer in which they occur. This may indicate that the same protein can serve opposing functions in different tumors (5). Approximately 20 proteins belong to the SOX family, and they are divided into 8 main groups denoted from A to H (6,7). Group F comprises the proteins SOX7 (8), SOX17 (9) and SRY-related HMG-box 18 (SOX18) (10), which are involved in the same pathways as the vascular endothelial growth factor (VEGF). The SOX18 protein is one of the most important proteins involved in the development of blood and lymphatic vessels during embryogenesis (11-17). Recent studies have also shown that the SOX18 protein may play a significant role in the progression of malignant diseases (6,17-21).

Based on the results of our previous research (3), we observed a differential SOX18 expression both at the mRNA and protein level in NSCLC and non-malignant lung tissues (NMLTs). We noted significantly lower mRNA expression levels of this transcription factor in paired tissues and

in all of the studied NSCLC tissues as compared to NMLTs. In contrast, increased SOX18 protein levels were observed in NSCLC cases compared to that noted in the NMLTs (3). Interestingly, the level of mRNA did not reflect in any way the level of protein, determined by western blot analysis. This allowed us to hypothesize that the *SOX18* transcript level could be controlled by microRNA (miRNA) molecules, since similar mechanisms are observed in many other types of tumors in relation to different types of proteins, as for example miRNA-34b in prostate cancer (22).

miRNAs are involved in many important biological processes, such as the regulation of cell proliferation, cell differentiation, apoptosis, embryogenesis and organogenesis (23-26). An increasingly visible role of miRNAs in the regulation of cell proliferation processes, cell differentiation and apoptosis has drawn the attention of scientists to the relationship between miRNAs and carcinogenesis (26). As evidenced, miRNAs not only regulate the expression of multiple oncogenes and tumor-suppressor genes, but may also act themselves as oncogenes and tumor suppressors. Those miRNAs with pro-apoptotic activity can function as tumor suppressors, inhibiting proliferation. The correlation between miRNAs and patient survival indicates the possibility to use miRNAs as potential tumor prognostic markers (24,27-30). A relationship between the expression level of 8 miRNAs and the survival of patients with lung adenocarcinoma (AC) has been shown (29). Patients with increased expression of miR-155, miR-17-3p, miR-106a, miR-93 or miR-21, or reduced expression of miR-7a-2, miR-7b or miR-145 exhibited a significantly lower survival rate (31). The prospects for the use of miRNAs in cancer therapy appear promising as well. It has been shown that inhibition of miRNAs may lead to a reduction in tumor cell proliferation *in vitro* (31).

The role of SOX18 expression in LSCC and other types of lung cancer is not fully understood. Yet, considering previous reports, this protein may be a significant factor in the development and progression of NSCLC. The determination of the role of specific miRNAs may be used in the future in cancer diagnosis, prognostic assessment and NSCLC-targeted therapy.

Materials and methods

Patients and clinical samples. The present study was carried out using paraffin blocks of LSCC and pairs of LSCC and NMLTs resected adjacent to the primary tumor. All samples were obtained during surgical resection from 2007-2014 at the Lower Silesian Centre of Lung Diseases in Wroclaw. Paraffin sections of the obtained LSCC samples were stained with hematoxylin and eosin (H&E) to verify the utility for immunohistochemical (IHC) analysis. The study group consisted of 25 formalin-fixed paraffin-embedded (FFPE) samples of LSCC used for further IHC analysis and 25 pairs of LSCC and NMLT which were collected in RNAlater solution (Qiagen, Hilden, Germany), and stored at -20˚C for RT-qPCR and droplet digital PCR (ddPCR) experiments. Additionally, the same samples were collected, frozen in liquid nitrogen and stored at -80˚C for western blot analysis. Clinical data were derived from hospital archives and are summarized in Table I.

Table I. Patient and tumor characteristics.

Parameters	Data
Total no. of LSCC cases	25
Age (years)	
Mean	67.52±8.82
Range	57-81
Gender, n (%)	
Male	19 (76.0)
Female	6 (24.0)
Tumor size, n (%)	
T1	9 (36.0)
T2	13 (52.0)
T3	3 (12.0)
T4	0 (0.0)
Lymph nodes, n (%)	
N0	20 (80.0)
N1, N2, N3	5 (20.0)
pTNM, n (%)	
1A	8 (32.0)
1B	6 (24.0)
2A	6 (24.0)
2B	4 (16.0)
3A	0 (0.0)
3B	0 (0.0)
4	1 (4.0)
Grade, n (%)	
G1	0 (0.0)
G2	21 (87.5)
G3	4 (12.5)

LSCC, lung squamous cell carcinoma. pTNM, pathological tumor-node-metastasis stage.

