High expression of lncRNA FOXD2-AS1 promotes epithelial to mesenchymal transition of non-small cell lung cancer

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Abstract

Non-small cell lung cancer (NSCLC) accounts for more than 80% of lung cancer and the high mortality probably be caused by early invasion and metastasis. LncRNA FOXD2-AS1 is involved in tumor proliferation, invasion and metastasis. Epithelial-to-mesenchymal transition (EMT) is the main critical process of progression and metastasis. Therefore, the aim of the study is to investigate the expression of FOXD2-AS1 in NSCLC and identify the effects of FOXD2-AS1 on EMT in lung cancer. The results showed that the expression of FOXD2-AS1 was significantly upregulated in NSCLC, and over-expression of FOXD2-AS1 promoted cell migration and invasion in lung cancer cells. In addition, over-expression of FOXD2-AS1 regulated EMT-related molecules, for instance, the mRNA and protein expression levels of CK18, α-SMA and FN were increased by FOXD2-AS1, while mRNA and protein expression levels of E-cadherin were decreased by FOXD2-AS1. In conclusion, FOXD2-AS1 promotes the invasion and metastasis of NSCLC, which might be a therapeutic target for the treatment of NSCLC.

Keyword: FOXD2-AS1, Epithelial-to-mesenchymal transition, NSCLC

Introduction

At present, lung cancer has become the main cause of cancer-related death. Non-small cell lung cancer (NSCLC) mainly includes adenocarcinoma, lung squamous cell carcinoma and large cell undifferentiated carcinoma, and accounts for more than 80% of lung cancer (1). The five-year survival rate remains poor due to the poor sensitivity of radiotherapy and chemotherapy (2). Recently, the progresses have been made in surgery, radiotherapy, chemotherapy and molecular targeted therapies have been achieved for NSCLC, especially in the molecular carcinogenesis and new targeted therapies(3-5). However, the overall survival rate of NSCLC disease still remains low (6, 7).

The high mortality in most tumor patients is probably caused by early invasion and metastasis (8). Invasion and metastasis of NSCLC is not only the sign of worsening state, but also an important reason for treatment failure (9). The mechanism was not clear probably owing to the complex process of metastasis in NSCLC (10). EMT referred to the transformation of epithelial cells in morphological mesenchymal cell phenotype and recently many studies reported that epithelial-to-mesenchymal transition (EMT) was the main critical process of progression and metastasis (11, 12). Cytokeratin 18 (CK18) and E-cadherin are classical epithelial phenotypic markers, while α-smooth muscle actin (α-SMA) and fibronectin (FN) are interstitial phenotypic markers (13-16). Therefore, the variation of CK18, E-cadherin, α-SMA and FN was used for the assessment of EMT variation.

It was found that the development and malignant transformation of lung cancer were influenced by genetic factors as well as the non-coding RNA of small molecules (17, 18). Therein, long noncoding RNA (lncRNA) exerted a significant role in the malignant transformation of lung cancer. LncRNA is consisted of over 200 nucleotides in length of transcript (19). Some lncRNAs have been illustrated to play regulatory roles in lung tumor, and were viewed as biomarkers for tumor diagnosis and prognosis (20-22). Previous studies have confirmed that FOXD2-AS1 was an

important modality of embryonic development, which performed abnormal expression in various tumors and was involved in tumor proliferation and, invasion and metastasis (23-25). FOXD2-AS1 plays a crucial role in carcinogenesis of gastric cancer (26), regulates cell proliferation of colorectal cancer (27), and promotes the progression and recurrence of bladder cancer (28). Moreover, the overexpressed FOXD2-AS1 can predict the poor prognosis of esophageal squamous cell carcinoma (29). In NSCLC, abnormally expressed FOXD2-AS1 induces the progression of NSCLC (30).

However, the potential mechanisms of FOXD2-AS1 in regulating the EMT of NSCLC have not been fully elucidated. The aim of the study was to investigate the expression of FOXD2-AS1 in A549 cells and identified the potential relationship between FOXD2-AS1 and EMT in NSCLC.

Materials and methods

Clinic samples collection.

