

Original Article

LncRNA FOXC2 antisense transcript accelerates non-small-cell lung cancer tumorigenesis via silencing p15

Zhe Sun^{1*}, Chaozhu He^{2*}, Miao Xiao², Binbin Wei², Yuanzhe Zhu², Guangxing Zhang², Huan Zhou², Jun Yuan², Xiaju Hu², Yuli Yi²

¹Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, China; ²Jiangxi Medical College, Nanchang University, Nanchang 330006, China. *Co-first authors.

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Abstract: Non-coding RNAs (ncRNAs) have been demonstrated to modulate the oncogenesis of non-small cell lung cancer (NSCLC), especially the long non-coding RNAs (lncRNAs). However, the role of lncRNA FOXC2-AS1 in the NSCLC is still unclear. In this research, we find that lncRNA FOXC2-AS1 is involved to NSCLC oncogenesis. The ectopic high-expression level of FOXC2-AS1 is closely correlated with the limited NSCLC patients' survival. In the functional experiments, the knockdown of FOXC2-AS1 dramatically suppressed the NSCLC cells' (A549, H460) proliferation, accelerated the apoptosis and induced the cycle arrest at G0/G1 phase. Mechanistic experiments revealed that FOXC2-AS1 repressed the p15 expression via recruiting the polycomb repressive complex 2 (PRC2) to the promoter of p15. The interaction within FOXC2-AS1 and p15 was validated using the rescue experiments. In conclusion, the results in this work confirmed that FOXC2-AS1 could aggravate NSCLC oncogenesis through repressing p15 expression via interacting EZH2, which provide new idea for the NSCLC therapeutic strategy.

Keywords: NSCLC, FOXC2-AS1, EZH2, p15, PRC2

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors worldwide and accounts for nearly 85 percentage of lung cancer [1-3]. NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, which is different from the small cell carcinoma [4]. Approximately 75 percentage of the NSCLC patients were diagnosed to be mid- or advanced-stage, causing the poor 5-year survival rate [5]. Undoubtedly, it is still urgent to explore the novel effective therapies and targets for NSCLC patients.

Long noncoding RNA (lncRNA) is a vital subgroup of ncRNA in the epigenetic regulation [6]. LncRNA is distinguished based on its length more than 200 nucleotides, different from the short traditional microRNA [7]. In the tumorigenesis, lncRNAs could participate multiple functions in the pathophysiological process, such

as proliferation, apoptosis and metastasis via transcriptional control or post-transcriptional control. PRC2 is a multisubunit complex, including EZH2, EED and SUZ12 [8, 9]. One mechanism by which lncRNA regulates the pathophysiological process is that lncRNAs could recruit the PRC2 [10]. For instance, HOXD-AS1 expression level is significantly up-regulated in osteosarcoma tissue and cells, and HOXD-AS1 epigenetically repressed p57 through recruiting enhancer of zeste homolog 2 (EZH2) to the promoter of p57 [11].

In this study, we focus on the role of lncRNA FOXC2-AS1 in the NSCLC tissue and cells and its cellular mechanism. FOXC2-AS1 has been reported to be oncogene in human cancer, including prostate cancer, breast cancer and osteosarcoma [12]. Present work indicated that FOXC2-AS1 might function as an oncogenic lncRNA in the NSCLC via repressing the p15 via recruiting PRC2.

Materials and methods

Clinical tissue collection

All the clinical tissue samples (thirty samples) were collected in the First Affiliated Hospital of Nanchang University. These volunteers underwent the surgical resection during which the tissue were obtained and then stored at -80°C for RNA isolation. All the participants had signed the informed consent form for this research.

Cell lines and culture

NSCLC cell lines (SK-MES-1, A549, H460, SPC-A1) were provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The human bronchial epithelial cell (NHBE) was purchased by Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in the RPMI Medium 1640 media added with heat-inactivated 10% FBS (Gibco, Carlsbad, CA, USA) and 100 mg/ml streptomycin, 100 U/ml penicillin (Gibco).

Cell transfection

The small silencing RNA (siRNA) of FOXC2-AS1 and EZH2 was synthesized by GenePharma (Shanghai, China). The siRNAs were transfected into cancer cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. All siRNA sequences are listed [Table S1](#). After 48 hours of transfection, the knocking down efficiency was measured.

