FOXF2 aggravates the progression of non-small cell lung cancer through targeting lncRNA H19 to downregulate PTEN

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Abstract. – **OBJECTIVE:** To illustrate the role of FOXF2 in the aggravation of the progression of non-small cell lung cancer (NSCLC) by targeting long non-coding RNA (lncRNA) H19 to down-regulate the gene of phosphate and tensin homolog deleted on chromosome ten (PTEN).

PATIENTS AND METHODS: The relative levels of FOXF2 and H19 in NSCLC tissues and adjacent normal tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between the expression levels of FOXF2 and H19 was analyzed. Kaplan-Meier curves were depicted for uncovering the prognostic value of FOXF2 in NSCLC patients. The proliferative and migratory abilities of A549 cells influenced by FOXF2 were also assessed. The interaction between FOXF2 and H19 was evaluated through chromatin immunoprecipitation (ChIP) assay and Western blot, so did the association between H19 and PTEN. Finally, rescue experiments were conducted to explore the role of FOXF2/H19/PTEN axis in regulating the viability and migration of A549 cells.

RESULTS: FOXF2 and H19 were upregulated in NSCLC and a positive correlation was observed between the two genes. High level of FOXF2 indicated a worse prognosis in NSCLC patients. The knockdown of FOXF2 attenuated the proliferative and migratory abilities of A549 cells. FOXF2 could bind to the promoter region of H19 and accelerated its transcription. Moreover, H19 could recruit EZH2 to bind to PTEN. The overexpression of H19 could reverse the regulatory effects of FOXF2 on the viability and migration of A549 cells.

CONCLUSIONS: FOXF2 was upregulated in NSCLC. It accelerated the proliferative and migratory abilities of the NSCLC cells by targeting H19 to downregulate PTEN, thus aggravating the progression of NSCLC.

Key Words:
Non-small cell lung cancer (NSCLC), FOXF2, H19, PTEN.

Introduction

Lung carcinoma is the most prevalent high-mortality cancer in developing countries[1]. Lung carcinoma can be pathologically classified into two categories: small cell lung cancer and non-small cell lung cancer (NSCLC), which accounts for about 13% and 87% incidence, respectively[2]. NSCLC includes three subgroups of squamous cell carcinoma, adenocarcinoma, and large cell carcinoma[3]. In the past few decades, the 5-year survival of lung carcinoma patients was only 18%[4]. Therefore, it is of great value to clarify the molecular mechanisms of NSCLC and provide new targets for clinical treatment.

Forkhead-box (FOX) is a superfamily of transcriptional regulators with multiple functions involving in development, proliferation, and tumorigenesis[5,6]. FOXF2 (also known as Freac-2 or Fkh16) is a member of the FOXF subfamily and was widely expressed in various mesenchymal tissues. It contains a nuclear-localized forkhead domain and two independent C-terminal activation domains[7]. Kundu et al[8] have revealed that FOXF2, as a key regulator in epithelial-mesenchymal transition (EMT), exerts an important role in the invasion and metastasis of NSCLC.

Long non-coding RNAs (lncRNAs) are non-coding RNAs over 200 nt in length and can regulate the gene expressions at the transcriptional and post-transcriptional levels[9]. The dysregulated lncRNAs are frequently observed in the development and progression of cancers[10-12]. Despite the lack of protein-encoding ability, lncRNAs are involved in cancer biological processes by regulating the oncogenes or tumor suppressors[13,14]. Therefore, cancer-related lncRNAs could be used as drug targets to develop therapeutic strategies. This study aimed to uncover the transcription fac-

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tor FOXF2 in influencing the progression of NSCLC and its potential mechanism.

Patients and Methods

Clinical Samples

A total of 48 paired tumor tissues and matched adjacent tissues (3 cm away from the tumor edge) were surgically resected from NSCLC patients admitted in Xi'an Chest Hospital from March 2016 to October 2018. They were pathologically diagnosed as NSCLC and did not receive preoperative anti-tumor therapy. The clinical data of the enrolled NSCLC patients were collected. All subjects volunteered to participate in the study and signed a written informed consent. This study was approved by the Ethics Committee of Xi'an Chest Hospital.