Immunohistochemistry. LSCC samples fixed in 10% buffered formalin and embedded in paraffin were used for the IHC reactions. In order to determine the SOX18 expression, the murine monoclonal mouse antibody directed against SOX18 (D-8, Sc-166025; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used in a dilution of 1:100 according to a previously established protocol (3). The IHC procedure was performed using the Autostainer Link 48 (DakoCytomation, Glostrup, Denmark) to provide reliable and repeatable conditions.

RNA extraction, cDNA synthesis and real-time PCR reactions. Total RNA was isolated from the RNAlater-fixed samples of LSCC and the corresponding NMLT samples with the use of the RNeasy Mini Kit (Qiagen). This total RNA was transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RT-qPCR was carried out in 20 μl volumes using the TaqMan Universal PCR Master Mix on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The TaqMan-specific probes used in the experiment (Hs00746079_s1 for *SOX18* and Hs00188166_m1 for *SDHA* as a reference gene) were also obtained from Applied

Table II. List of TaqMan® microRNAs used in this study.

miRNA transcript	Assay ID	Mature miRNA sequence
hsa-miR-7-5p	000268	UGGAAGACUAGUGAUUUUGUUGU
hsa-miR-20a-3p	002437	ACUGCAUUAUGAGCACUUAAAG
hsa-miR-24-3p	000402	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-202	002362	UUCCUAUGCAUAUACUUCUUUG
hsa-miR-335-5p	000546	UCAAGAGCAAUAACGAAAAAUGU
hsa-miR-374a	000563	UUAUAAUACAACCUGAUAAGUG
hsa-miR-374b	001319	AUAUAAUACAACCUGCUAAGUG
hsa-miR-488	001106	CCCAGAUAAUGGCACUCUCAA
hsa-miR-499-5p	001352	UUAAGACUUGCAGUGAUGUUU
hsa-miR-548ab	463573_mat	AAAAGUAAUUGUGGAUUUUGCU
hsa-miR-548ak	463366_mat	AAAAGUAACUGCGGUUUUUGA
hsa-miR-548i	002909	AAAAGUAAUUGCGGAUUUUGCC
hsa-miR-764	241115_mat	GCAGGUGCUCACUUGUCCUCCU
hsa-miR-1205	002778	UCUGCAGGGUUUGCUUUGAG
hsa-miR-1909	121123_mat	UGAGUGCCGGUGCCUGCCCUG
hsa-miR-3128	244506_mat	UCUGGCAAGUAAAAAACUCUCAU
hsa-miR-3973	464008_mat	ACAAAGUACAGCAUUAGCCUUAG
hsa-miR-4645-5p	463591_mat	ACCAGGCAAGAAAUAUUGU
hsa-miR-4716-3p	462953_mat	AAGGGGGAAGGAAACAUGGAGA
hsa-miR-4802-3p	462011_mat	UACAUGGAUGGAAACCUUCAAGC

miRNA, microRNA.

Biosystems. All reactions were performed in triplicates under the following conditions: activation of polymerase at 50˚C for 2 min, initial denaturation at 94˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 15 sec and annealing and elongation at 60˚C for 1 min. The relative mRNA expression of the studied markers was calculated with the ΔΔCq method.

miRNA quantification using ddPCR. Small RNA fractions containing miRNAs from the RNAlater-fixed samples of LSCC and NMLT were isolated with the use of the mirVana miRNA Isolation kit (Ambion, Waltham, MA, USA) according to the manufacturer's instructions. For reverse transcription (RT-PCR), the TaqMan MicroRNA Reverse Transcription kit was used, as well as miRNA-specific stem-loop primers (both from Applied Biosystems), 20 primers for *SOX18* (Table II) and 2 as a reference for miRNA genes (Table III). An input of 30 ng of RNA from each sample was reversely transcribed using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The miRNAs that most probably interact with the *SOX18* transcript were selected from miRNA libraries and repositories available online: miRBase, TargetScanHuman 6.2, miRanda and RepTar database (date of access, 15 May 2015). The thermocycler parameters were as follows: hold for 30 min at 16˚C, for 30 min at 42˚C, and for 5 min at 85˚C.

The ddPCR reaction mixtures contained: 1.33 µl of RT product, 1 µl of TaqMan miRNA-specific probe (Life Technologies), 7.67 µl of molecular biology-grade water and 10 µl of 2x ddPCR™ Master Mix for Probes (Bio-Rad). A total of 20 µl of the reaction mixtures was loaded into a plastic cartridge with 70 µl of Droplet Generation Oil for Probes in the QX100 Droplet Generator (all from Bio-Rad). The droplets obtained from each sample were then transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany). PCR amplifications were carried out in the C1000 Touch Thermal Cycler at 95˚C for 10 min, followed by 40 cycles at 95˚C for 3 sec and 60˚C for 1 min, and 1 cycle at 98˚C for 10 min ending at room temperature (RT). Finally, the plate was loaded on a Droplet Reader (Bio-Rad) and read automatically. Absolute quantification (AQ) of each miRNA was calculated from the number of positive counts per panel using Poisson distribution. The quantification of the target miRNAs is presented as the number of copies/µl of the PCR reaction mixture.