Quantitative real-time RT-PCR

RNA was extracted from the tissue and cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized using SuperScript First-Strand Synthesis system and PCR was performed on AB7300 thermo-cycler (Applied Biosystems, Carlsbad, CA, USA). Relative data was calculated using the $\Delta\Delta C_t$ method normalized to GAPDH. Primers used for quantitative real-time PCR were listed in the [Table S1](#).

CCK-8 viability assay

NSCLC cells' viability was analyzed using the Cell Counting Kit-8 system (Dojindo Laboratory, Kumamoto). Briefly, A549 and H460 cells were

seeded into 96-well plates at 5×10^3 per well density. After 72 h of incubation, cell viability was tested by a Benchmark microplate spectrometer (Bio-Rad).

Flow cytometry analysis

A549 and H460 cells (5×10^5 per well) were transfected with siRNAs and then seeded into 6-well plates as described above. The apoptosis was tested using an Annexin V-FITC and PI kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were administrated with 5 μ l of FITC Annexin V and 5 μ l of propidium iodide (PI) for 15 min at room temperature in the dark. The cycle distribution analysis was performed when the cells (2×10^5 per well) were seeded at in 6-well plates. Cells were labeled with propidium iodide (PI) and determined by flow cytometry. Flow cytometry analysis was analyzed using a BD FACSCanto II system (BD Biosciences).

Western blot analysis

Protein lysate was extracted from NSCLC cells as previously described and subjected to 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The bands on the polyvinylidene fluoride (PVDF) membrane (Amer-sham Pharmacia, Piscataway, NJ, USA) were electro-transferred and then blocked with 5% non-fat skimmed milk. Anti-bodies were administrated, including anti-p15 (Abcam, appropriate 1:1000 dilutions), and anti-GAPDH (CWBIO, Beijing, China). Subsequently, the membrane was incubated with the of the corresponding secondary antibodies at 37°C for 1 hours. The quantification for immunocomplexes was measured with the Imagequant 5.1 software.

Subcellular fractionation

The FOXC2-AS1 nucleus fraction and cytoplasmic fraction were isolated from nuclear and cytoplasm using the Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA).

RNA immunoprecipitation (RIP)

RNA immunoprecipitation was performed to detect the endogenous Ago2 binding to RNA using the anti-Ago2 antibody with Magna RIP RNA-Binding Protein Immunoprecipitation Kit

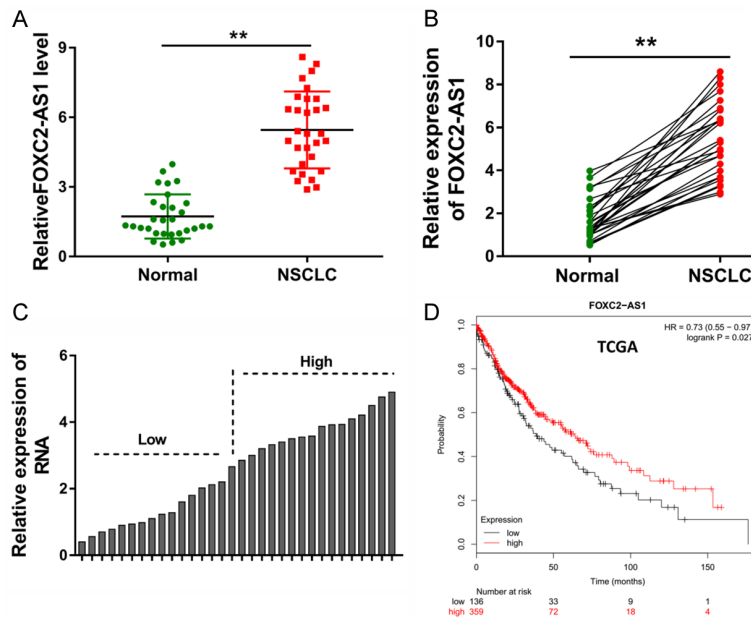


Figure 1. LncRNA FOXC2-AS1 is over-expressed in the NSCLC tissue samples and indicates the poor prognosis. A. RT-PCR analysis revealed the lncRNA FOXC2-AS1 in this clinical NSCLC tissue samples. B. Intra-group analysis showed the lncRNA FOXC2-AS1 expression levels were higher in the NSCLC tissue than that of corresponding normal tissue. C. These samples were divided into high and low group based on the median value. D. The Cancer Genome Atlas (TCGA) database revealed the long-term prognosis of lung cancer patients with high and low FOXC2-AS1 expression. **Presents the p -value less than 0.01.

