Influence of miR-7a and miR-24-3p on the *SOX18* transcript in lung adenocarcinoma

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Abstract. The molecular pathogenesis of the development of non-small cell lung carcinomas (NSCLCs) is extremely complex. Understanding the molecular basis of the development of this malignant tumor may enable the use of targeted therapy, which may result in a better treatment outome for these patients. Adenocarcinoma (AC) is the most common NSCLC subtype, equally common among smokers and non-smokers, and its pathogenesis remains unknown. The SOX18 protein is an important protein that plays a role in the development of blood and lymphatic vessels during the process of embryogenesis. Recent studies have also shown that the SOX18 protein may play a significant role in tumors, including lung cancers. In the present study, we analyzed the expression of the SOX18 protein and the mRNA level in postoperative samples of AC and non-malignant lung tissues (NMLTs), and a disparity in both levels was observed. Based on our previous observations that miR-7a and miR-24-3p are able to modulate SOX18 expression in NSCLC, the main aim of this study was to verify the miRNA modulation of the *SOX18* transcript with the use of the MirTrap System in established lung cancer cell lines NCI-H1703, NCI-H522 and A549. The SOX18 mRNA expression level was significantly lower in AC than that noted in the NMLTs ($P<0.0001$). However, the protein levels were higher in AC cases compared to levels noted in the NMLTs ($P<0.0001$). Additionally, correlations between the RQ values of SOX18 in NMLT and AC cases ($r=0.8195$, $P=0.0001$), and between miR-7a and miR24-3p in AC cases ($r=0.4344$, $P=0.0016$), were noted. In conclusion, we confirmed that miR-7a and miR-24-3p are more highly expressed in NMLTs than in the AC samples, and that they modulate the *SOX18* transcript in NSCLC cells.

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Introduction

Lung cancer is the most common malignant tumor (13% of all cancers) worldwide, both in terms of morbidity and mortality. It is more common among men (18% of all cancers), while in women it occupies the fourth place (9.5%). The morbidity of this type of tumor shows a growing trend (1). Furthermore, a greater percentage of cases in both sexes is observed in developing countries (2). The primary reasons for this include, among others, an aging population and a change in lifestyle observed over the past few decades, in which an inadequate diet, the lack of physical activity and smoking are often found (1,3,4).

Recent research on lung cancer in never-smokers has led to the distinction of a separate disease classification referred to as lung cancer in never smokers (LCINS) (5-7). The etiology of this cancer is not fully understood. However, a number of possible risk factors have been described, e.g. lung disease family history, passive smoking, diet or work-related exposure to harmful substances (8). When it comes to histological classification, two main groups of lung cancer can be observed: small cell lung cancers (SCLCs) and non-small cell lung cancers (NSCLCs) (9).

Adenocarcinoma (AC) is the most common NSCLC subtype (approximately 50% of all diagnosed cases). It develops in the smaller bronchi and bronchioles and in the alveolar epithelium (10), and it is most often located in the peripheral segments of the lung. It is equally common among smokers and non-smokers, which indicates that its etiopathogenesis is to a greater extent linked to factors other than tobacco smoke. Among the different NSCLCs, AC has the most unfavorable prognosis (10). Due to the complexity of the molecular mechanisms initiating the development of NSCLCs, it is necessary to investigate new potential cancer markers. In this sense, the SOX18 protein appears to be an auspicious element in future anticancer therapies.

The SOX family proteins (*SRY*-related HMG-box) are important transcription factors involved, for example, in the

development of the cardiovascular system and the lymphatic ducts (11,12). The SOX family is composed of approximately 20 proteins divided into 10 groups, from A to J (13,14). There is a low sequence homology between the groups. However, proteins within the same group show at least 80% homology in the HMG domain, and they possess other conservative domains inside the group (13,15). Group F proteins (SOX7, SOX17, SOX18) are primarily involved in the development of the cardiovascular system during embryogenesis (16-18).