Cell Culture

A549, NCI-H1650, and HCC827 were provided by Cell Bank (Shanghai, China). The cells were maintained with Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 UI/mL penicillin and 0.1 mg/mL streptomycin at 37°C, in a 5% CO_2 incubator.

Cell Transfection

The cells were cultured until they reached 60% of confluence and subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 h of incubation, the complete medium was replaced. The cells transfected for 24-48 h were harvested for the following experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The extraction of total RNA in the cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed in Table I.

Table I. Primer sequences.

Gene	Primer sequences
FOXF2	F: 5'-TCGCTGGAGCAGAGCTACTT-3' R: 5'-CCCATTGAAGTTGAGGACGA-3'
PTEN	F: 5'-CCAGTCAGAGGCGCTATGTG-3' R: 5'-ACTTGTCTTCCCGTCGTGTG-3'
H19	F: 5'-AGCGGGTCTGTTTCTTTACTT-3' R: 5'-GTCGTGGAGGCTTTGAATCT-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membranes were incubated with the secondary antibody after rinsing with the Tris-Buffered Saline and Tween (TBST). The bands were exposed by electrochemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Wound Healing Assay

The cells were seeded into 24-well plates at 5.0×10^5 cells/well. An artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. The wound closure images were taken at 0 and 24 h using an inverted microscope, respectively. Percentage of wound closure was then calculated.

Chromatin Immunoprecipitation (ChIP)

The cells were subjected to 10-min cross-link with 1% formaldehyde at room temperature. Subsequently, the cells were treated with lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immunoprecipitated with the corresponding antibodies or anti-IgG.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. Survival analysis was conducted using the Kaplan-Meier method, followed by the log-rank test to compare the differences. $p<0.05$ was considered as statistically significant.

Results

Upregulation of FOXF2 in NSCLC

Totally 48 paired NSCLC and adjacent normal tissues were collected in this study. QRT-PCR results revealed higher abundances of FOXF2 and H19 in NSCLC tissues, compared to those of normal tissues (Figure 1A, 1C). Through the Kaplan-Meier analysis, a worse prognosis was observed in NSCLC patients with a high level of FOXF2 (Figure 1B). A positive correlation between the expressions of FOXF2 and H19 was identified in NSCLC tissues (Figure 1D).

Silence of FOXF2 Suppressed Proliferative and Migratory Abilities of NSCLC

FOXF2 was identically upregulated in NSCLC cells (Figure 2A). A549 cell line was selected for the following experiments. The transfection of si-FOXF2 in A549 cells markedly down-regulated FOXF2 level (Figure 2B). The viability in A549 cells was remarkably suppressed after transfection of si-FOXF2 (Figure 2C). Besides, the wound closure was also significantly inhibited after the silence of FOXF2 (Figure 2D). It could be concluded that the knockdown of FOXF2 inhibited the proliferation and migration of NSCLC cells.

FOXF2 Mediated H19/PTEN (Gene of Phosphate and Tensin Homolog Deleted on Chromosome Ten) Axis

Subsequently, our results further indicated that the transfection of si-FOXF2 markedly down-regulated the H19 level in A549 cells (Figure 3A). The ChIP assay was then conducted to explore the interaction between FOXF2 and H19. H19 was mainly enriched in the anti-FOXF2 group while not in the anti-IgG group, indicating that FOXF2 could bind to the promoter region of H19 (Figure 3B). We thereafter constructed si-H19 to knockdown the expression of H19 in A549 cells (Figure 3C). The Western blot analysis showed that PTEN expression was markedly increased after the transfection of si-H19 in A549 cells (Figure 3D). Besides, the silence of H19 attenuated the recruitment ability of EZH2 to PTEN (Figure 3E). The above data demonstrated that FOXF2 stimulated the transcription of H19 to further regulate PTEN level in A549 cells.