(Millipore, Bedford, MA). IgG was used for the endogenous control. The RNA expression after the immunoprecipitation was determined by qRT-PCR relative to input.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out using the EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA, USA). Briefly, chromatin was sheared to small fragments and incubated with the antibody (anti-EZH2, anti-H3K27me3) to immunoprecipitate DNA-protein complexes. Immunoglobulin G (IgG) was used as a negative control. The quantified level was detected using the qRT-PCR with SYBR-Green kit.

Statistics

All these data are presented as mean \pm SD pattern. The statistical methods include Student t -test and one-way ANOVA test. P -value was set at 0.05 for the statistical significance. Data was calculated using the SPSS software (SPSS Inc, Chicago, IL, USA) and graphed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

LncRNA FOXC2-AS1 is over-expressed in the NSCLC tissue samples and indicates the poor prognosis

In this clinical NSCLC tissue samples, RT-PCR analysis revealed that lncRNA FOXC2-AS1 was up-regulated in the collected NSCLC tissue (Figure 1A, Table 1). Besides, the intra-group analysis showed that lncRNA FOXC2-AS1 expression levels were higher in the NSCLC tissue than that of corresponding normal tissue (Figure 1B). Based on the median value, these samples were divided into high and low group (Figure 1C). The Cancer Genome Atlas (TCGA) database revealed the long-term prognosis of FOXC2-AS1 in lung cancer, indicating the poor survival rate of the over-expression of FOXC2-AS1 for NSCLC (Figure 1D). Therefore, above data demonstrate that

lncRNA FOXC2-AS1 is over-expressed in the NSCLC tissue samples and indicates the poor prognosis.

FOXC2-AS1 silenced expression inhibits the NSCLC cells' tumorous biological behavior

The high level of lncRNA FOXC2-AS1 in the NSCLC clinical tissue specimens implies its potential oncogene. After that, the loss-of-functional experiments were carried out to verify it. Firstly, the lncRNA FOXC2-AS1 level was increased in the candidate NSCLC cells (Figure 2A). These synthesized siRNAs targeting FOXC2-AS1 were transfected into A549 and H460 cells to knock down the FOXC2-AS1 expression (Figure 2B). CCK-8 proliferative ability assay demonstrated the inhibition of NSCLC cells' proliferation in the FOXC2-AS1 knocking down (Figure 2C). Flow cytometry analysis revealed the apoptotic increasing of NSCLC cells induced by the FOXC2-AS1 knocking down (Figure 2D). The cell cycle analysis demonstrated the cycle distribution and arrest at the G0/G1 phase, indicating the inhibitive role of FOXC2-AS1 knocking down on the NSCLC cells

Table 1. Relationship between FOXC2-AS1 expression and clinicopathological characteristics of NSCLC patients

	Total = 30	FOXC2-AS1		P value
		Low (15)	High (15)	
Gender				
Male	17	9	8	0.708
Female	13	6	7	
Age				
≤65	14	8	6	0.524
>65	16	7	9	
Smoking				
Yes	14	6	8	0.613
No	16	9	7	
Size of tumor				
≥3 cm	19	7	12	0.019*
<3 cm	11	8	3	
Differentiation				
Well, Moderate	19	9	10	0.638
Poor	11	6	5	
TNM stage				
I/II	18	12	6	0.071
III/IV	12	3	9	
Lymph node metastasis				
Positive	17	8	9	0.419
Negative	13	7	6	

*P<0.05 represents statistical difference.

progression (**Figure 2E, 2F**). The in vivo xeno-graft assay indicated that the FOXC2-AS1 knocking down repressed the NSCLC cells tumor growth (**Figure 2G, 2H**). Together, our results indicated that FOXC2-AS1 silenced expression inhibits the NSCLC cells' tumorous biological behavior.