It has been observed that SOX18 takes part in wound healing processes and the development of atherosclerosis (19). Likewise, an increased level of this protein has been found in melanomas and malignant pancreatic, stomach and breast tumors (21). Furthermore, high expression of SOX18 in gastric cancer stromal cells has been correlated with a poorer patient prognosis (22). In breast cancer, SOX18 is associated with tumor malignancy grade (G) and Ki-67 proliferation index; therefore, it can be of particular significance in future prognosis (21). It has been shown in additional *in vitro* studies that the SOX18 protein stimulates the migration and proliferation of human umbilical vein endothelial cells (HUVECs), the result of which is intensive angiogenesis. Moreover, it was demonstrated that inhibition of SOX18 expression in the MCF-7 human breast cancer cell line resulted in the weakening of the ability of these cells to migrate due to the destabilization of the actin cytoskeleton structure, which further confirms the role of SOX18 in cell migration (23).

Studies aiming to determine the prognostic significance of the SOX18 expression have been conducted in gastric, breast and lung cancer to date (24-27). An increased expression of all of the genes from the SOX F group has been shown in gastric tumor tissue compared to unaltered stomach tissue. Furthermore, it was discovered that cases with high SOX18 expression are characterized by a shorter recurrence-free survival time. The results of these studies suggest that an increase in the SOX18 protein expression may be an adverse prognostic factor in gastric cancer (22).

The role of SOX18 expression in NSCLC is not yet fully understood. However, taking into account previous reports, this protein may be an important factor in the development and progression of NSCLCs. Our preliminary studies have shown a disparity in the amount of *SOX18* mRNA relative to the quantity of protein (25-27), which may be associated with the regulation of the translation by microRNAs (miRNAs). This has allowed us to hypothesize that the *SOX18* transcript level is subject to control by 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 (28,29).

miRNAs are involved in many important biological processes, such as proliferation, cell differentiation, apoptosis, embryogenesis and organogenesis. An increasingly visible role of miRNAs in the regulation of cell proliferation and cell differentiation and apoptosis has drawn the attention of scientists to the relationship of miRNAs and tumor processes (30-34). As discovered, miRNAs do not only regulate the expression of multiple oncogenes and tumor-suppressor genes, but may also act themselves as oncogenes and suppressors. The correlation between miRNA expression profiles and patient survival may reveal their potential role as prognostic markers. A relationship between the expression level of different miRNAs and the survival of patients with lung AC has been shown (31,34-36). As is the case with miR-9500 molecules in lung cancer, miRNAs affecting the transcription of the *SOX18* gene may also be of great prognostic importance (37). Discovering a method for a sensitive and efficient determination of the expression level of miRNA molecules could be useful in elucidating the pathogenesis of lung cancer, and thereby also in the reduction of lung cancer mortality.

Materials and methods

Patients and clinical samples. The present experiments were carried out using archival paraffin blocks of lung AC as well as pairs of AC and non-malignant lung tissue (NMLT) resected adjacent to the primary tumor. The samples were obtained during surgical resection in 2012-2016 at the Lower Silesian Lung Diseases Centre in Wroclaw. The paraffin sections of the AC samples were stained with haematoxylin and eosin (H&E) in order to verify the appropriateness of the immunohistochemical (IHC) analyses. The study group consisted of 50 pairs of AC and NMLTs collected into RNAlater solution (Qiagen, Hilden, Germany) and stored at -20˚C for RT-qPCR and 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.

Cell lines. For the present study we used three lung cancer cell lines (NCI-H1703, NCI-H522, A549) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The NCI-H1703 cell line was derived from a stage I LSCC, the NCI-H522 cell line from a stage II AC, and the A549 cell line from a highly malignant AC. The NCI-H1703 and NCI-H522 cell lines were cultured in RPMI-1640 medium with the addition of 2 mM L-glutamine (Lonza, Basel, Switzerland). The A549 cell line was grown in a high glucose DMEM medium with the addition of 2 mM L-glutamine (Sigma, St. Louis, MO, USA). All media were supplemented with FBS (Sigma) up to a final concentration of 10%. The cell lines were cultured at 37˚C in 5% CO_2.

