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Original Article

miRNA-124 down-regulates SOX8 expression and suppresses cell proliferation in non-small cell lung cancer

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Abstract: Non-small lung cell carcinoma (NSCLC) is a leading lethal disease and a global health burden. The function of the Sex determining region Y (SRY)-related high mobility group box (SOX) family gene in cancer has attracted the attention of more and more scientists recently, yet there are few reports regarding the role of SOX in NSCLC. Our study aimed to investigate the expression of SOX8, a protein belonging to the E group of the SOX family, as well as SOX9, in non-small cell lung cancer (NSCLC) and the relationship of gene expression to clinicopathological factors and prognosis in patients. Immunohistochemical analysis was used to measure the expression of SOX8 in 80 NSCLC and 7 adjacent normal tissues. SOX8 expression was detected as elevated in tumor samples and correlated to tumor size ($P < 0.001$), lymph node metastasis ($P = 0.001$), differentiation classification ($P = 0.015$), and clinical stage ($P = 0.013$) significantly. Moreover, Kaplan-Meier survival analysis demonstrated that shorter survival time for patients who had higher SOX8 expression ($P < 0.001$). In addition, our experiments indicate that miRNA-124 functions as a tumor suppressor in NSCLC. We also demonstrate miRNA-124 directly targeted and decreased SOX8 in NSCLC cell lines, suggesting smiRNA-124 may regulate NSCLC cell proliferation via decreasing SOX8 (oncogenicity of biomarker in NSCLC).

Keywords: NSCLC, miRNA-124, Sox8, IHC, lung cancer, survival analysis, cell proliferation

Introduction

Non-small lung cell carcinoma (NSCLC), one of heaviest global disease burdens, is a non-curable disease that impacts thousands of people every year. While accumulated efforts to develop new molecular strategies and targeted therapies for patients with NSCLC become more and more promising, patient survival time remains poor [1]. Standard of care therapy in the clinical arena often involves resection with or without chemo-radiotherapy where the type of drugs administered is determined by the tumor, node and metastasis (TNM) system and clinical stage. Lung cancer, however, is actually a heterogenetic disease where each patient may harbor varying genetic tumor profiles although the TNM and clinical stage presents similarly. The genetic complexity of NSCLC calls for better models to determine appropriate treatment options for each patient. Our studies correlating tumor-related biomarker identification to patient prognosis may help physicians to improve the therapeutic regimen for NSCLC patients.

SOX (Sex determining region Y (SRY)-related high mobility group box) family proteins have been reported to be involved in the pathological and physiological processes of numerous tissues [2-4], such as reprogramming and regeneration [5]. So far, twenty different SOX proteins that share an HMG domain with more than 80% sequence identity are divided into different

Table 1. Correlation between expression of SOX8 and clinical parameters of 80 NSCLC

	Cases	*SOX8 expression*		*p-value*
		Low	*High*	
Gender				
Male	*60*	*22*	*38*	0.796
Female	*20*	*8*	*12*	
Age				
≥	*48*	*21*	27	0.238
<	*32*	*9*	23	
Alcohol[$]				
Yes	*50*	*8*	17	0.615
NO	25	20	30	
Cigarette[$]				
Yes	55	19	36	0.165
NO	18	10	8	
Differentiation				
Well	28	16	12	0.015*
Moderate/poor	52	14	38	
Histology				
Squamous	64	34	30	0.822
Adenoma	16	9	7	
T stage				
T1-T2	33	20	13	< 0.001*
T3-T4	47	10	37	
N stage				
N0	28	18	10	0.001*
N+	52	12	40	
Clinical stage				
I-II	43	12	37	0.013*
III-IV	37	16	15	

[$]Means some patients were not in neither of subgroup due to undetermined record; *Indicates *P* value < 0.05.

groups termed A to H [6]. SOX8 belongs to group E of SOX family proteins, which also includes SOX9 and SOX10 [7]. It is known that SOX8 plays important role in mammalian testis development [8, 9]. Recently, over-expressed SOX8 protein was observed in hepatocellular carcinomas [3] and brain tumors [10, 11]. However, there is little known about SOX8 in lung cancer. Given that sox8 shares a similar structure and genomic organization as SOX9, which is up-regulated in lung cancer [12, 13], we tested the clinical significance of SOX8 expression in 87 human tissue samples including 80 NSCLC patients' cancer and 7 normal bronchia tissues.