FOXC2-AS1 is responsible for the epigenetic repression of p15 by interacting with PRC2

For the deepgoing mechanism, we found that FOXC2-AS1 was markedly located in the nucleus (**Figure 3A**). Moreover, existing references have reported that lncRNAs in the nucleus could suppress its targeting gene by recruiting the Polycomb Repressive Complex 2 (PRC2). Biologically, PRC2 is a multimeric enzymatic complex including the set-domain-containing methyltransferase EZH2. Then, the RNA-RIP assay was carried out to verify that whether FOXC2-AS1 could regulate the target gene expression at the transcriptional level. Data showed that FOXC2-AS1 could be pulled down

using the EZH2 and SUZ12 antibodies in A549 cells (**Figure 3B**). In the FOXC2-AS1 silencing transfection, RT-PCR revealed that the p15 level was increased among these CDK inhibitors (CKIs), including p15, p16, p21, p27 (**Figure 3C**). In A549 cells, western blot assay revealed that p15 protein was increased in the FOXC2-AS1 silencing transfection (**Figure 3D**). Besides, p15 protein level also was increased in the EZH2 silencing transfection (**Figure 3E**). To validate whether FOXC2-AS1 regulated the transcriptional repression of p15 via the enrichment of PRC2, chromatin immunoprecipitation (ChIP) was performed and revealed that FOXC2-AS1 silencing decreased the binding ability (**Figure 3F**). Therefore, it is observed that FOXC2-AS1 is responsible for the epigenetic repression of p15 by interacting with PRC2.

FOXC2-AS1 regulates the NSCLC tumorigenesis by partly regulating p15

Above data had indicated the transcriptional repression of FOXC2-AS1 on the p15 in NSCLC cells. Then, rescue experiments were performed using A549 cells to verify the whether p15 was related to the regulation modulated by FOXC2-AS1. Western blot revealed that the FOXC2-AS1 silencing recovered the inhibition of p15 induced by the p15 siRNA (**Figure 4A**). The apoptosis analysis stated that p15 siRNA reduced the apoptotic rate, which was recovered by the FOXC2-AS1 silencing co-transfection (**Figure 4B**). Proliferative analysis illustrated that p15 siRNA promoted the proliferative absorbance of NSCLC cells and then the co-transfection with FOXC2-AS1 silencing restored it (**Figure 4C**). Cycle analysis revealed that the p15 siRNA accelerated the cycle progression, which was negatively correlated with the G0/G1 phase arrest induced by FOXC2-AS1 silencing (**Figure 4D**). These data suggest that FOXC2-AS1 regulates the NSCLC tumorigenesis by partly regulating p15.

Discussion

To advance our understanding of the biological roles of long noncoding RNA (lncRNA), we investigate the expression level of lncRNA FOXC2-AS1 in the NSCLC both tissue and cells, moreover, its aberrant high-expression is closely correlated with the poor clinical prognosis. In