Immunohistochemistry (IHC). The AC and NMLT samples fixed in 10% buffered formalin and embedded in paraffin were used for the IHC reactions. In order to determine SOX18 expression, the murine monoclonal mouse antibody directed against SOX18 (D-8, Sc-166025; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:100 according to a previously established protocol (25). The IHC procedure was performed on the Autostainer Link 48 (DakoCytomation, Glostrup, Denmark) so as 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 AC and their corresponding NMLT samples with the use of the RNeasy Mini kit (Qiagen). It was later transcribed to cDNA by means of the High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. RT-qPCR was carried out in

Table I. AC patient and tumor characteristics (N=50).

Parameters	Data
Age (years)	
Mean	64.58±7.72
Range	52-81
Sex, n (%)	
Male	24 (48.0)
Female	26 (52.0)
Tumor size, n (%)	
T1	21 (42.0)
T2	22 (44.0)
T3	3 (6.0)
T4	4 (8.0)
Lymph nodes, n (%)	
N0	28 (56.0)
N1, N2, N3	22 (44.0)
pTNM, n (%)	
1A	16 (32.0)
1B	7 (14.0)
2A	13 (26.0)
2B	0 (0.0)
3A	12 (24.0)
3B	0 (0.0)
4	2 (4.0)
Grade, n (%)	
G1	2 (4.0)
G2	21 (42.0)
G3	27 (54.0)

20 μl volumes using the TaqMan Universal PCR MasterMix (Applied Biosystems) on a 7900HT Fast Real-time PCR System. The TaqMan specific probes used in the experiment (Hs00746079_s1 for *SOX18*, 000268 for hsa-miR-7a, 000402 for hsa-miR-24-3p as well as Hs00188166_m1 for *SDHA* and 000490 for hsa-miR-191 as reference genes) were also obtained from Applied Biosystems. The reactions were all 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 markers studied was calculated using the $\Delta\Delta$Ct method.

miRNA quantification using Droplet Digital PCR™ (ddPCR). Small fractions of RNA containing miRNAs from the cell lines studied and from the RNAlater-fixed samples of AC and NMLT were isolated with the use of the mirVana miRNA Isolation kit (Ambion, Waltham, MA, USA) according to the manufacturer's protocol. For reverse transcription (RT-PCR), the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) was used together with the aforementioned miRNA-specific stem-loop primers (Applied Biosystems). An input of 30 ng of RNA from each sample was reverse-transcribed using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The thermocycler parameters were as follows: hold for 30 min at 16˚C, for 30 min at 42˚C, and finally for 5 min at 85˚C.

The ddPCR reaction mixtures contained 1.33 μl of RT product, 1 μl of TaqMan miRNA specific probe (Thermo Fisher Scientific, Walthman, MA, USA), 7.67 μl of molecular biology-grade water and 10 μl of 2X ddPCR™ MasterMix for Probes (Bio-Rad). The 20 μl of the reaction mixtures were loaded into a plastic cartridge (Bio-Rad) with 70 μl of Droplet Generation Oil for Probes (Bio-Rad) in the QX100 Droplet Generator (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 a C1000 Touch thermal cycler at 95˚C for 10 min, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 1 min as well as 1 cycle of 98˚C for 10 min ending at room temperature (RT). Finally, the plate was loaded onto a Droplet Reader (Bio-Rad) and read automatically. The absolute quantification of each miRNA was calculated from the number of positive counts per panel by using the Poisson distribution. The quantification of the target miRNAs is presented as the number of copies/μl in the PCR reaction mixture.

SDS-PAGE and western blotting. Whole cell lysates were obtained from the AC and NMLT samples by using the T-PER Tissue Protein Extraction kit (Thermo Fisher Scientific) with the addition of a cocktail of inhibitors (Sigma), 250 units of Benzonase® (Merck Millipore, Bedford, MA, USA) and 2 mM PMSF (phenylmethanesulfonyl fluoride). 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 Gel Electrophoresis System (Thermo Fisher Scientific). After the protein transfer, the membrane was incubated in a blocker solution (4% BSA in TBST buffer) for 1 h at RT followed by overnight incubation at 4˚C with anti-SOX18 monoclonal mouse antibody, diluted 1:100 (D-8, Sc-166025; Santa Cruz Biotechnology). Subsequently, the membrane was washed with TBST buffer and incubated for 1 h at RT with secondary donkey anti-mouse antibody conjugated with HRP, diluted 1:3000 (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, Danvers, MA, USA), diluted 1:1000, was used as an internal control. The western blotting results were analyzed using the ChemiDoc MP System (Bio-Rad).