Unlike siRNA or shRNA, endogenous microRNAs (miRNAs) are abnormally expressed in human tumors and are nature's way of imposing transcriptional and translational regulation of targeted genes [14]. Due to above advantages, some miRNAs, notably in cancer such as miRNA-34a and let-7, are close to the stringent criteria for becoming ideal therapeutic intervention [15]. Recently, publications also indicated multiple functions of miRNAs in NSCLC. For example, microRNA-99b acts as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3 [16]. miRNA-124 is a brain-enriched miRNA and related to breast carcinogenesis and metastasis [17]. It is also adversely associated with the over-expression of SOX8 in glioma, and hepatocellular carcinoma [3, 11, 18, 19]. However, the function of miRNA-124 in NSCLC and its relationship with SOX8 remains to be uncovered.

Our study indicates that SOX8 expression correlates with survival time and other clinical parameters in 80 unselective NSCLC patients. In addition, we illustrate Sox8 is one of miRNA-124 targeted genes via dual luciferase reporter assay. In addition, miR-124 is under-expressed in 9 fresh NSCLC samples comparing to adjacent normal tissues. Ectopic expression of miR-124 can regulate NSCLC cell line proliferation; SOX8 protein decreased along with over-expression of miRNA-124 in H1299 and A549 cells. Thus, our work, from hospital ward to lab bench, may provide a new prognostic biomarker to help physicians select an appropriate therapeutic regimen for patients. Further, our research also indicates that if confirmed by future pre-clinical studies in animals, introduction of miRNA-124 maybe another tool to suppress NSCLC.

Methods and materials

Patients and specimens

From June 2007 to March 2008, 80 NSCLC patients tumors combined with 7 normal bronchial tissue (with > 5 cm distance from the primary tumor's edge) were collected into paraffin specimen from Linyi hospital. **Table 1** summarizes all clinical information. Nine pairs of newly diagnosed NSCLC samples used for miRNA extraction were from the same hospital. All samples were diagnosed by pathologists and collected before surgery, chemotherapy or