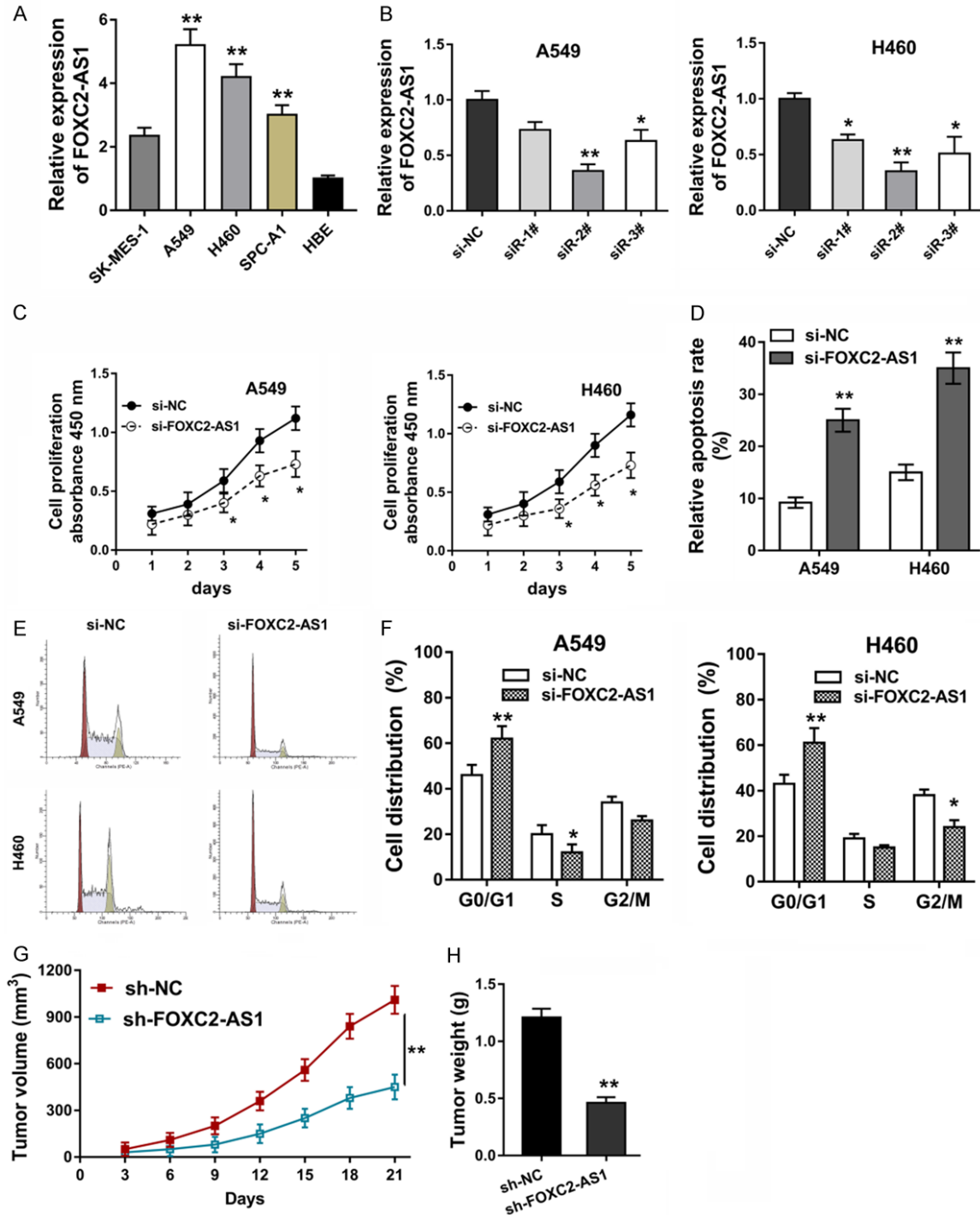


Figure 2. FOXC2-AS1 silenced expression inhibits the NSCLC cells' tumorous biological behavior. A. RT-PCR demonstrated the lncRNA FOXC2-AS1 level in the candidate NSCLC cells. B. The loss-of-functional experiments were carried out using the siRNAs targeting FOXC2-AS1. C. CCK-8 proliferative ability assay demonstrated the inhibition of NSCLC cells' proliferation in the FOXC2-AS1 knocking down. D. Flow cytometry analysis revealed the apoptotic increasing of NSCLC cells induced by the FOXC2-AS1 knocking down. E, F. The cell cycle analysis demonstrated the cycle distribution and arrest at the G0/G1 phase. G, H. The in vivo xenograft assay indicated the tumor growth with FOXC2-AS1 knocking down. **Presents the *p*-value less than 0.01. *Presents the *p*-value less than 0.05.

this research, we find the ponderable regulation by which FOXC2-AS1 modulate the NSCLC

tumorigenesis via sponging the miRNA, thereby targeting the target protein.