In vitro studies - MirTrap System. In order to identify that miR-7a and miR-24-3p interact with the *SOX18* gene transcript, an *in vitro* test was conducted using the MirTrap System kit (Clontech Laboratories, Mountain View, CA, USA). The MirTrap System is based on the DYKDDDDK (FLAG epitope) tag on the dominant-negative subunit of the RISC protein, which allows for the capture and isolation of the entire Ago/RISC complex containing the miRNA/target mRNA pair of

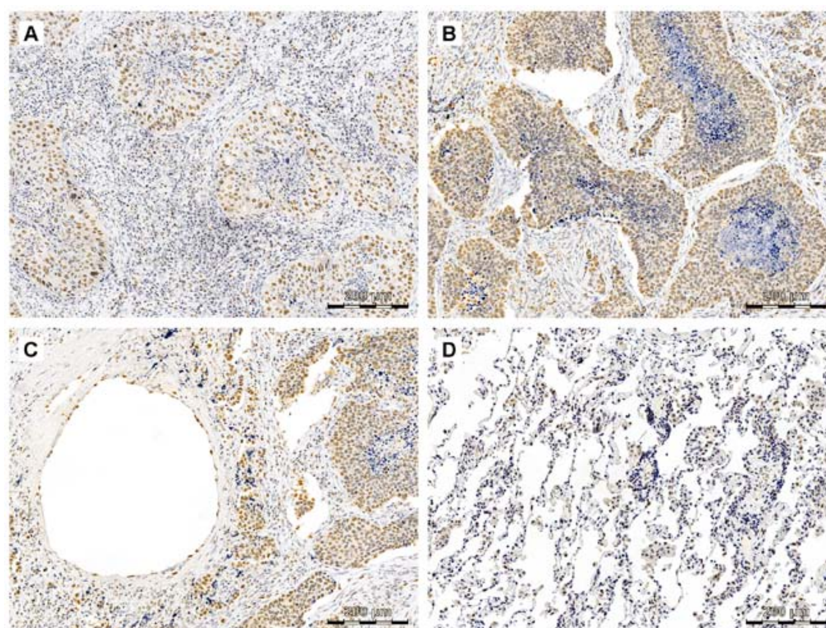

Figure 1. Differentiated expression of SOX18 protein in lung adenocarcinoma (AC). All samples presented either weak (A) or strong (B) nuclear expression of SOX18 protein. SOX18 expression was also noted in the nuclei of endothelial cells of vessels - indicated with arrows (C). No SOX18 expression was noted in healthy lung tissue (D). Original magnification, x200 and x100.

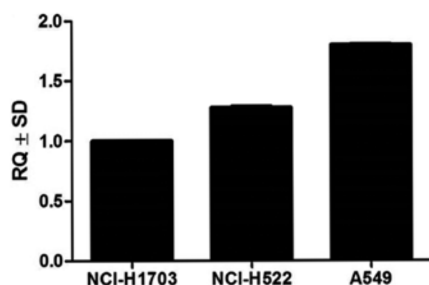

Figure 2. SOX18 mRNA expression profiles. SOX18 mRNA expression in the cell lines studied (NCI-H1703, NCI-H522 and A549). All the experiments were performed in triplicates. Error bars represent SD.

interest. After performing the *in vitro* experiments (according to the manufacturer's protocol), the miR-7a's and miR-24-3p's target in the lung cancer cell lines was quantified by using the RT-qPCR technique. In order to perform the fold enrichment analysis of the *SOX18*/miR-7a and *SOX18*/miR-24-3p pairs, the positive (AcGFP1/miR-132) and negative (hPlod3/miR-132) controls were analyzed according to the manufacturer's protocol.