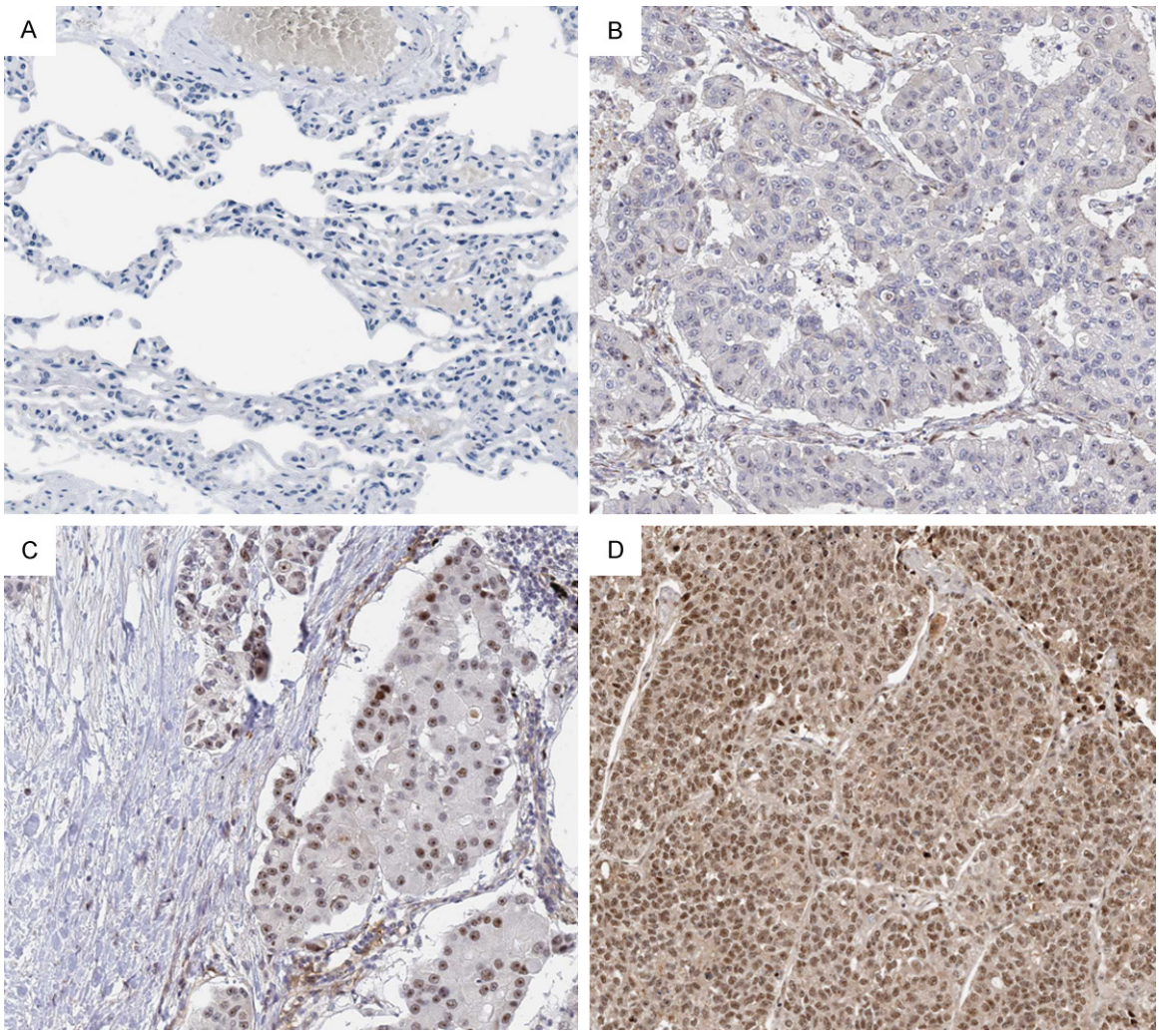

Figure 1. Representative Immunohistochemical pictures of SOX8 expression showed in 80 NSCLC samples and 7 normal adjacent tissues: A. Normal lung tissue; B. Negative staining in tumor; C. Moderate staining in tumor; D. Strongest staining in tumor.

radiotherapy. Informed consent was obtained from every patient according to the guidelines of Shandong Academy of Medical Sciences Ethics Committee.

Immunohistochemical staining and evaluation

Resected specimens were fixed with 10% neutral-buffered formalin and embedded in paraffin blocks according to instruction [20]. Briefly, 4-μm tissue slices were incubated with anti-SOX8 (1:100; rabbit antibody, Sigma-Aldrich, USA) with the PowerVision™ Two-Step Histostaining Reagent (Zhongshan Goldenbridge Bio, Beijing, China). For the negative controls, slides were probed with normal goat serum under the same experimental conditions.

The slides were evaluated and scored separately by two independent pathologists who were blinded to the patients' information. The staining scores of SOX8 were determined by a combination of the extent and intensity of staining. The intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining, referred to SOX9 (due to lack of SOX8 IHC staining methods reported), was scored as 1 (< 33%), 2 (33-66%), 3 (> 66%). The percentages were evaluated by the ratio of positive stained areas to the entire carcinoma-involved area. Final score was generated from the multiplication of the intensity and extent scores. Low expression and high expression were separated by final staining score of 0-4 and 5-9, respectively.