60 NSCLC cases, including 30 pairs of NSCLC tissues and adjacent normal tissues were collected from the patients hospitalized at Chengdu Second People's Hospital from Jan 16, 2014 to Oct 30, 2017. All the patients have no chemotherapy and radiotherapy before. All the patients agreed and signed the confirmed consent. The study has been approved by the Ethics Committee of Chengdu Second People's Hospital. The tissue samples were stored in liquid nitrogen preservation at -80℃. The baseline data (age, BMI, gender, ect.), tumor size, grade and stage were recorded (Table I).

Table I. The expression of FOXD2-AS1 in lung carcinoma patients' tissues.

Factors	Case	FOXD2-AS1 (mean)	*P* value
Gender			0.421
Male	17	2.192 ±0.054	
Female	13	2.119 ±0.075	
Age(years)			0.191

<60	17	2.226 ±0.075	
≧60	13	2.074 ±0.085	
Histological grade			0.002
Well-intermediately differentiation	12	2.259 ±0.038	
Poor differentiation	18	2.080 ±0.033	
Metastasis			0.02
NO	21	2.072 ±0.038	
YES	9	2.224 ±0.032	

Cell culture.

NSCLC cell line A549 was obtained from American type culture collection (ATCC). The cell lines were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) culture medium supplemented with 10 % fetal bovine serum (FBS) (HyClone, Camarillo, CA, USA) as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator with a humidified atmosphere of 5% CO_2 at 37℃. Cell propagation was conducted every other day.

Quantitative real time PCR.

Total RNA of cells was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Then Primer-Script™ One Step RT-PCR Kit (TaKaRa, Dalian, China) was used for cDNA synthesis. Real time-qPCR was performed to amplify cDNA template using the SYBR® Premix Dimmer Eraser kit (TaKaRa, Dalian, China) and carried out on ABI 7500 system (Applied Biosystems, CA, USA) using a SYBR Premix Ex Taq II kit (TaKaRa) according to the manufacturer's instructions. The expressive differences of lncRNA FOXD2-AS1 were compared between cancer and para-carcinoma tissue using RT-PCR. LncRNA FOXD2-AS1 and mRNA values were normalized to GAPDH.

Grouping and transfection.

The A549 cells on logarithmic stage were used for the experiment and divided

into blank group, control group and LncRNA group. The blank group was treated with nothing. Full length FOXD2-AS1 cDNA was amplified from cDNA of BEAS-2B and cloned into pcDNA-3 plasmid. The transient over-expression of FOXD2-AS1 was achieved by transfection of pcDNA-3- FOXD2-AS1 into A549 cells using Lipofectamine 2000 (Invitrogen), and A549 cells tranfected with empty plasmid served as control group.

Wound scratch assay.

Cells A549 were planted into 6-well plate (4×105 cells/well) until the cells reached 80-90% of confluence. Then cells were scratched with a 10-μl pipette tip. After this, cells were washed with FBS and serum-free medium. Subsequently, the wound was visualized with a microscope (magnificantion ×100; Olympus, Tokyo, Japan) at the time points of 0 h and 24 h after scratching.

Transwell assay.

Cells were collected and re-suspended in serum-free RPMI-1640 and placed in the upper chamber of 24-well Transwell plates which were pre-coated with Matrigel (8 μm-pore; Corning Incorporated, NY, USA). The concentration of cells was adjusted to 2×10^5 cells/ml. Then cells in lower chamber were cultured with RPMI 1640 culture medium supplemented with 10% FBS, and incubated for 24 h at 37℃. After this, cells were washed with PBS. Later cells were fixed with 4% paraformaldehyde. Then cells were stained with 0.1% crystal violet. Subsequently, the cells in the bottom of the chamber were calculated by using an optical microscrope (magnificantion ×100).

Western blot analysis.