The NCI-H1703, NCI-H522 and A549 cell lines expressing the MirTrap protein as well as the mRNA fusion of AcGFP1 with a miR-132 target sequence were cotransfected with the microRNA mimics for miR-7a and miR-24-3p. The RISC/miRNA/*SOX18* complexes were immunoprecipitated via anti-DYKDDDDK (FLAG) beads. RNA was isolated, and the fold-enrichment of the miRNA target in the complex was determined with the use of the qRT-PCR technique. A 2.5-fold enrichment was considered to be a positive result. The enrichment was calculated by using the *GAPDH* transcript for normalization purposes. The levels of mRNA were determined with the quantitative RT-qPCR method with the use of the SYBR-Green kit (Bio-Rad) and the 7900HT Fast Real-time System (Applied Biosystems).

Statistical analysis. The Shapiro-Wilk test was used so as to evaluate the normality assumption of the groups examined. In order to compare the differences between the LSCC and the NMLT groups, the Wilcoxon signed-rank test was used. Additionally, the Spearman correlation test was carried out for the analysis of 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 $P<0.05$.

Results

Immunohistochemistry. In the presented results, SOX18 expression was observed mostly in the nuclei of both cancer and endothelial cells of vessels (Fig. 1). A nuclear localization of the SOX18 protein was observed only in AC cells and in the epithelial cells of vessels, but no SOX18 protein expression was noted in the tumor stromal and NMLT samples.

SOX18 mRNA expression levels in AC and NMLT samples - RT-qPCR. Analysis of SOX18 mRNA levels using the RT-qPCR technique in the studied cell lines revealed increased expression in the NCI-H1703, NCI-H522 and A549 cancer cell lines (Fig. 2). The *SOX18* mRNA expression level was determined in all 50 (100%) cases of AC and all 50 (100%) cases of NMLT (data not shown).

SOX18 protein level - western blotting. Bands of SOX18 protein were observed at 41 kDa in the whole-cell fractions of all 50 (100%) cases of AC, but only in 4 (8%) cases of NMLT (Fig. 3A). The expression of the SOX18 protein was significantly higher in all of the analyzed cases of AC compared to NMLT