FOXF2 Accelerated Proliferative and Migratory Abilities of NSCLC Through H19/PTEN Axis

It is speculated that FOXF2 influenced the progression of NSCLC by targeting H19/PTEN axis. Here, we constructed a pcDNA-H19 vector to up-regulate H19 level in A549 cells (Figure 4A).

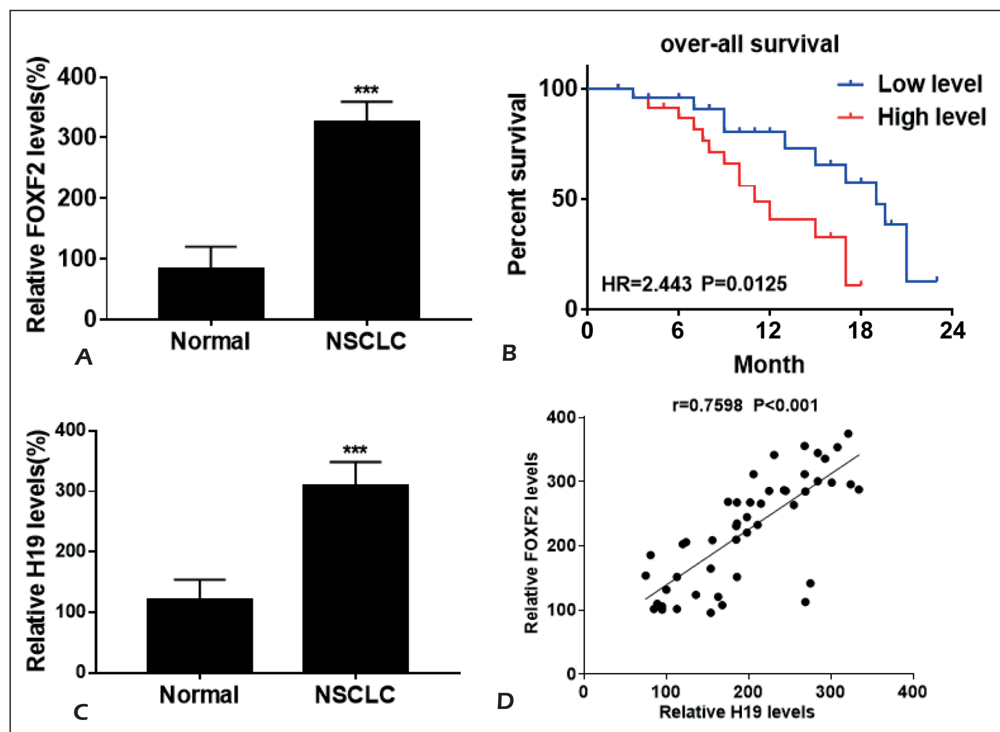

Figure 1. The upregulation of FOXF2 in NSCLC. **A**, Relative level of FOXF2 in NSCLC tissues and adjacent normal tissues. **B**, The Kaplan-Meier curves depicted the overall survival in NSCLC patients with high level or low level of FOXF2. **C**, The relative level of H19 in NSCLC tissues and adjacent normal tissues. **D**, A positive correlation between the expressions of FOXF2 and H19 in NSCLC tissues.