After 72-hour transfection, cells were collected and lysed with RIPA Lysis buffer (Beyotime, China) on the ice. The supernate was obtained to detect its protein concentration. Equivalent amount of protein (50 μg) from each sample was resolved by using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% skimmed milk for 2 h at room temperature, washed three times in TBS-Tween-20, and incubated with primary antibodies. The primary antibodies were

as follows: anti-CK18 (1:200, SAB4501653, Sigma-Aldrich, St. Louis, MO, USA); anti-E-cadherin (1:100, SAB4503751, Sigma-Aldrich, St. Louis, MO, USA); anti-α-SMA (1:100, AV40209, Sigma-Aldrich, St. Louis, MO, USA); anti-FN (1:100, F3648, Sigma-Aldrich, St. Louis, MO, USA); anti-GAPDH (1:100, G9545, Sigma-Aldrich, St. Louis, MO, USA). The membranes were incubated with the primary antibodies overnight at 4℃ and then incubated with the secondary antibody (1:5000) for 1 h at room temperature. Finally, the protein bands were visualized using the ECL detection system (Thermo Fisher, MA, USA) and quantified by densitometry using Gel-Pro analyzer software. GAPDH was used as a loading control.

MTT assays.

MTT assay was conducted to detect the proliferation effect of A549 cells caused by the change of FOXD2-AS1level in LncRNA. A549 cells (1×10^5 cells per well) were seeded in 96-well plates at 37℃ in an incubator containing 5% CO_2. After 24 h transfection, cells were treated with 20 μl 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, 5μg/μl, Sigma) 10 nM for 4 h. Then cells were treated with dimethylsulfoxide (DMSO, 150μl/hole) with the final concentration of 0.5 mg/mL. The absorbance at the wavelength of 490 nm was detected using micro-plate reader. The ratio of absorbance in the other groups to the control group was calculated as the multiplication rate.

Statistical analysis.

Data were expressed as mean ± SD. SPSS 20.2 was performed to analyze the data. Two-tailed student T-test was performed to compare differences between two groups. ANNOVA followed by Newman-Keuls post-hoc analysis was conducted to evaluate the differences among 3 groups. $p<0.05$ was considered as statistical significance.

Results

LncRNA FOXD2-AS1 was overexpressed in tissue samples from NSCLC patients.

We profiled NSCLC tumor and matched normal samples using qRT-PCR to identify the expression of novel lncRNAs in NSCLC. The results noted that FOXD2-AS1 was of higher expression in tumor tissues (n=30) than in normal tissues (n=30) ($p < 0.001$), which illustrated that lncRNA FOXD2-AS1 was abnormally high expressed in lung cancer tissues (Figure. 1A). We validated that higher lncRNA FOXD2-AS1 expression levels were closely associated with poor differentiation of histological grade and metastasis in NSCLC patients (Table I, $p<0.05$). However, we didn't see any other agents affecting the expression of lncRNA FOXD2-AS1 (Table I, $P > 0.05$).

For further confirmation of the expressive results of FOXD2-AS1, pcDNA-3-FOXD2-AS1 for overexpression was transfected into 1549 cells. The results validated that the expression of FOXD2-AS1 in lung cancer cells transfected with pcDNA-3-FOXD2-AS1 was significantly increased compared with blank group and control group (Figure 1B), indicating the successful transfection of pcDNA-3- FOXD2-AS1.