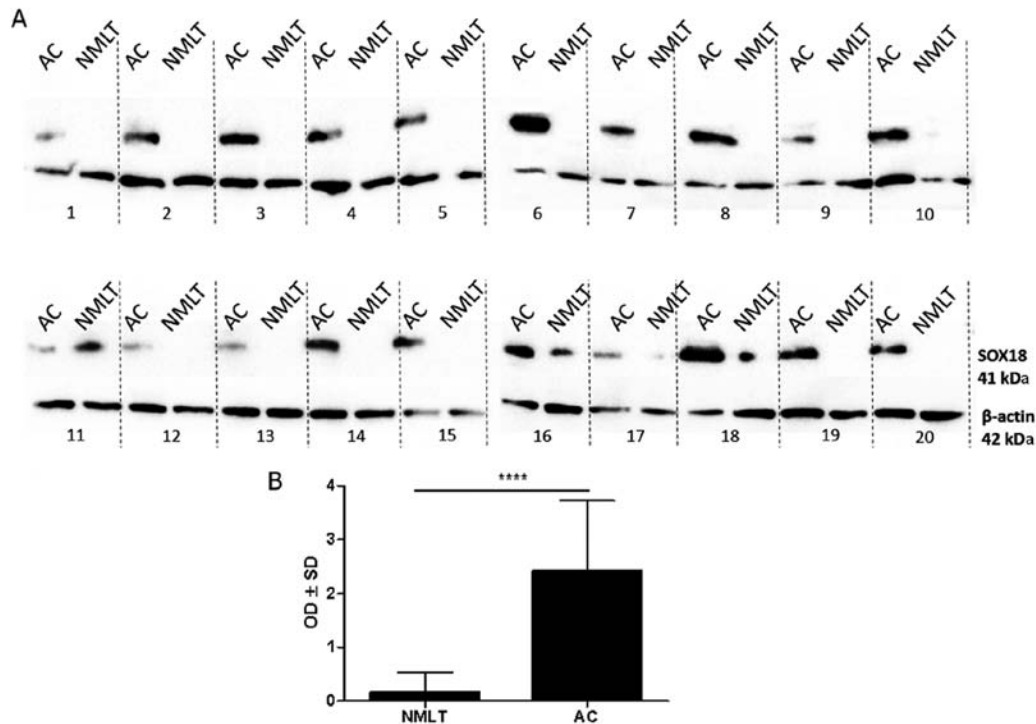

Figure 3. SOX18 protein expression in samples of lung adenocarcinoma (AC) and non-malignant lung tissue (NMLT). (A) The tissue pairs are indicated by the numbers below each blot. (B) Densitometric measurement of the SOX18 bands revealed a significantly higher SOX18 expression in AC samples than in NMLTs (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. Western blot analysis was performed in duplicates. Error bars represent SD.

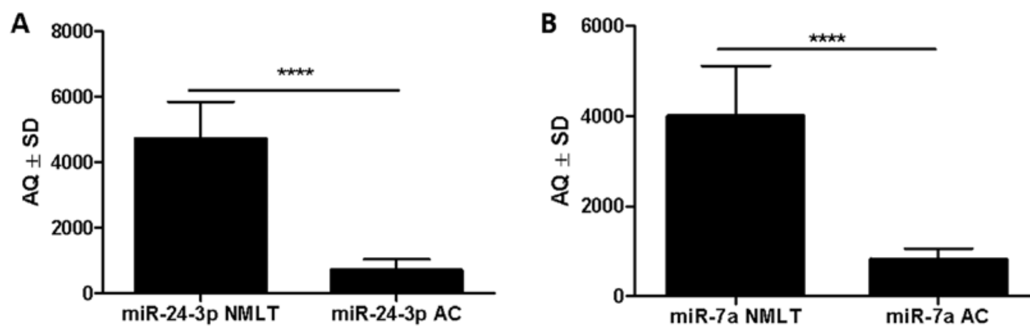

Figure 4. miRNA expression levels were measured by ddPCR. AQ of miRNA levels revealed a statistically higher expression of (A) miR-24-3p (P<0.001 Wilcoxon signed-rank test) and (B) miR-7a (P<0.001 Wilcoxon signed-rank test) in NMLT samples compared to AC samples. SOX18-specific miRNAs show a significant downregulation in AC samples compared to NMLTs. All the experiments were performed in triplicates. Error bars represent SD.

(mean OD±SD, 2.42±1.31 vs. 0.16±0.38, respectively; P<0.0001 Wilcoxon signed-rank test) (Fig. 3B).

miRNA expression levels - ddPCR. According to the ddPCR absolute quantification method, miR-7a was significantly more highly expressed in 48 NMLT cases (96%) compared to that noted in AC (4%) (mean AQ±SD 4,008±1,104 vs. 827±234, respectively; P<0.0001, Wilcoxon signed-rank test) (Fig. 4B). The same observation was made for miR-24-3p: there was a higher expression in 48 cases of NMLT (96%) compared to AC (4%) (mean AQ±SD 4,726±1,114 vs. 708±325, respectively; P<0.0001, Wilcoxon signed-rank test) (Fig. 4A).

Overall, both miR-7a and miR-24-3p had a significantly higher copy number in NMLT samples compared to the AC tissues. By using the Spearman correlation test, positive correlations were observed between AQ values of miR-7a and miR-24-3p in AC cases (r=0.4344, P=0.0016), AQ values of miR-7a and SOX18 mRNA in AC samples (r=0.3813, P=0.0063), miR-7a and SOX18 mRNA in NMLT cases (r=0.4029, P=0.0023) and AQ values of miR-7a in NMLT and AC samples (r= -0.3443, P=0.0144).

In vitro cell line studies - MirTrap System. MirTrap System experiments followed by RT-qPCR analyses validated SOX18 mRNA enriched against the miR-7a/MirTrap and miR-24-3p/MirTrap complexes. The RT-qPCR results are shown as relative fold-change between the studied miRNAs and miR-132 (positive control). The results presented in Fig. 5 show that miR-24-3p is classified as highly enriched, and miR-7a as moderately enriched.