Table III. TaqMan® probes used as reference genes in this study.

miRNA transcript	Assay ID	Mature miRNA sequence
hsa-miR-103	000439	AGCAGCAUUGUACAGGGCUAUGA
hsa-miR-191	000490	CAACGGAAUCCCAAAAGCAGCU

miRNA, microRNA.

SDS-PAGE and western blot analysis. Whole cell lysates of LSCC and NMLT samples were obtained by using the T-PER Tissue Protein Extraction kit (Thermo Fisher Scientific, Walthman, MA, USA) with the addition of a

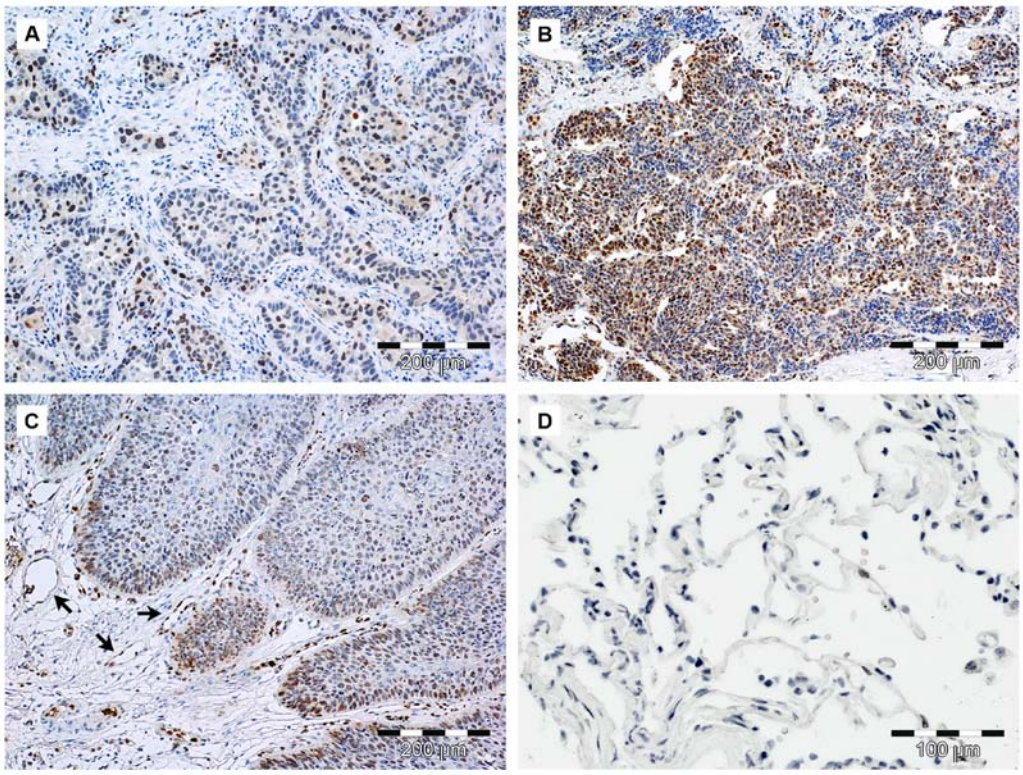

Figure 1. Differential expression of SOX18 protein in the LSCC tissues. All samples presented either (A) weak or (B) strong nuclear expression of SOX18 protein. (C) SOX18 expression was also noted in nuclei of endothelial cells of vessels (indicated with arrows). (D) No SOX18 expression was noted in the healthy lung tissue. Original magnifications, x200 and x100. SOX18, SRY-related HMG-box 18; LSCC, lung squamous cell carcinoma.

cocktail of inhibitors (Sigma, St. Louis, MO, USA), 250 U of Benzonase (Merck Millipore, Bedford, MA, USA) and 2 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were mixed with 4X SDS-PAGE gel loading buffer (200 mM Tris-HCl - pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol), loaded on 10% acrylamide gel and separated by SDS-PAGE under reducing conditions, and then transferred onto a PVDF membrane in the XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific, Santa Clara, CA, USA). After protein transfer, the membrane was incubated in blocker solution (4% BSA in TBST buffer) for 1 h at RT followed by overnight incubation at 4˚C with the anti-SOX18 monoclonal mouse antibody, diluted at 1:100 (D-8, Sc-166025; Santa Cruz Biotechnology). Next, the membrane was washed with TBST buffer and incubated for 1 h at RT with the secondary donkey anti-mouse antibody conjugated with HRP, diluted at 1:3,000 (709-035-149; Jackson ImmunoResearch, Mill Valley, CA, USA), then rinsed and treated with the Immun-Star HRP Chemiluminescent kit (Bio-Rad). Rabbit anti-human β-actin monoclonal antibody (#4970; Cell Signaling Technology, Inc., Danvers, MA, USA) diluted at 1:1,000 was used as an internal control. The western blotting results were analyzed in the ChemiDoc MP System (Bio-Rad).