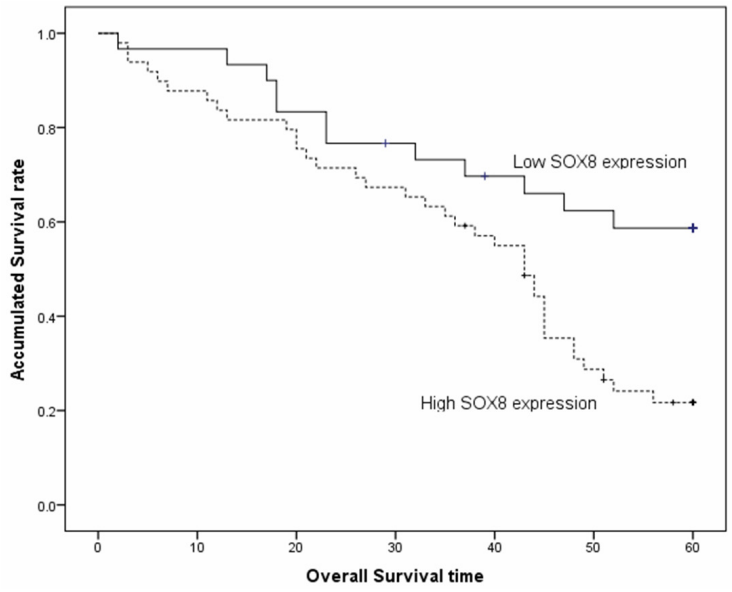

Figure 2. Kaplan-Meier curves with univariate analyses (log-rank) for patients with high and low SOX9-expressing in NSCLC patients. $P = 0.006$.

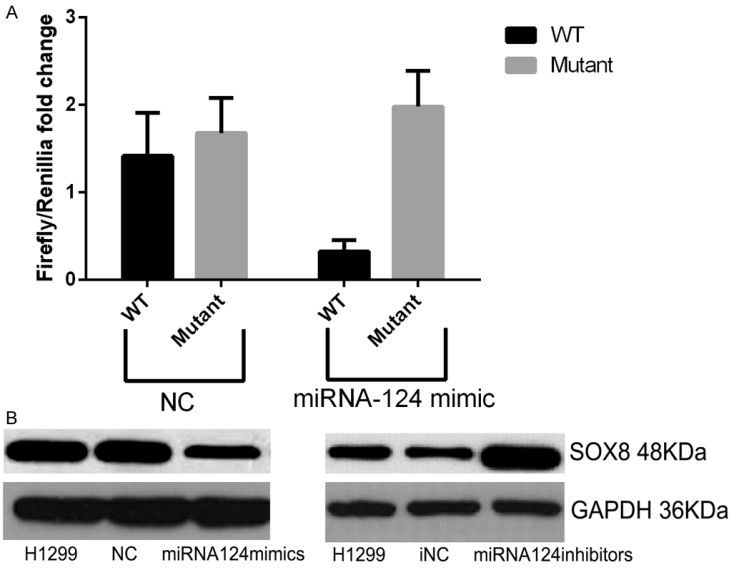

Figure 3. miRNA124 directly regulate the expression of SOX8. A. Dual luciferase reporter assay indicate miRNA124 reduced the relative luciferase activity of wild type rather than mutant of 3'UTR of SOX8 mRNA ($P = 0.002$). miRNA vector didn't alter the relative luciferase activity between them. Renillia was used to be internal control. B. SOX8 protein expression was correlated to the over- and down-regulated miRNA124 in H1299.

Follow-up

Follow-up data, ranged from 3 to 60 months, were collected after the surgery for these 80 patients, three patients of whom could not be contacted due to changes in address or phone number. The overall survival (OS) and disease-free survival (DFS) were calculated from the day of surgery to the date of death or tumor relapse.