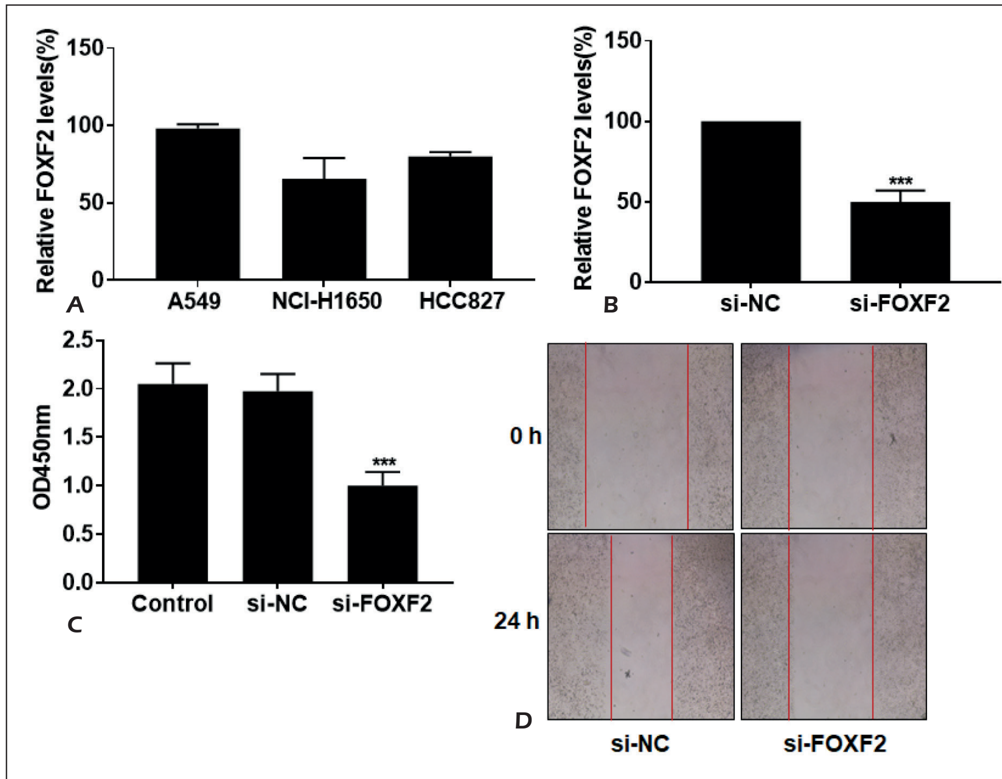

Figure 2. The silence of FOXF2 suppressed the proliferative and migratory abilities of NSCLC. **A**, Relative level of FOXF2 in A549, NCI-H1650, and HCC827 cells. **B**, Transfection efficacy of si-FOXF2 in A549 cells. **C**, Viability in A549 cells transfected with si-NC or si-FOXF2. **D**, Wound closure of A549 cells transfected with si-NC or si-FOXF2. (magnification: 10×).

The knockdown of FOXF2 could greatly up-regulate the PTEN level, which was then partially reversed by the co-transfection of pcDNA-H19 (Figure 4B). Moreover, the reduced viability of A549 cells transfected with si-FOXF2 was elevated by the H19 overexpression (Figure 4C). The