Statistical analysis. The Shapiro-Wilk test was used for the evaluation of the normality assumption of the groups examined. In order to compare the differences between the LSCC and NMLT groups, the Wilcoxon signed-rank test was used. Additionally, the Spearman's correlation test was carried out to analyze the existing correlations. All the statistical analyses were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). The results were considered statistically significant at $p<0.05$.

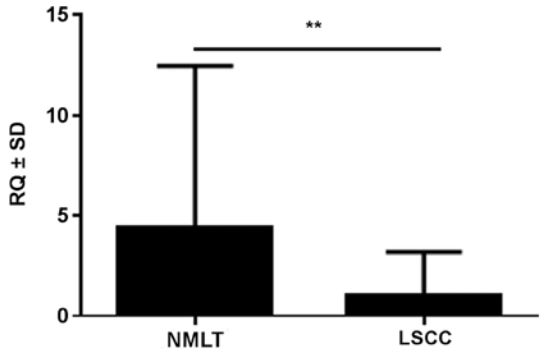

Figure 2. SOX18 mRNA expression profiles. SOX18 mRNA expression in samples of LSCC and NMLT (**p<0.01, Wilcoxon signed-rank test). SOX18, SRY-related HMG-box 18; LSCC, lung squamous cell carcinoma; NMLT, non-malignant lung tissue.

Results

Immunohistochemistry. In total, 25 cases of LSCC were tested in this study. There were 19 men (76%) and 6 women (24%), and the mean ± SD age at surgery was 67.52±8.82.

In the presented results, SOX18 expression was observed mostly in the nuclei of both cancer and endothelial cells (Fig. 1). The nuclear localization of the SOX18 protein was observed