Cell culture and transfection

Human NSCLC cell lines A549 and H1299 were kindly gifted by Dr. Le (provincial hospital affiliated to Shandong university) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. For transfections, cells were seeded in 6-wells plate to reach 70% confluency overnight. Lipofectamine 2000 (Invitrogen™, CA, USA) was used the transfection reagent. miRNA-124 mimics and inhibitors (Genepharma, Shanghai, China) were used to overexpress miRNA-124 in NSCLC cell lines.

Real time PCR

Total RNA was extracted from tissues using TRIZOL (Invitrogen, CA, USA) and miRNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. Quantitative detection of mature miRNA-124 was performed with All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia Inc., MD, USA). The expression assay was calculated by normalizing to U6 small nuclear RNA. All reactions were performed in triplicate for each sample.

Cell viability assay

Cell proliferation was detected by Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instructions. In brief, 2.5×10^3 of different treated cells were cultured in 96-well plates. Culture medium was changed every three days. Each experiment was performed in triplicate and then averaged.

Western blot

Proteins were extracted using RIPA lysis buffer (Beyotime, China). Proteins from whole cell

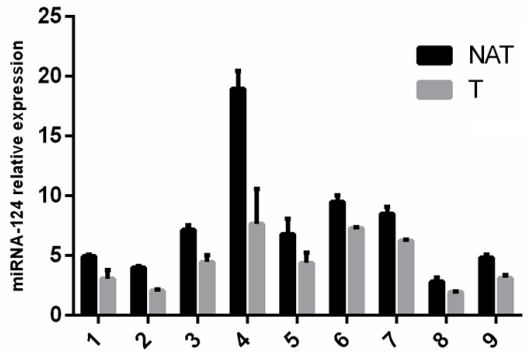

Figure 4. miRNA124 expression was decreased in tumor versus to normal tissues. q-PCR was performed to test the expression of miRNA124 in 9 fresh pairs of NSCLC tumors (T) and adjacent tissues (NAT). The significant difference ($P < 0.05$) happened to all pairs except the fifth one.

lysates were resolved by 10% SDS-PAGE gel, then transferred to the PVDF membrane, blocked in 5% non-fat milk in TBS/Tween-20, and blotted with the antibody against Sox8 (1:500, Sigma-Aldrich, USA), and according secondary antibody (1:1000, Beyotime, China). Film was developed using a SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientic. USA). β-Actin was used as an internal loading control.

Luciferase activity assay

1×10^5 HEK293T cells were seeded in 24-well plates. After 24 h, the mixture with 1 μg of firefly luciferase reporter vector containing 3'UTR of SOX8 genes, 0.1 μg of Renilla luciferase and miR-21 mimic or normal control were incubated with Lipofectamine 2000 about 15 min and aliquot into each well equally. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) 48 h after transfection. The firefly luciferase activity was normalized to the Renilla luciferase intensity.

Statistical analysis

The statistical software SPSS 13.0 (SPSS, Chicago, IL, USA) and GraphPad Prism6.00 (California USA) was used for analyses. The chi-square test was used to determine the correlation between SOX8 expression and clinicopathologic factors. The Kaplan-Meier method was used to estimate the probability of patient survival, and the differences between survivals of subgroups of patients were compared by using Mantel's log-rank test. Other results were analyzed using the Student's t-test. *P* value of < 0.05 indicates statistical significance.

Results

SOX8 over-expression in NSCLC tissues

Immunohistochemical analysis indicates that SOX8 was rarely detected in normal tissue adjacent to the tumor while SOX8 expression was observed well in tumor slides (**Figure 1**), which may reveal SOX8 over-expressed in NSCLC patients ($P < 0.01$). Furthermore, SOX8 was mainly expressed with nuclear staining. 62.5% (50 of 80) of tumor samples were considered to be stained with high expression of SOX8 within 86.25% (69 of 80) positively staining.