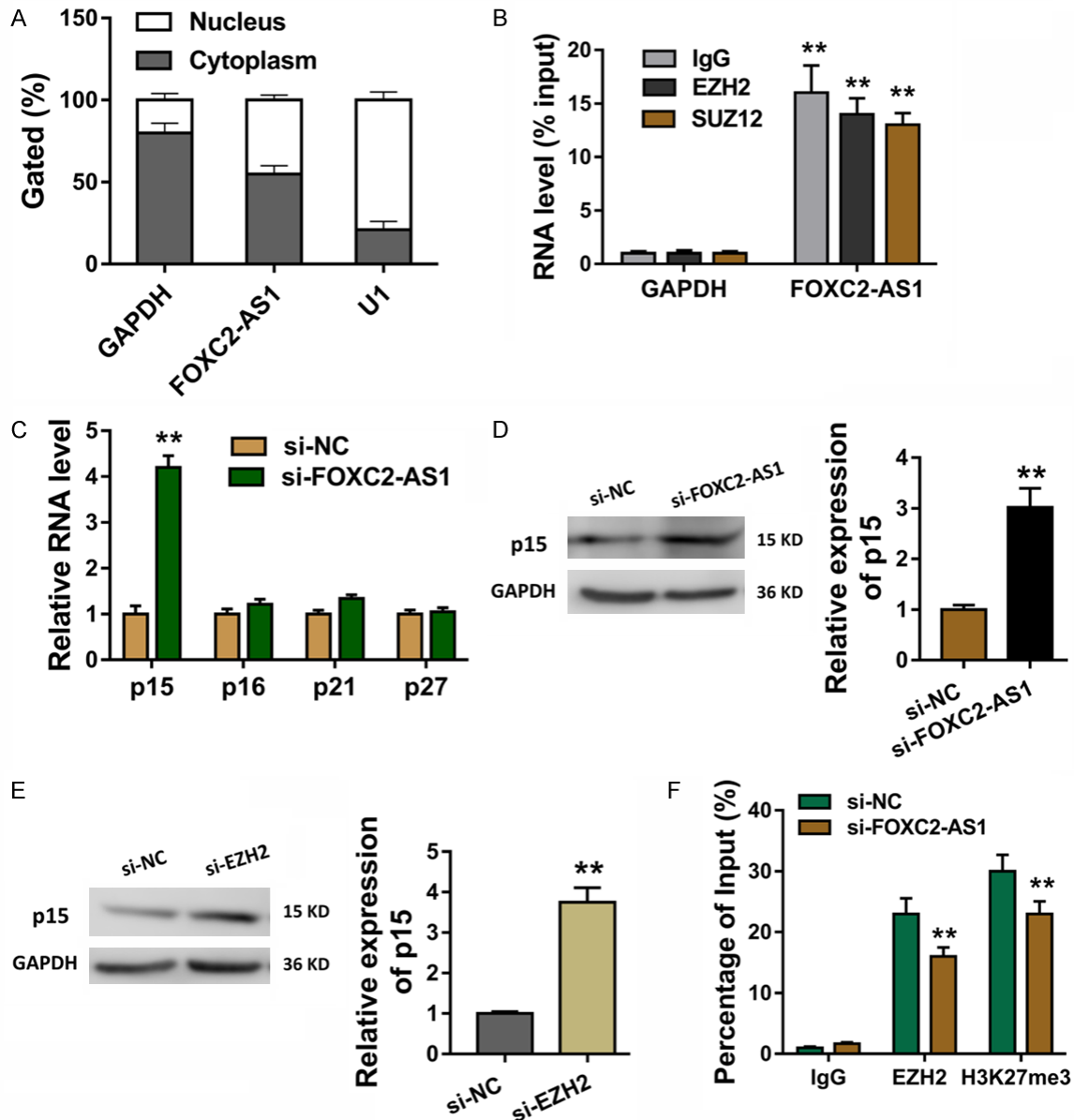


Figure 3. FOXC2-AS1 is responsible for the epigenetic repression of p15 by interacting with PRC2. **A.** The location of nucleus or cytoplasmic location for FOXC2-AS1 in A549 cells. **B.** RNA-RIP assay was carried out to verify that whether FOXC2-AS1 could be pulled down using the EZH2 and SUZ12 antibodies in A549 cells. **C.** RT-PCR revealed the p15 level was increased among these CDK inhibitors (CKIs), including p15, p16, p21, p27. **D.** Western blot assay revealed the p15 protein in the FOXC2-AS1 silencing transfection in A549 cells. **E.** Western blot assay revealed the p15 protein level in the EZH2 silencing transfection. **F.** Chromatin immunoprecipitation (ChIP) was performed to validate whether FOXC2-AS1 regulated the transcriptional repression of p15 via the enrichment of PRC2. **Presents the *p*-value less than 0.01.