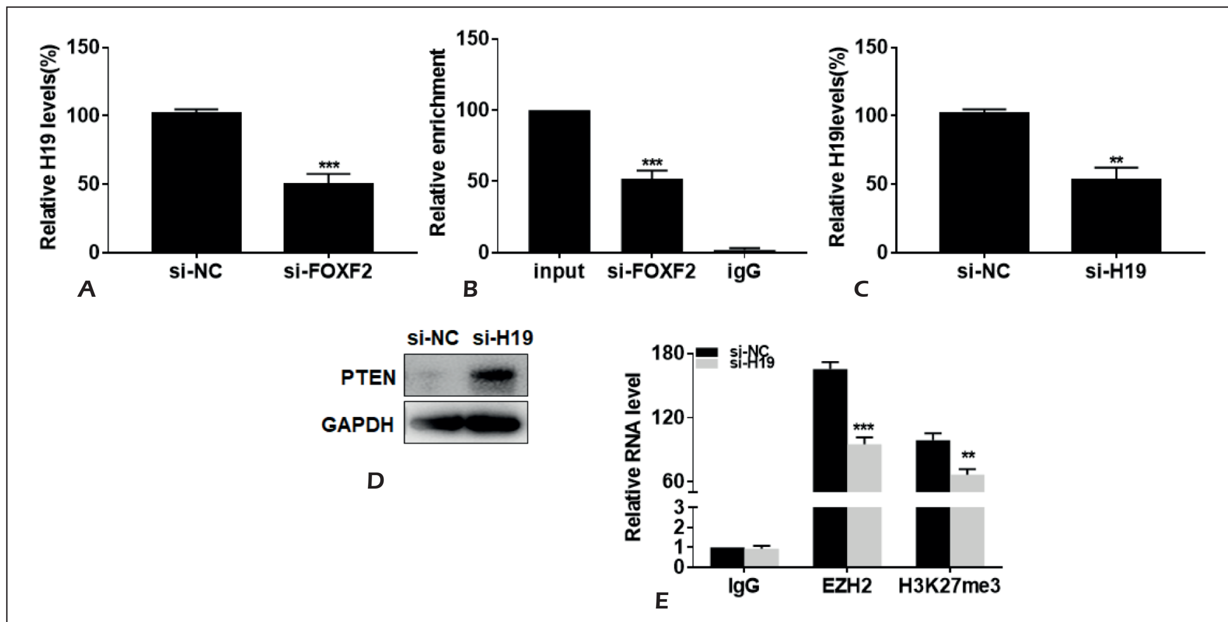

Figure 3. FOXF2 regulated H19/PTEN axis. **A**, Relative level of H19 in A549 cells transfected with si-NC or si-FOXF2. **B**, Enrichment of H19 in the input, anti-FOXF2, and anti-IgG group. **C**, Transfection efficacy of si-H19 in A549 cells. **D**, The protein level of PTEN in A549 cells transfected with si-NC or si-H19. **E**, Enrichment of PTEN in anti-IgG, anti-EZH2 and anti-H3K27me3 group.