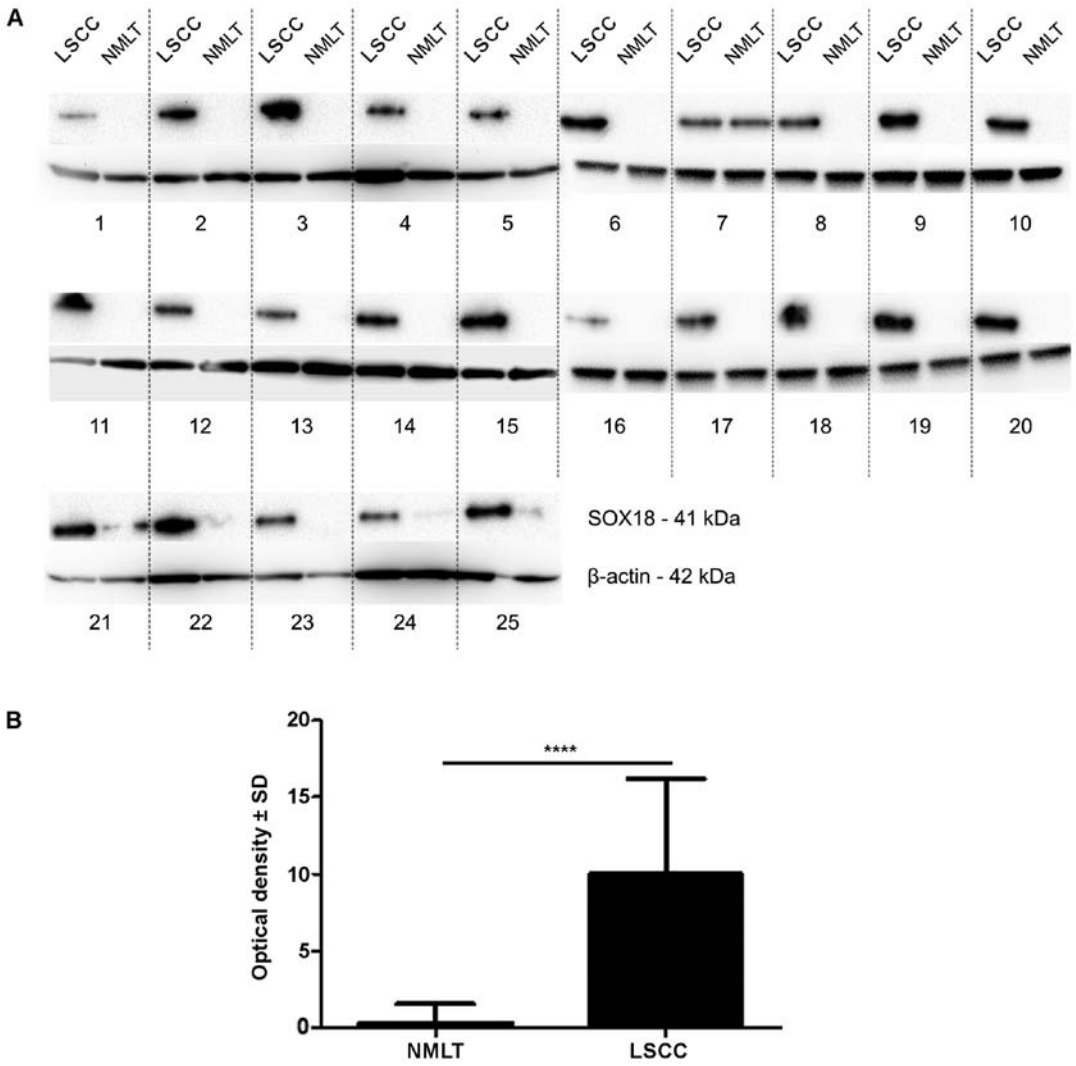

Figure 3. SOX18 protein expression in samples of LSCC and NMLT. Pairs are indicated by the numbers below. Densitometric measurement of SOX18 bands revealed a significantly higher SOX18 expression in LSCC samples than that noted in the NMLTs (****p<0.0001, Wilcoxon signed-rank test). SOX18 levels were normalized against β-actin and one of the NMLT samples was used as a calibrator. SOX18, SRY-related HMG-box 18; LSCC, lung squamous cell carcinoma; NMLT, non-malignant lung tissue.

in 23 cases (92%), and cytoplasmic expression was noted in 1 case (4%). The quantitation of the IHC analysis was based on scoring for the number of positively stained nuclei. In the case of SOX18 (nSOX18) expression in LSCC cancer cells, a semi-quantitative scale based on tumor cell positivity in the whole tissue section was employed. This scale is encoded as: 0 (0% cells stained), 1 (1-10% cells stained), 2 (11-25% cells stained), 3 (26-50% cells stained) and 4 (51-100% cells stained). We were not able to observe SOX18 protein expression in the fibroblastic-like cells of the tumor stroma and healthy lung tissue. By using the Spearman's correlation test, significant correlations were observed between IRS SOX18 and RQ-values of SOX18 in LSCC samples (r=0.43, p=0.041), IRS SOX18 and AQ-values of miR-24-3p (r=-0.48, p=0.02), and nSOX18 and miR-7a in the NMLT cases (r=-0.49, p=0.018).

SOX18 mRNA expression levels in LSCC and NMLT - RT-qPCR. *SOX18* mRNA expression level was determined in 21/25 cases (84%) of LSCC and in all 25 cases (100%) of NMLT. We observed a lower expression of *SOX18* in LSCC as compared to NMLT in 22 cases (88%) (mean RQ ± SD, 1.05±1.26 vs. 4.44±4.99, respectively). The difference was statistically significant for the analyzed pairs (p<0.01, Wilcoxon signed-rank test) (Fig. 2). *SOX18* expression in NMLT was positively correlated with *SOX18* expression in the LSCC samples (r=0.48, p=0.019; Spearman's correlation test).

SOX18 protein level - western blot analysis. The bands of SOX18 protein were observed at 41 kDa in the whole cell fractions of all 25 cases (100%) of LSCC and in only 3 cases (12%) of NMLT. The expression of SOX18 protein was significantly higher in all of the analyzed cases of LSCC compared to that noted in the NMLT (mean OD ± SD, 9.97±6.24 vs. 0.32±1.20, respectively; p<0.0001, Wilcoxon signed-rank test) (Fig. 3).

miRNAs expression levels - ddPCR. From all the 20 potentially miRNAs that could interact with the SOX18 transcript, we could justify a closer examination of only two of them that were variably expressed: miR-7a and miR-24-3p (Fig. 4).