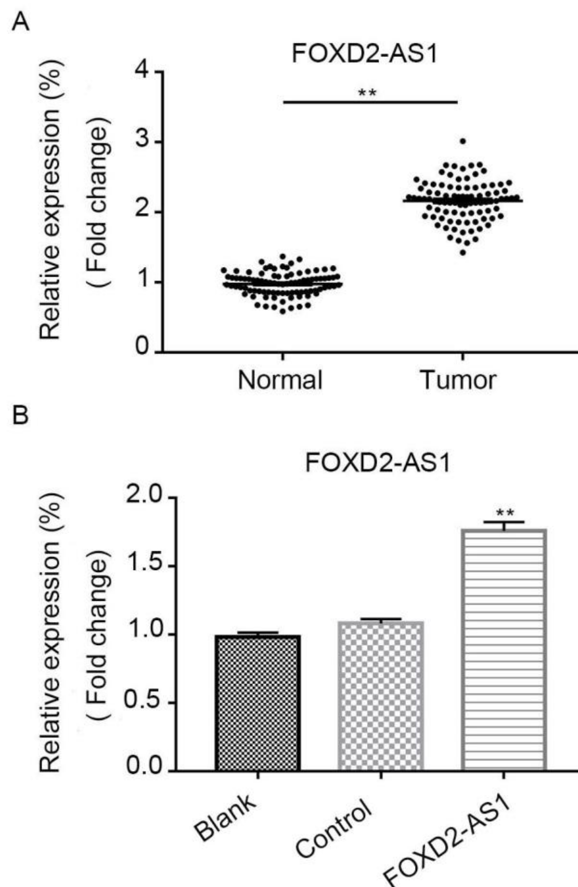

Figure 1. LncRNA FOXD2-AS1 was overexpressed in NSCLC tumor tissues.

(A) FOXD2-AS1 was overexpressed in tumor tissues (n=30) than normal tissues (n=30) detected by qRT-PCR. (B) Relative FOXD2-AS1 expression in A549 was analyzed by qRT-PCR after pcDNA-3-FOXD2-AS1 transfection. The expression of FOXD2-AS1 was significantly increased after treatment of pcDNA-3-FOXD2-AS1 compared with blank group and control group. Data are mean ±SD. **, p < 0.01.

FOXD2-AS1 promoted cell proliferarion of NSCLC cells.

To further investigate the function of FOXD2-AS1 in NSCLC, MTT assays showed that FOXD2-AS1 overexpression promoted NSCLC cell growth (Figure 2).

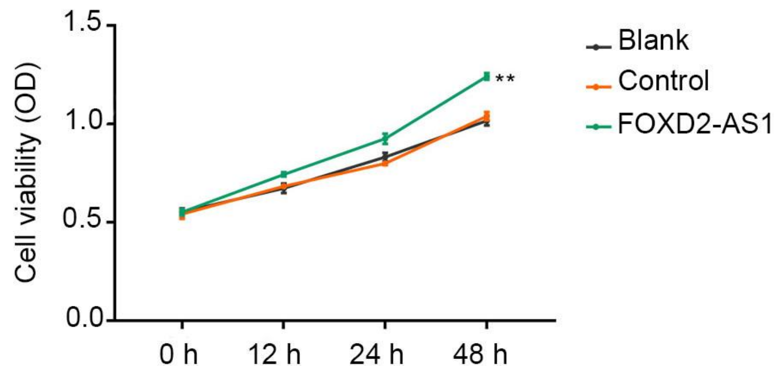

Figure 2. FOXD2-AS1 promoted the cell proliferation of NSCLC cells.

The result showed that overexpressed FOXD2-AS1 promoted the proliferation of NSCLC. Data are mean ±SD. **, p < 0.01.

FOXD2-AS1 promoted cell migration of NSCLC cells.

After 24 h, the percent of wound in the cells treated with FOXD2-AS1 was significantly decreased, which suggested that FOXD2-AS1 could facilitate the migration of NSCLC cells (Figure 3A and B, p<0.01).

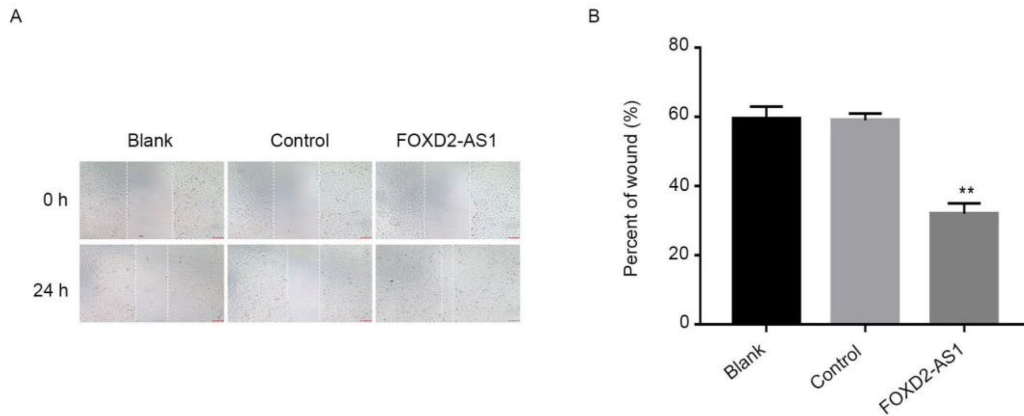

Figure 3. FOXD2-AS1 promoted the cell migration of NSCLC cells.