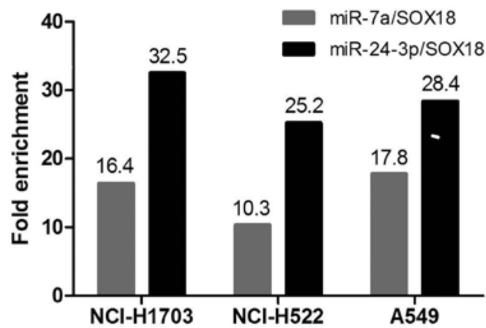

Figure 5. Fold enrichment of miR-7a/SOX18 and miR-24-3p/SOX18 complexes identified using MirTrap System. The RT-qPCR analysis using miR-7a and miR-24-3p TaqMan probes revealed that these miRNAs interact with the SOX18 transcript in lung cancer cell lines NCI-H1703, NCI-H522 and A549. GAPDH levels served as internal controls. A minimum of 2.5-fold enrichment by RT-qPCR was used as a positive identification of a microRNA target.

Discussion

SOX family genes (*SRY*-related HMG-box) were isolated in mammals in 1990 on the basis of the presence of the conservative HMG (high mobility group box) protein domain, primarily occurring in the sex-determining region Y (SRY) (11). SOX proteins can be found in many tissues at different stages of development, where they fulfill important functions in a variety of processes occurring in the body, such as embryonic development, disease processes e.g. arteriosclerosis, carcinogenesis (14,17,38-40). In recent years, their role in tumors has been intensively studied, which has allowed for the demonstration of their participation in the pathogenesis of many malignant tumors (41).

Based on the results of our previous research, we observed a differential SOX18 expression both at the mRNA and protein levels in NSCLC (25,42). Of note, the level of mRNA did not reflect in any way the level of protein, determined by the western blot method. This allowed us to hypothesize that the *SOX18* transcript level is subject to control by miRNA molecules, because similar mechanisms are observed in many other types of tumors (28,30,35,52).

There are some speculations on the role of the methylation increment of CpG islands within the *SOX18* gene promoter in NSCLC progression (43). The presence of an altered methylation profile of the *SOX18* gene promoter in tumor and tumor-surrounding tissue compared to unchanged tissue has been shown by Azhikina *et al* in NSCLC cases (44). The authors suggest that the changes in the methylation pattern of the *SOX18* promoter in the tissues surrounding the tumor (which do not exhibit malignancy features in the morphological and histological image) may indicate early genetic changes leading to carcinogenesis.

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 (20). For example, in a glioblastoma multiform cell line, a significant overexpression of the SOX2 protein has been observed, while the silencing of the SOX2 gene has been proved to result in reduced invasiveness and mitigation of cell migration (45). In turn, a decreased SOX2 expression has been observed in gastric cancer. All this resulted in the inhibition of the cell growth by blocking the cell cycle and the induction of apoptosis in the cells (46).

The molecular mechanisms responsible for the disparity observed between *SOX18* mRNA and the protein level in NSCLCs are not well known. Even though the hypermethylation of the *SOX18* promoter has been described, the complexity of the role of SOX18 in NSCLC progression has not yet been fully explained. Another important feature of lung cancer development, besides the epigenetic changes, are miRNAs, whose role in NSCLC has been already well proven (28,31,34,47-52). Based on our recent studies on lung squamous cell carcinoma (LSCC), we have been able to determine two miRNA molecules from the potential panel of miRNAs that most probably interact with the *SOX18* transcript (42). We observed statistically higher expression levels of miR-7a and miR-24-3p in the NMLT samples in comparison to the LSCC samples.

In the present study, we aimed to ascertain whether the *SOX18* transcript modulation mechanism observed in LSCC cells is also characteristic for adenocarcinoma (AC) cells and, with the use of the RISC-Trap technique, we finally provide evidence of the fact that miR-7a and miR-24-3p interact with the *SOX18* transcript in lung cancer cell lines.