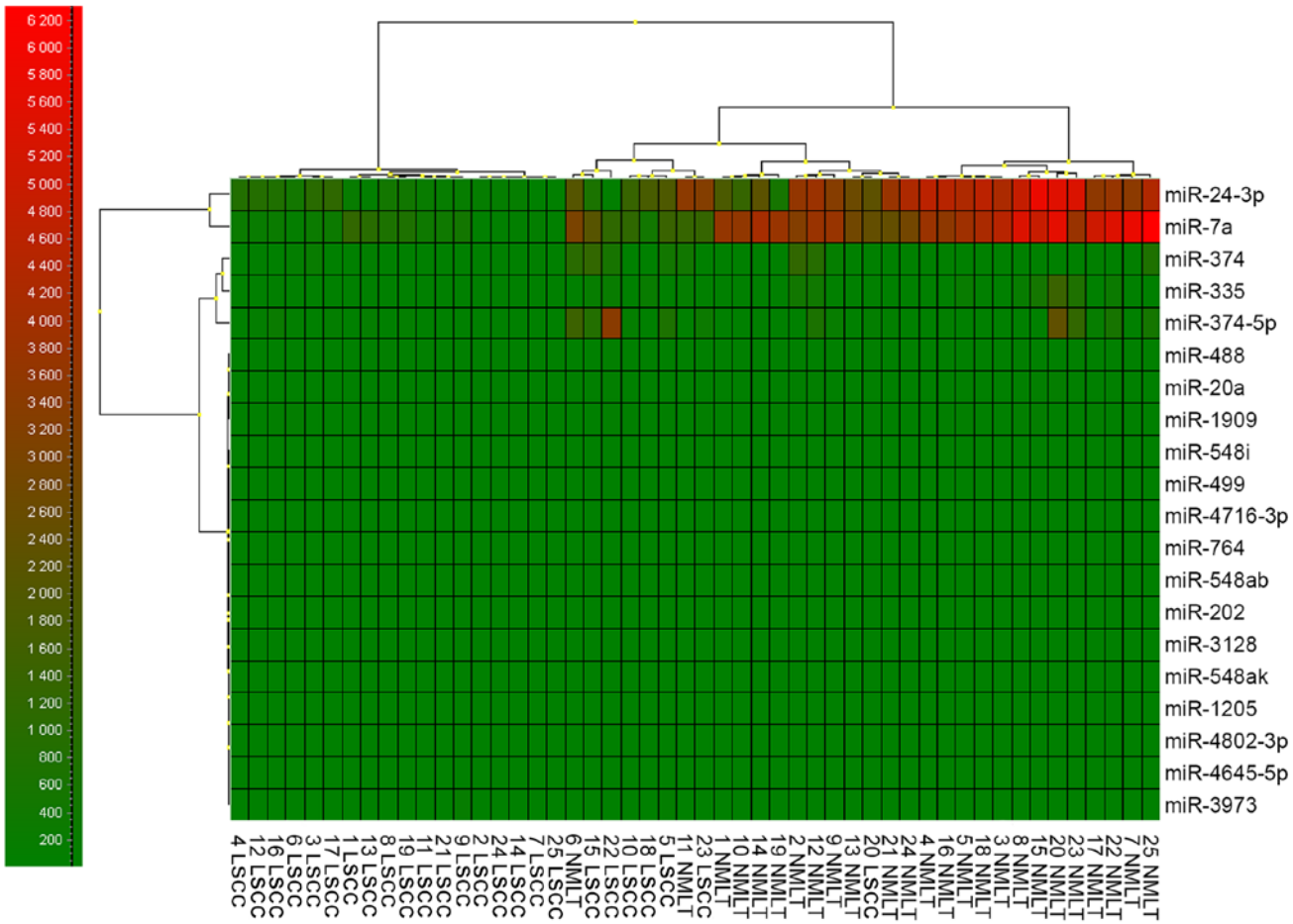

Figure 4. Clustering of LSCC and NMLT miRNA expression profiles. This heat map revealed three groups: tumor (n=18), normal (n=13) and mixed (n=19) clusters. Hierarchical clustering was performed using GenEx 6 for absolute miRNA levels, and is color-coded from bright green (undetected) to red (high expression). miR-7a and miR-24-3p were found to be significantly more highly expressed in the NMLT samples compared to the LSCC samples. LSCC, lung squamous cell carcinoma; NMLT, non-malignant lung tissue; miRNA, microRNA.

According to the ddPCR AQ method, miR-7a was significantly more highly expressed in 23 cases (92%) of NMLT compared to LSCC (mean AQ ± SD, 4026±1,158 vs. 658.1±670, respectively; $p<0.0001$, Wilcoxon signed-rank test). The same observation was made for miR-24-3p: there was a higher expression in 18 cases (72%) of NMLT compared to LSCC (mean AQ ± SD, 3,674±1,304 vs. 735.9±835.7, respectively; $p<0.0001$, Wilcoxon signed-rank test) (Fig. 5).

However, only one of the miRNAs used as a reference gene showed a relatively constant and invariant expression in all examined samples - miR-191 (5,654±764 copies/μl), as previously described (28,32). The reference genes were not required for analysis purposes, but helped us to ensure the quality and relevance of the chosen samples.