SOX8 expression associates with clinical pathological factor in NSCLC

To further investigate the significance of SOX8 expression in NSCLC, we analyzed the association of SOX8 with the patients' clinical parameters (**Table 1**). There was a significant correlation between SOX8 expression with tumor size, lymph node metastasis, differentiation classification and clinical stage. On the contrary, the SOX8 expression was not related to histology, gender and alcohol or cigarette history. Similar to these, age is not correlated to SOX8 expression in NSCLC patients.

High Sox8 expression indicates poor prognosi

As an over-expressed protein, survival analysis was also performed to assess the effect of SOX8 on patient survival time. With the follow up of NSCLC patients, high SOX8 expression significantly indicated reduced time of life after receiving surgery ($p = 0.006$, **Figure 2**).

Sox8 as a target gene of miRNA-124

Due to the cardinal role of SOX8 in NSCLC and the advantages of microRNA in cancer development and metastasis, we further analyzed the sequences of 3'UTR in Sox8 mRNA and some predicted miRNAs through the website service Targetscan [21]. Given the number of publications of miRNA-124 in cancer [22-26], we chose miRNA-124 for further validation. In our dual luciferase reporter assay, we found miRNA-124 decreased the relative luciferase activity of firefly carrying the wild type, but not mutant, 3'UTR

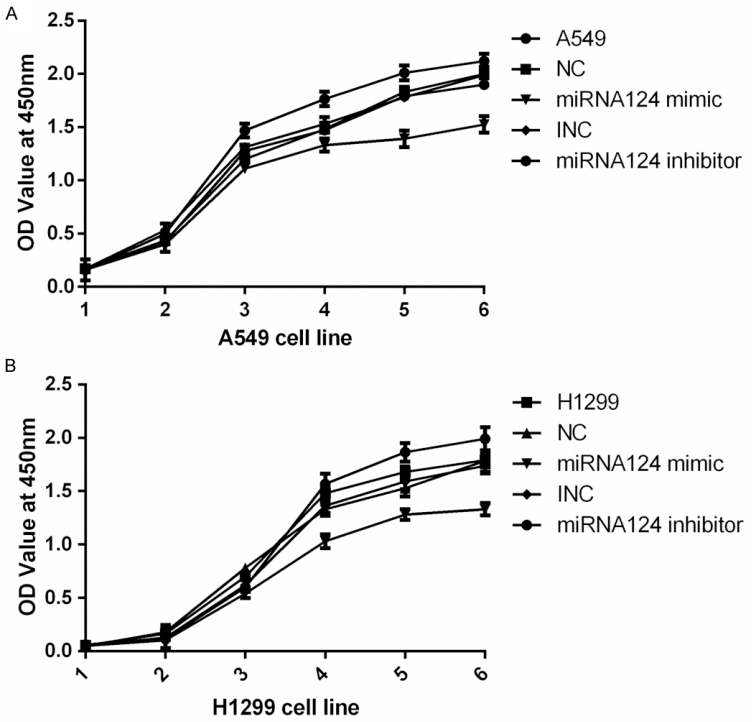

Figure 5. Cell growth was significantly interrupted by deregulated the expression of miRNA124 in A549 (A) and H1299 (B).

of SOX8 mRNA (P = 0.002, **Figure 3A**). Additionally, SOX8 protein was decreased in the H1299 cells with miRNA-124 mimics compared to vector control, supporting our validation of SOX8 as a target gene of miRNA-124 (**Figure 3B**).

Decreased expression of miRNA-124 in NSCLC patients

It is established that miRNA-124 is down-regulated in various kinds of tumors [25, 27, 28]. Real time PCR was performed to test whether miRNA-124 also decreased in NSCLC patients. Significantly reduced expression of miRNA-124 was observed in 9 NSCLC samples comparing with normal adjacent tissues (**Figure 4**).

Cell proliferation is regulated by miRNA-124 in NSCLC cell lines

With information about the potential role of SOX8 and the relationship between SOX8 and miRNA-124 in patient samples, we further analyzed whether miRNA-124 truly related to some functions of NSCLC in cell-based assays. miRNA-124 mimics and inhibitors in two types of NSCLC cells provided more evidence to indicate that miRNA-124 can regulate NSCLC cell growth (**Figure 5**).