The results obtained with the RT-qPCR technique show a statistically higher expression level of *SOX18* mRNA in NMLT compared to that in the corresponding AC. Moreover, we observed a statistically higher expression level of the SOX18 protein in AC samples compared to the NMLT ones. It appears that the same mechanism exists both in LSCC and AC cells, which could be characteristic of the progression mechanism in NSCLC cells. The SOX18 transcription factor is expressed during embryonic development, but its presence has been observed in different cells of many organs such as the lungs, the skeletal muscles, the stomach and the heart (21). Therefore, we previously postulated that the *SOX18* transcript may be modulated by miRNA molecules after embryonic development. This could explain the disparity observed between the mRNA and protein levels of the SOX18 transcription factor.

During our several years of research, we have been able to demonstrate the role of the SOX18 transcription factor in several types of cancer: invasive ductal breast carcinoma, ovarian cancer and non-small cell lung cancer (25-27). The contribution of the SOX18 transcription factor in tumors is currently being intensively studied on the grounds of the demonstrated role of this protein in the processes of angiogenesis and lymphangiogenesis as well as in cell proliferation (17,18,23,40,53-56).

Taking into account the fact that miR-7a and miR-24-3p suppress the *SOX18* transcript in NSCLC cells, the variable levels of these miRNA molecules may be detected in the blood of patients, which increases the availability and universality of the use of these molecules in lung cancer diagnosis, as they could provide information not only about the type, but also about the stage of the tumor. A relationship between the expression level of 8 miRNAs and the survival of patients with lung AC has been shown (35). Patients with an increased expression of miR-155, miR-17-3p, miR-106a, miR-93 or miR-21 or with a reduced expression of miR-7a-2, miR-7b or miR-145 exhibited a significantly lower survival rate (52). The prospects for the

use of miRNAs in cancer therapy seem promising as well. It has been shown that miRNA inhibition can lead *in vitro* to a reduction in tumor cell proliferation (33,51,57).

Performing the experiments with the MirTrap System gave us final proof of the miR-7a and miR-24-3p properties for binding with the *SOX18* transcript in lung cancer cells. The cell lines studied in this study represent not only different types of NSCLC (NCI-H1703 and A549, lung AC; NCI-H522, lung squamous cell carcinoma), but also exhibit different potential for lung cancer invasiveness. High values of fold enrichment of RISC/miRNA complexes confirmed that the *SOX18* transcript is supressed by miR-7a and miR-24-3p in NSCLC cells.

The results presented in the present study are similar to those describing the role and function of miR-7a and miR-24-3p in NSCLC cells, where miR-7a was suppressed, BCL-2 (B-cell lymphoma 2 protein) was identified as a possible target and miR-24-3p regulated the autophagy process (28). The fact that a single miRNA molecule is able to control many different mRNAs is not new. Similar observations have already been noted in many different types of cancer (30,31,34-36). Therefore, it is possible that miR-7a and miR-24-3p suppress the *SOX18* transcript together with some other transcripts in NSCLCs.

As mentioned in our previous study, miR-7a and miR-24-3p are successfully downregulated in NSCLC cells in order to express the SOX18 transcription factor necessary for the tumor to develop new blood vessels. This mechanism is still unclear, but most probably miR-7a and miR-24-3p molecules are inhibited by some endogenous siRNA molecules called antagomirs (anti-miRs). These antisense transcripts (NATs) are transcribed from the opposite strand of other protein-coding or non-protein-coding genes (58-60). To date, it has not been confirmed that the same suppression mechanism which has been identified in Alzheimer's disease occurs in NSCLC pathology (61).

In the present study, we demonstrated that the disparity between the mRNA and protein levels of the SOX18 transcription factor was caused by miR-7a and miR-24-3p, although the mechanism through which lung cancer cells downregulate miRNA molecule levels is still unclear. The molecular pathogenesis of the development of non-small cell lung carcinoma is extremely complex and complicated. Understanding the molecular basis of the development of this malignant tumor may enable the use of targeted therapy, which may result in a better patient outome. It is therefore important to search for new and more effective therapeutic strategoes as well as new proteins that could be potential targets. In this sense, the SOX18 protein and the SOX protein family appear to be auspicious elements for anticancer therapy.

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