Overall, both miR-7a and miR-24-3p had a significantly higher copy number in the lung tissue samples (NMLTs) compared to the cancer samples (LSCC). Statistically higher copy numbers per μl of miR-7a were observed in the NMLTs rather than in the LSCC, both for all the analyzed samples and the paired cases. By using the Spearman's correlation test, positive correlations were observed between AQ-values of miR-7a and miR-24-3p in the NMLT cases ($r=0.4$, $p=0.057$), AQ-values of miR-7a and miR-24-3p in the LSCC samples ($r=0.51$, $p=0.012$), and AQ-values of miR-24-3p in the NMLTs and miR-24-3p in the LSCC samples ($r=0.4$, $p=0.017$).

Discussion

The proteins encoded by *SOX* genes act as transcription factors in cells mostly at the embryonic stage of development. SOX proteins can be found in many tissues at different stages of development, fulfilling important functions in a variety of processes occurring in the body, such as embryonic development and disease processes - atherosclerosis or carcinogenesis (33). In recent years, their role in tumors has been intensively studied, as a result of which it has been possible to demonstrate the participation of these transcription factors in the pathogenesis of many malignant tumors (34).

In previous study we demonstrated that the cytoplasmic expression of the SOX18 protein could be a new prognostic marker in NSCLC patients and that it plays a possible role in the regulation of lung cancer cell proliferation (3). The molecular mechanisms that explain the observed disparity between the mRNA and the protein levels of SOX18 have not been fully discovered yet. Previous observations by Azhikina *et al* and Dammann *et al* considered the methylation of promotors as a mechanism of regulation of variable genes in NSCLC (35,36). Although the hypermethylation of promotors in lung carcinomas can be observed quite often, there is also some evidence for the role of miRNAs in lung cancer pathology (23,24,27,28). Balakrishnan *et al* identified two miRNA molecules that

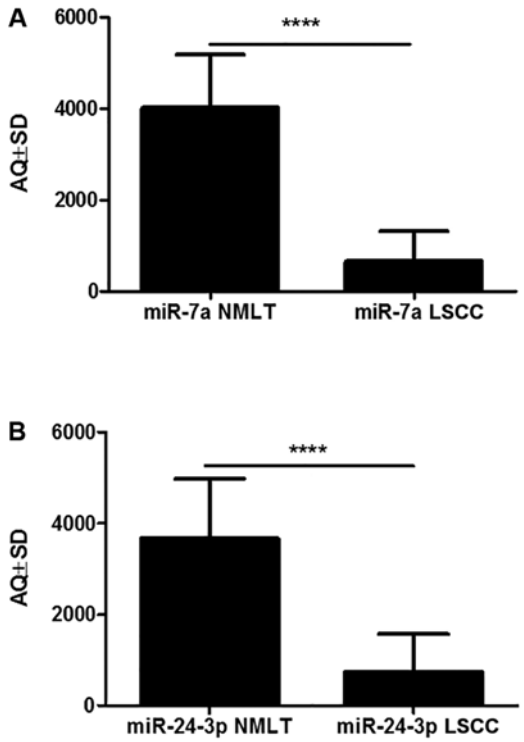

Figure 5. miRNA expression levels were measured by ddPCR. AQ of miRNA levels revealed a statistically higher expression of (A) miR-7a and (B) miR-24-3p in NMLT samples when compared to LSCC samples (****p<0.001, Wilcoxon signed-rank test). SOX18-specific miRNAs showed a significant downregulation in LSCC samples compared to that in the NMLTs. All the experiments were performed in triplicates. miRNA, microRNA; ddPCR, droplet digital PCR; AQ, absolute quantification; NMLT, non-malignant lung tissue; LSCC, lung squamous cell carcinoma; SOX18, SRY-related HMG-box 18.

interact with the mRNA of the SOX18 gene (37). In the present study, we aimed to identify the miRNAs that are responsible for the observed disparity between *SOX18* mRNA and the protein level in NMLT and LSCC cells.

The results obtained with the RT-qPCR technique showed a statistically higher expression level of *SOX18* mRNA in NMLTs compared to the corresponding LSCC samples. Moreover, we observed a statistically higher expression level of the SOX18 protein in LSCC samples compared to the NMLTs.

In addition to the involvement of the SOX18 transcription factor in a series of embryonic development processes, its expression has also been shown in cells of many organs, such as the heart, the lungs, the skeletal muscles, the stomach or the jejunum, in mature organisms (10,38). In this study, the RT-qPCR data showed that the *SOX18* mRNA level was at an approximately average level in the mature lung tissues, but that in almost all cases of NMLT (88%) there was a lack of its protein. We postulated that miR-24-3p together with miR-7a could play a role in the mechanism of *SOX18* transcript inhibition. It is very probable that after the embryonic development of lung tissue, the *SOX18* gene product could be inhibited or even degraded via miRNAs. Their role in blood vessel development, vascular adaptations and arterial occlusions in normal and tumor tissues has already been firmly confirmed (39,40). To date, many studies have demonstrated that members of the *SOX* gene family play important roles in the development and maintenance of the lung (5). Moreover, the expression of SOX2 in neural progenitor cells (NPCs) is proven to be controlled by miRNAs (41), as well as SOX4 and SOX15 in cancers (42). It has been also confirmed that miR-124 downregulates *SOX8* expression and suppresses cell proliferation in NSCLC (43).