Discussion

Recently, lines of evidences indicate SOX proteins play important role in tumor development and tumor stem cell maintenance [5]. Although, both SOX8 and SOX9 are belonging to the E group of SOX family proteins, there are little reports about SOX8 in cancer, while SOX9 was investigated in various types of tumor including NSCLC [29-31]. Taking two clues into account (high sequence and structure similarity to SOX9 and SOX8 over-expression in glioma and hepatocellular carcinoma [3, 12]), we tested SOX8 expression in 87 human tissue samples including 80 NSCLC patients cancer and 7 normal bronchia tissues. SOX8 protein expressed obviously in tumor samples while there is nearly no staining in normal tissues. This indicates that over-expressed SOX8 may help normal cells to transform into cancer cells. We also found that SOX8 expression significantly related to the NSCLC tumor size, lymph node metastasis and cell differentiation, further supporting that SOX8 may be involved in NSCLC carcinogenesis and metastasis. It was reported that SOX8 may increase cell growth by regulating the activity of beta-catenin, a protein involved in tumor metastasis [3, 32]. However, the detailed mechanism of the role of SOX8 in cancer still remains to be elucidated. In addition, our clinical data revealed that higher expression of SOX8 correlated to less survival time for patients, supporting SOX8 as a potential oncogene in NSCLC. If the results can be confirmed by a larger sample size and animal experiments, SOX8 will be a promising biomarker for helping physician to make better clinical decisions.

A line of evidence demonstrates that miRNAs can regulate multiple cellular pathways by modulating the expression of various target genes in NSCLC [33]. TargetScan service was used to predict the upstream miRNAs that target SOX8 mRNA via sequence blast between 3'UTR of

SOX8 mRNA and sequence of miRNAs. Together with the role of miRNA-124 depressed SOX9 [34], we chose miRNA-124 to further validate. In our study, miRNA-124 can decrease the relative luciferase activity of wild type, but not mutant, 3'UTR of SOX8, which supported the hypothesis that SOX8 is one of the directly targeted genes of miRNA-124. This finding may explain partly why miRNA-124 can decrease the expression of SOX9 [34] due to it has the same site (5'CUGCUGA3') where miRNA-124 binds to the 3'UTR of SOX8 mRNA. miRNA-124 is reported as a tumor suppressor in human cancers such as oral, hepatocellular and gastric cancer [22, 24, 35]. In our experiment, reduced expression of SOX8 was detected in tumors compared with normal adjacent tissues through quantitative PCR, confirming previous reports in literature [35-37].

miRNA-124 was reported to inhibit breast cancer cell proliferation by targeting CD151 [38], decrease hepatocellular tumor growth by regulating STAT3 [22], and reduce colorectal carcinoma through decreasing iASPP [36]. Here, we over-/under-expressed miRNA-124 in H1299 and A549 cell lines to measure the effect on cell proliferation. Our results illustrated that elevated miRNA-124 could significantly reduce NSCLC cell growth while inhibited miRNA-124 expression could promote cell growth. Interestingly, down-regulated SOX8 protein expression was observed along with over-expressed miRNA-124, which further supports that miRNA-124 can directly regulate the expression of SOX8 and decreased SOX8 may be involved in one of mechanisms of miRNA-124 suppressing the cell proliferation.

To our knowledge, the study offers the first report about the significance of SOX8 protein in NSCLC patients and the effect of miRNA-124 in NSCLC cell lines. In addition, we also confirmed that SOX8 was one of miRNA-124 target genes.

In conclusion, the study provides the first convincing clue that miRNA-124 may suppress tumor proliferation of NSCLC via down-regulating SOX8. This correlation to tumor progression suggests SOX8 may be a potential prognostic marker for NSCLC.

Disclosure of conflict of interest

None.

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