With the rapid progression of next-generation sequencing technology (NGS), more and more lncRNAs with aberrant expression profile have been identified in the human cancer tissue [13-16]. Anterior references about the FOXC2-AS1 have illustrated that it has been closely correlated with the tumorous progression and prolif-

eration, such as breast cancer and prostate cancer, and dramatically regulates the molecular progress [17, 18]. In the prostate cancer, FOXC2-AS1 high-expression promotes the proliferation and tumor growth of cells in vitro and in vivo, and mechanically, miR-1253 targeted FOXC2-AS1 3'-UTR to regulate EZH2 abundan-

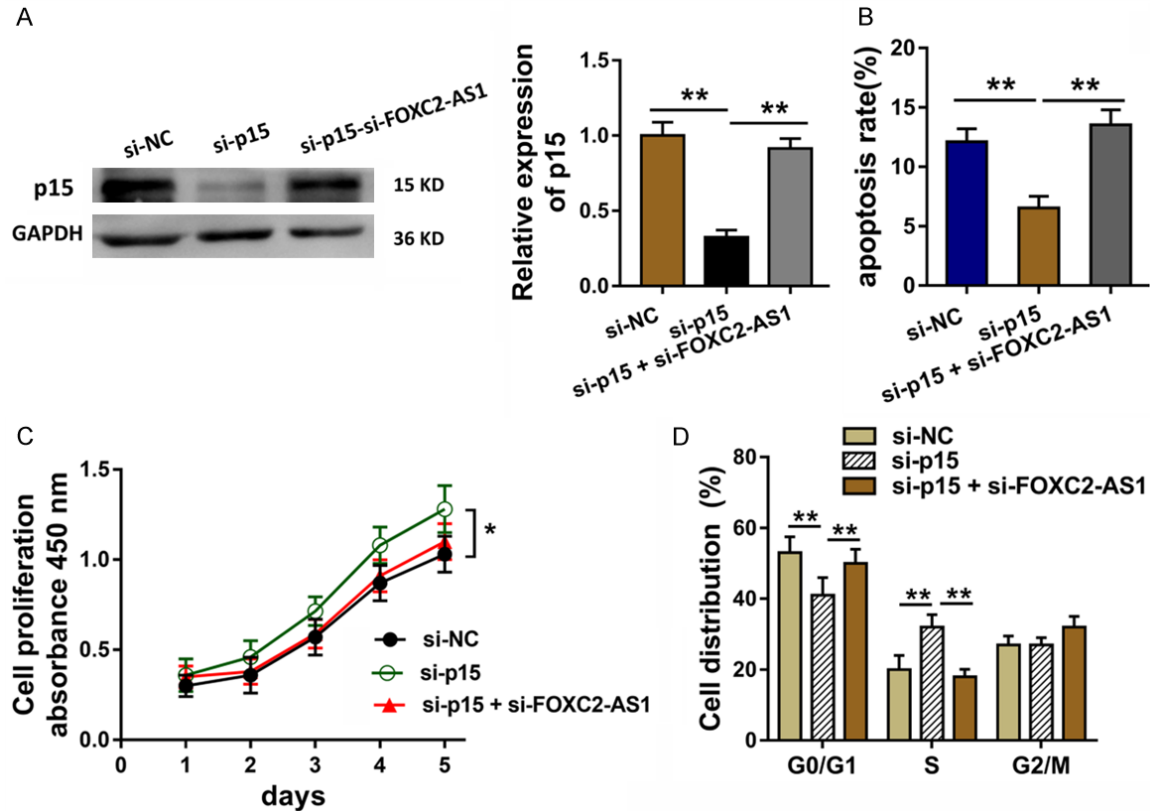


Figure 4. FOXC2-AS1 regulates the NSCLC tumorigenesis by partly regulating p15. A. Western blot revealed the p15 level in A549 cells transfected with FOXC2-AS1 silencing and p15 siRNA. B. Apoptosis analysis stated the apoptotic rate of NSCLC cells transfected with FOXC2-AS1 silencing and p15 siRNA. C. Proliferative CCK-8 analysis illustrated the proliferative absorbance of NSCLC cells with the co-transfection with FOXC2-AS1 silencing and p15 siRNA. D. Cycle analysis revealed the cellular distribution of NSCLC cells with the co-transfection with FOXC2-AS1 silencing and p15 siRNA. **Presents the *p*-value less than 0.01. *Presents the *p*-value less than 0.05.