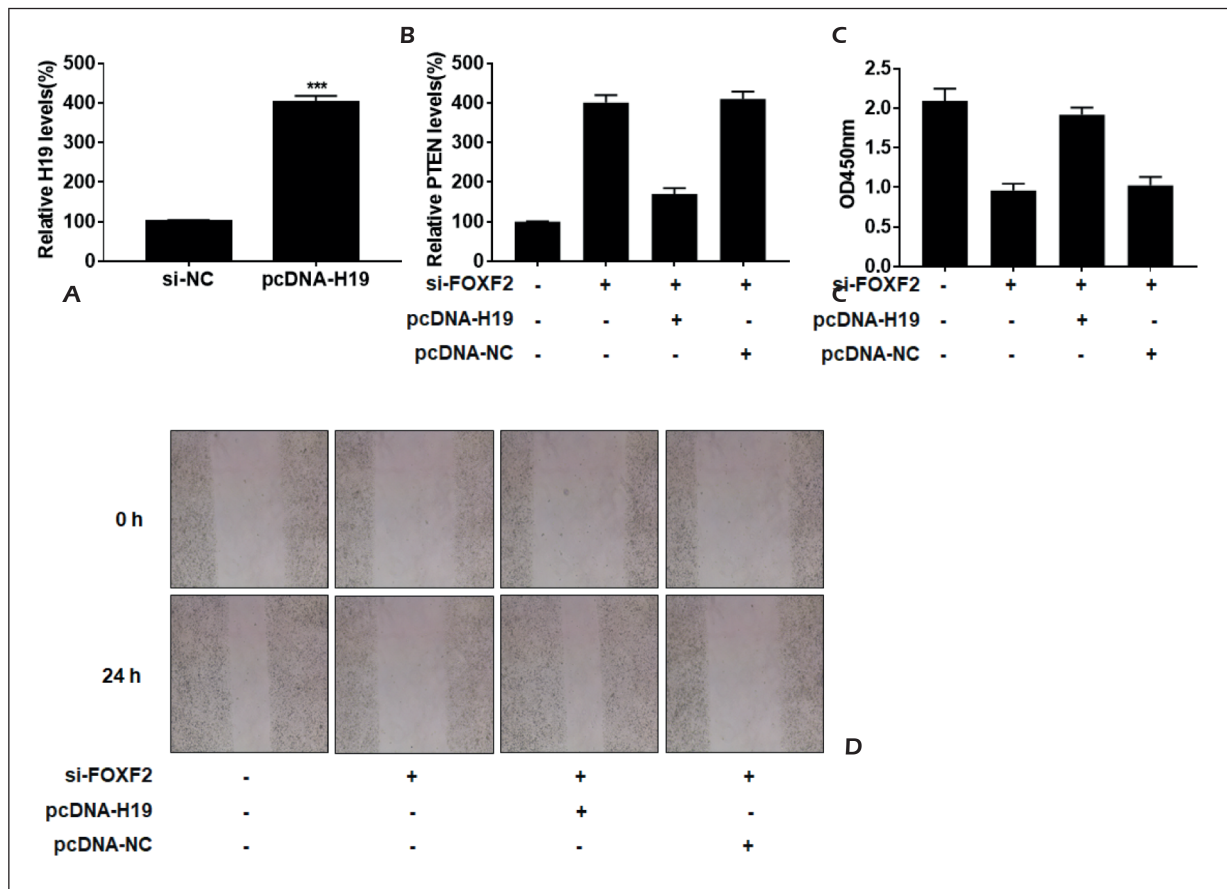

Figure 4. FOXF2 accelerated proliferative and migratory abilities of NSCLC through H19/PTEN axis. **A**, Transfection efficacy of pcDNA-H19 in A549 cells. **B**, Relative level of PTEN in A549 cells with different treatments. **C**, Viability in A549 cells with different treatments. **D**, Wound closure of A549 cells with different treatments (magnification: 10×).

decreased percentage of the wound closure due to FOXF2 knockdown was also reversed after the overexpression of H19 in A549 cells (Figure 4D). Hence, it is suggested that FOXF2 accelerated the proliferative and migratory abilities of the cancer cells by targeting H19 to down-regulate PTEN.

Discussion

The molecular basis driving the development of NSCLC is unclear. FOXF1, a member of the FOXF family, has been reported to induce EMT, invasion, and metastasis cancer cells in breast cancer and colorectal cancer[15,16]. Lo et al[17] pointed out that FOXF2 participated in the development of breast cancer by regulating the genes involved in the cell cycle progression and EMT. In this study, FOXF2 was found to be highly expressed in NSCLC and positively correlated with the expression level of H19. The silence of FOXF2 attenuated the proliferative and migratory abilities of NSCLC, indicating a vital role of FOXF2 in the progression of NSCLC.

LncRNA H19 locates on the chromosome 11p15.5 with 2.3 kb in length[18]. H19 is normally expressed in the fetal period, while downregulated in adult tissues. The upregulation of H19 could be observed in cancer tissues[19]. In many types of solid tumors, the abnormally expressed H19 is closely related to the invasion, metastasis, recurrence, and poor prognosis[20,21]. Our study demonstrated that the H19 expression was increased in NSCLC. Moreover, FOXF2 bound to the promoter region of H19 and further stimulated its transcription.

EZH2 is an important component of the complex of polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of lysine 27 on histone H3 protein subunit to silence the target genes[22-24]. In bladder cancer, H19 silenced E-cadherin expression by recruiting EZH2, thus stimu-

lating tumor growth[21]. PTEN is the most common tumor-suppressor gene[25]. PTEN has diverse functions in tumor biology, including the regulation of tumor cell biology, DNA damage repair and etc[26]. Our results detected that H19 silenced PTEN by recruiting EZH2, thereafter aggravating the progression of NSCLC.

Conclusions

We found that FOXF2 was upregulated in NSCLC. Additionally, FOXF2 accelerated the proliferative and migratory abilities of NSCLC cells by targeting H19 to downregulate PTEN, thus aggravating the progression of NSCLC. Our results indicated that FOXF2 might be a promising therapeutic and prognostic marker for NSCLC.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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