Up until now, there have been many reports that strengthen the role of a variety of miRNAs in lung tissue and the epigenetics of lung cancers (28,29,31,44-46). Li *et al* showed that miR-7a was suppressed in NSCLC cells, and B-cell lymphoma 2 (BCL-2) protein was identified as a possible target (47). Furthermore, miR-24-3p was also found to be significantly downregulated in NSCLC samples, where it regulates the autophagy process. Since miRNAs interact with many different mRNA targets, it is not surprising that miR-7a and miR-24-3p could also bind to the *SOX18* transcript. Therefore, we propose that miR-7a together with miR-24-3p can act as major factors that control *SOX18* expression in LSCC.

The results presented in our study correspond to those of Balakrishnan *et al*, where miR-7a and miR-24-3p were confirmed to interact with the *SOX18* transcript (37). In their study, they used immunoprecipitation as a technique to confirm the binding properties of those miRNAs with the SOX18 mRNA.

It has been firmly confirmed that miRNAs can be down-regulated or upregulated during the development of lung cancers. On the one hand, most of the miRNAs that are down-regulated are essential to inhibit the growth and survival of tumor cells (23). On the other hand, the genes that are upregulated by miRNAs are essential for cell adhesion, mobility and development. Tavazoie *et al* demonstrated that miR-335, also examined in our study, regulated metastasis and invasion through the suppression of the SOX4 gene in the breast cancer cell line MDA-MB-231 (48). Our data do not confirm these properties for miR-335 in the case of NMLT and LSCC, but *SOX18* suppression is most probably caused by miR-7a and miR-24-3p molecules.

In the present study, we have, most probably, a situation where the cancer cells successfully downregulate miR-7a and miR-24-3p, which leads to higher expression of the SOX18 protein. The mechanism involved in these modulations is not fully understood and requires further analysis, but we postulate that the downregulation of these miRNAs is due to two different models: chromatin remodeling or natural antagomiRs (anti-miRs). Although the chromatin remodeling process modulates the expression of miRNAs in dendritic cells, it can only be hypothesized that miRNAs, in particular miR-7a, are also downregulated via this mechanism in LSCC (49). We believe that miR-7a and miR-24-3p expression in LSCC and other types of lung cancer is effectively modulated via natural anti-miRs. Until recently, anti-miRs were considered as artificial particles that could be used as a new and highly specific weapon against pro-oncogenic miRNAs in many diseases (50-55), but the latest studies discovered natural antisense transcripts (NATs), which are natural endogenous RNA molecules transcribed from the opposite strand of other protein- or non-protein-coding genes (56). The mechanisms by which NATs regulate gene expression are highly incomprehensible. Faghihi *et al* proved that in Alzheimer's disease the β-secretase 1 (BACE1) protein expression is modulated via the competition of miR-485-5p and BACE1-AS (NATs) for a binding site in the exon region of BACE1 mRNA (56).

The opposing effects of BACE1-antisense and miR-485-5p on BACE1 protein were proven *in vitro*. They also demonstrated that the expression of both BACE1-antisense and miR-485-5p are dysregulated in RNA samples from Alzheimer's disease subjects compared to control individuals.

It has not been verified yet whether the same suppression model takes place in lung cancer pathology, especially in regards to SOX18 and its role in cancer angiogenesis. Yet, results from ddPCR (data not shown) indicate that, in LSCC samples, we can observe populations of unspecific products that compete with miR-7a and miR-24-3p for *SOX18* mRNA binding sites. Those 'unspecific products' could be similar to those NATs that were discovered by Faghihi *et al*, but further analyses are required to prove this theory (56).

In conclusion, our data, along with our previous findings, indicate that the disparity between the mRNA and protein levels of the SOX18 transcription factor in NSCLC and NMLT could be caused by the abilities of miR-7a and/or miR-24-3p to bind to the transcript. The proper mechanism via which it is carried out remains unknown and will be our next research goal. Yet, it is most probable that NATs are involved in the suppression of these miRNAs allowing cancer cells to express SOX18 protein. The presence of SOX18 is highly desirable for cancer cells mostly due to its role in angiogenesis and the intensification of the metastasis process. However, further studies are required in order to fully understand the role of SOX18 in cancer development and progression.

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