(A-B) The migration of NSCLC was significantly increased after the transfection of pcDNA-3-FOXD2-AS1 compared with blank and control groups. Data are mean ±SD. **, $p < 0.01$.

FOXD2-AS1 promoted cell invasion of NSCLC cells.

Transwell assay was conducted to examine the invasion of A549 cells. As shown in Figure 4A and B, cells transfected with pcDNA-3- FOXD2-AS1 significantly increased the number of invaded cells ($p<0.01$).

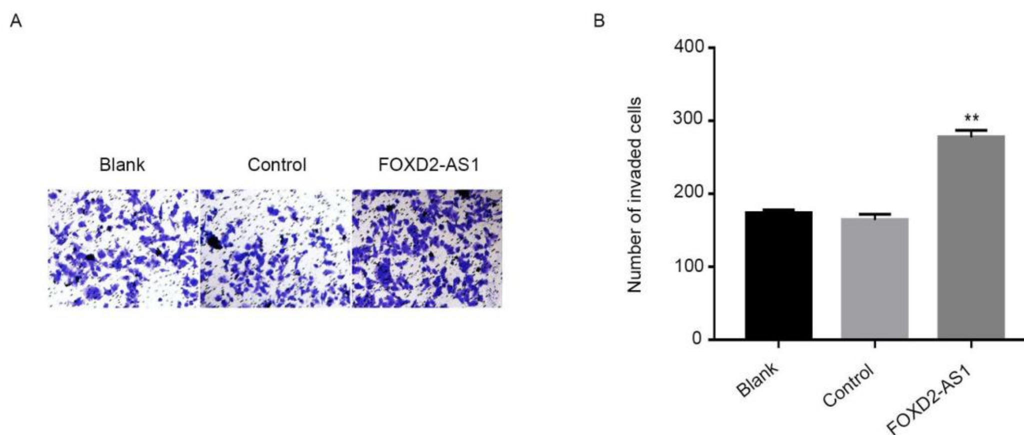

Figure 4. FOXD2-AS1 strengthened the invasion ability of NSCLC cells.

Cells transfected with pcDNA-3-FOXD2-AS1 showed higher invasion ability than in control group (A). The statistical data were presented in B. Data are mean ±SD. **, $p < 0.01$.

FOXD2-AS1 regulates EMT in lung cancer cells.

Furthermore, we observed the effects of up-regulated lncRNA FOXD2-AS1 expression on cell EMT phenomenon. We found that, compared with control group, the overexpression of lncRNA FOXD2-AS1 increased the expression levels of FN, CK18, and α-SMA, but decreased the expression of E-cadherin in A549 cells. (Figure 5A-D, $p<0.01$).