ce [19]. In breast cancer, FOXC2-AS1 is associated with poor prognosis and the silenced FOXC2-AS1 suppresses the proliferation ability and cell cycle and apoptosis rate [20]. These data evidence that lncRNA FOXC2-AS1 is likely to act as an oncogene in the human tumor.

In present work, our data demonstrated that the ectopic high-expression level of lncRNA FOXC2-AS1 was correlated with the poor prognosis of NSCLC patients from the TCGA database. The knocking down of lncRNA FOXC2-AS1 repressed the proliferation, induced the apoptosis and caused the cycle arrest at G0/G1 phase. Thus, at the cellular plane, the function of FOXC2-AS1 on the NSCLC might be an oncogenic RNA. This finding is not only in accord with the previous reference, but also reveals the feasible regulation of FOXC2-AS1 for the epigenetic regulation of NSCLC.

The mechanical manner by which lncRNAs regulate the tumorigenesis is dependent on its

subcellular localization given that the nucleus and cytoplasmic distribution of lncRNAs [21-23]. The consensus is that cytosolic lncRNAs are responsible for the post-transcriptional regulation, e.g. microRNA sponge and mRNA stability, and nuclear lncRNAs modulate the gene transcription through transcription factors or modification enzymes complex [24-26]. In this study, we found that the FOXC2-AS1 is located both in the nuclear and the cytoplasm, suggesting the potential regulation via transcriptional and post-transcriptional regulation. Existing references have reported that lncRNAs in the nucleus could suppress its targeting gene by recruiting the Polycomb Repressive Complex 2 (PRC2) [27-29]. Biologically, PRC2 is a multi-meric enzymatic complex including the set-domain-containing methyltransferase EZH2. Then, the data showed that FOXC2-AS1 could be pulled down using the EZH2 and SUZ12 antibodies in A549 cells, indicating the binding within FOXC2-AS1 and EZH2. After the confir-

mation using the rescue experiments, in which the p15 recovers the inhibition of FOXC2-AS1 silencing, reveals the authentic modulation of lncRNA FOXC2-AS1 on the p15 via recruiting the EZH2. p15 acts as a anti-oncogenic element in the NSCLC, thereby being regulated by the FOXC2-AS1/EZH2 [30].

Taken together, the results we found in this work suggest the oncogenic role of lncRNA FOXC2-AS1 on the NSCLC tumorigenesis. FOXC2-AS1 accelerates the NSCLC cells proliferation and cycle progression at the tumor phenotype. Moreover, the mechanism finding reveals the vital axis of lncRNA FOXC2-AS1 regulating the p15 via recruiting the EZH2, providing a therapeutic strategy for NSCLC.

Disclosure of conflict of interest

None.

Address correspondence to: Yuli Yi, Jiangxi Medical College, Nanchang University, Nanchang 330006, China. E-mail: yiyuli777jx@163.com

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Table S1. qRT-PCR primers and siRNA sequences

	Sequences 5'-3'
FOXC2-AS1	Forward, 5'-CACAGTGTAGGAGAGTAATCAG-3' Reverse, 5'-AAGCTCCATTTCGCAACCTTAC-3'
p15	Forward, 5'-TTGATTGAAGGGCAGAGGA-3' Reverse, 5'-TTGGTCAGCACAGATCATCG-3'
GAPDH	Forward, 5'-CTAAGGCCAATGACCGAAAG-3' Reverse, 5'-ACCAGAGGCGGATACAGACA-3'
si-FOXC2-AS1-1#	5'-GCCTAATATAACCGGACTTT-3'
si-FOXC2-AS1-2#	5'-GCTTATAGTACATACCCCAT-3'
si-FOXC2-AS1-3#	5'-CGTGATATAACTCCTAACCT-3'
p15 siRNA	5'-UGUUCGGCUUAGCAGUGGUUG-3'