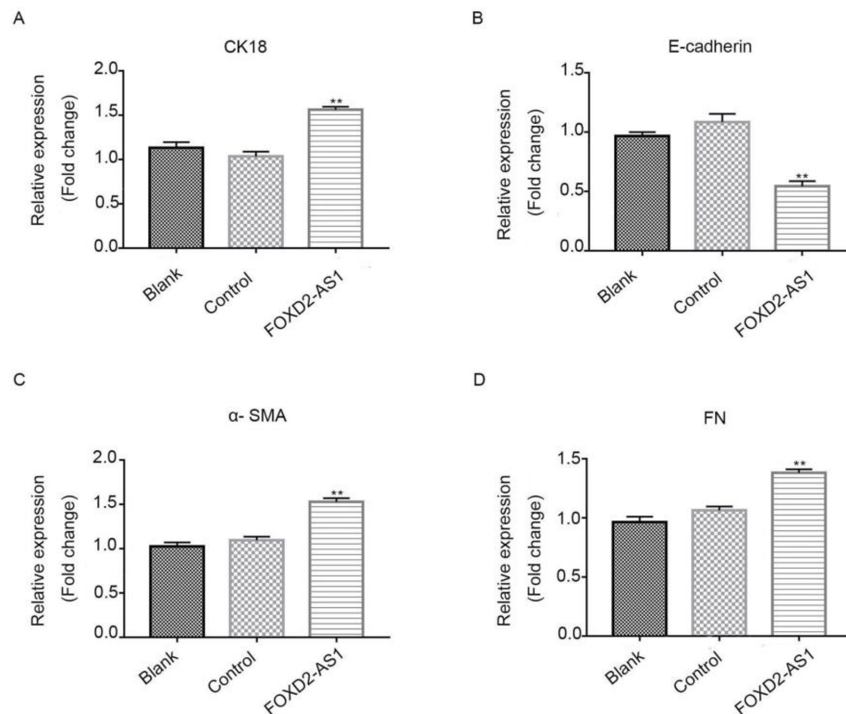

Figure 5. FOXD2-AS1 increased the mRNA levels of CK18, FN, and α-SMA, and decreased the mRNA level of E-cadherin,

(A-D) The overexpression of lncRNA FOXD2-AS1 increased the mRNA levels of CK18, FN, and α-SMA, while decreased the mRNA level of E-cadherin in A549 cells. Data are mean ± SD. **, $p < 0.01$.

In order to further validate the FOXD2-AS1 expressive mechanism, western blot were performed to analyze the protein levels of CK18, E-cadherin, α-SMA and FN after transfection and GAPDH was used as an internal control. The gray analysis showed that the overexpressed FOXD2-AS1 significantly increased the expression of

CK18, α-SMA, and FN in A549 cells, while remarkably reduced E-cadherin expression (Figure 6A-E, p<0.01).

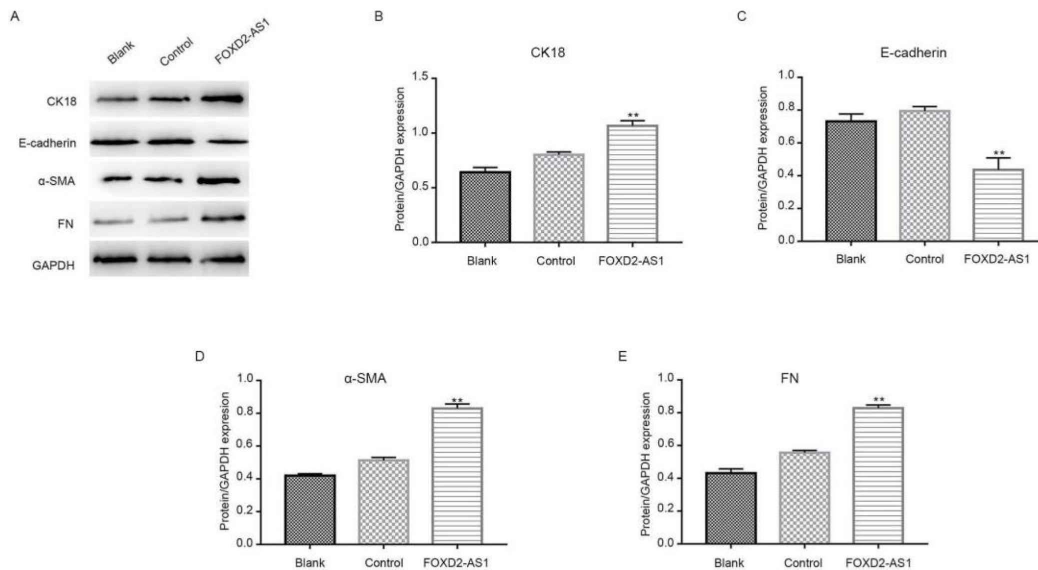

Figure 6. FOXD2-AS1 increased the protein levels of CK18, FN, and α-SMA, and decreased the mRNA level of E-cadherin,

(A) FOXD2-AS1 overexpression increased the protein levels of CK18, FN, and α-SMA, while decreased the protein level of E-cadherin in A549 cells. (B-E) The statistical data were also exhibited. Data are mean ± SD. **, $p < 0.01$.

Discussion

Accumulating studies have indicated that lncRNAs performed an important function in the cancer pathogenesis. Many studies reported lncRNA contributed to tumorigenesis, tumor progression, and regulating tumor recurrence and metastasis (31). Lung cancers mainly including NSCLC were the most common malignancy (32). Jia et al. found that lncRNA HIT combined with ZEB1 promotes the migration and invasion of NSCLC (33). Tian et al. showed that overexpressed ZFAS1 decreases survival of human NSCLC patients (34). Fang et al. reported that lncRNA XIST acts as an oncogene in NSCLC by epigenetically repressing KLF2 expression (35). The expression of lncRNAs FOXD2-AS1 is associated with the tumor aggression and regulates tumor progression and metastasis (23, 25). Previous studies suggested that

migration, invasion and metastasis were the key factors for therapeutic failure of NSCLC (36, 37). In our study, we detected the FOXD2-AS1 level in NSCLC tissue, which performed higher expression than that in normal tissue. Moreover, MTT assays also indicated that up-regulated FOXD2-AS1 promoted the proliferation of NSCLC cells. Therefore, lncRNA FOXD2-AS1 acted as an oncogene in NSCLC.

EMT is of complex mechanism and plays an important role in pathological process including invasion and migration of neoplasms (38). On one hand, EMT can alter characteristics of carcinoma cell to weaken the expression of junction markers and improve the movement of carcinoma cell and thus enhances its invasion and migration ability; on the other hand, EMT can enhance the ability of invasion and migration through altering the microenvironment of tumor proliferation, invasion and metastasis as well as angiopoiesis (39, 40). Recently, lncRNAs regulate EMT via adjusting gene expression, which provides a novel direction. Growing evidence reported the relationship between EMT and lncRNA in tumor proliferation, metastasis, and invasion. For instance, Cao et al. illustrated that lncRNA UBE2CP3 promoted tumor metastasis by inducing EMT in hepatocellular carcinoma (HCC) (31). Miao et al. demonstrated that downexpression of lncRNA FOXF1-AS1 regulates EMT, stemness and metastasis of NSCLC (41). Li thought BCAR4 promotes proliferation, invasion and metastasis by affecting EMT (42). However, the potential roles of FOXD2-AS1 in NSCLC are not clear. In our study, we explored the potential effects of FOXD2-AS1 on the cell migration and invasion of NSCLC. The results showed that up-regulated FOXD2-AS1 enhanced the migration and invasion ability of NSCLC cells.

To further explore the potential mechanisms of FOXD2-AS1 in NSCLC, we examined the effects of FOXD2-AS1 on EMT markers: CK18, E-cadherin, α-SMA, and FN. Increasing evidence showed that The E-cadherin and CK18 are associated with epithelial markers and the FN and α-SMA are mesenchymal markers (43, 44). Increasing evidence showed that CK18, α-SMA, and FN are positively correlated with ETM (45). Consistently, our study showed the expression of CK18, α-SMA and FN in

the cells transfected with pcDNA-3- FOXD2-AS1 was increased, which may further promoted the cell migration and invasion rate of NSCLC E-cadherin is an important marker of maintaining epithelial phenotype, the loss of which in cells will weaken adhesion ability and enhance the invasive ability (46). The suppressed expression of E-cadherin promoted the process of EMT and thus enhanced invasion and migration ability (47). In this study, we found that overexpressed FOXD2-AS1 downregulated the expression of E-cadherin, which further showed that FOXD2-AS1 may play an important role in promoting the progression of EMT of NSCLC cells and enhancing the migration and invasion ability of NSCLC cells.

Taken together, in the present study, lncRNA FOXD2-AS1 was upregulated in NSCLC tumor tissues and the up-regulated FOXD2-AS1 promoted the progression of EMT as well as the proliferation, migration, and invasion in NSCLC. In general, our study provide a new basis for further studies and identified a critical of FOXD2-AS1, which is a potential therapeutic target of NSCLC and shows a critical role in EMT.

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