IncRNA PART1, manipulated by transcriptional factor FOXP2, suppresses proliferation and invasion in ESCC by regulating the miR-18a-5p/SOX6 signaling axis

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Abstract. An increasing number of studies have demonstrated that long non-coding (lnc)RNAs are associated with tumor invasion, metastasis and the prognosis of patients with a variety of different tumors. However, the roles of lncRNA prostate androgen regulated transcript 1 (PART1) in esophageal squamous cell carcinoma (ESCC) remain unknown. In the present study, reverse transcription-quantitative PCR was performed to investigate the levels of PART1, SRY-box transcription factor 6 (SOX6) and miR-18a-5p in ESCC tissues and cells. The functions of PART1 in ESCC were demonstrated using Cell Counting Kit-8 and Matrigel assays. Promoter activity and dual-luciferase reporter assays, RNA immunoprecipitation and western blot analyses were also used to determine the potential mechanisms of PART1 in ESCC cell lines. It was found that PART1 and SOX6 were both downregulated in ESCC tissues and cells, and their low expression levels were associated with TNM stage, lymph node metastasis and poor prognosis in patients with ESCC. Forkhead box protein P2 (FOXP2) exhibited low expression level in ESCC tissues, and its expression was positively correlated with PART1 expression level in ESCC tissues. FOXP2 was found to bind to the promoter region of PART1 to regulate its expression in ESCC cells. Functionally, PART1 overexpression suppressed cell proliferation and invasion, whereas PART1 downregulation promoted cell proliferation and invasion in the ESCC cell lines. Mechanistically, PART1 functions as a competing endogenous (ce)RNA by sponging miR-18a-5p, resulting in the upregulation of the downstream target gene, SOX6, coupled with the inactivation of the β-catenin/c-myc signaling axis, to suppress ESCC cell proliferation and invasion. In conclusion, data from the present study unveil a potential ceRNA regulatory pathway, in which PART1 affects SOX6 expression level by sponging miR-18a-5p, to ultimately suppress ESCC development and progression.

Introduction

Esophageal cancer (ESCA), as a complex disease harboring numerous pathogenic factors, is the sixth leading cause of cancer-associated death worldwide (1). There are two main histological types, including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, which exhibit completely distinct geographical distributions and primary risk factors (2). Patients with ESCC account for >90% of all patients with ESCA in China (3). The prognosis of patients with ESCA remains poor, with a 5-year survival rate of only 25% (4), which is primarily due to the late-stage at diagnosis and distant metastasis. Thus, there is an urgent requirement to identify novel prognostic markers and new molecular targets to improve the prognosis and therapeutic efficacy in patients with ESCC.

Long non-coding (inc)RNAs are considered to be a novel class of transcripts, that are >200 nucleotides in length, do not have any protein-coding potential (5), and are typically transcribed by RNA polymerase II, which mirrors that of protein-coding genes (6). Currently, IncRNAs consist of five different molecular types according to their genomic location on the chromosome, including sense, antisense, intronic, intergenic and bidirectional IncRNAs (7-9). IncRNAs have been associated with the regulation of numerous essential biological processes, such as allosterically manipulating enzymatic activity, shaping chromosome conformation, functioning as regulators of histone methylation and imprinting genomic loci (9-11). Recently, IncRNAs have been associated with tumor initiation, progression and metastasis (12,13), and play important roles in the survival, growth, differentiation, apoptosis and regulating signal pathways in a number of tumors (14-18). Notably, IncRNAs have been found to be novel prognostic markers and therapeutic targets for numerous types
of cancer (19,20). Prostate androgen regulated transcript 1 (PART1) is predominately expressed in the prostate and is transcriptionally regulated by androgens in prostate cancer cells (21). At present, PART1, as a novel IncRNA, has been associated with tumor progression, metastasis and chemotherapy resistance (22,23). In addition, PART1 has been found to be involved in the regulation of several signaling pathways, such as the PTEN/AKT signaling pathway (24), the microRNA (miR)-150-5p/LRG1 axis (22) and the miR-429/SHCBP1 regulatory network (25), suggesting that targeting PART1 may be a novel and promising therapeutic strategy in patients with cancer. Therefore, elucidation of the function of PART1 and its molecular mechanisms, may provide further understanding and enable treatment in a clinical setting for different types of cancer. However, to date, the functions and molecular mechanisms of PART1 in ESCC remain unclear.

Recently, forkhead box protein P2 (FOXP2) has been verified to be a tumor suppressor (26). Notably, FOXP2 suppresses the transcriptional activity of numerous downstream target genes, via its zinc finger domain, further affecting tumor progression (26,27). In addition, FOXP2 was found to be essential for the suppression of cell growth by regulating p21 in osteosarcoma cells (28). These findings suggest that FOXP2 may function as a key regulator in various types of cancer by regulating its target genes.

In the present study, the expression level of PART1 in ESCC tissues and cells was investigated, and the clinical value of PART1 in TNM stage, lymph node metastasis and the prognosis of patients with ESCC was also determined. In addition, FOXP2 was found to be a transcription factor, that regulated the expression level of PART1 by binding to its promoter region and acted as a competing endogenous (ce)RNA by sponging miR-18a-5p to promote the expression of SOX6, which further resulted in the suppression of cell proliferation and invasion in ESCC. Collectively, the PART1/miR-18a-5p/SOX6/β-catenin signaling axis uncovered in the present study may be a novel therapeutic target for patients with ESCC.

Materials and methods

Bioinformatics analysis. The GEO GSE111011 dataset (https://www.ncbi.nlm.nih.gov/geo/?term=GSE111011) was downloaded to analyze the levels of PART1 and SOX6 in patients with ESCC, from tumor and paired normal samples. The GEO GSE43732 (29) was used to investigate the miR-18a-5p expression in ESCC samples. The StarBase online database v2.0 (30) was also used to investigate the levels of PART1 and miR-18a-5p in patients with ESCA and in normal samples. Correlation analysis between PART1 and SOX6, as well as the prognostic value of SOX6 was also examined using the StarBase database. The upstream promoter sequence of PART1 was detected from three independent repeated experiments.

Cell lines and cell culture. The human ESCC cell lines (Eca109, EC9706, TE1, KYSE70 and KYSE450) and the normal Het-1A esophageal epithelial cell line were purchased from the Chinese Academy of Sciences Cell Bank, and were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO$_2$.

Cell transfection. Small interfering (si)RNAs (50 nM) (Table I), miR-18a-5p mimic (50 nM, forward (F), 5'-UAAGGUCCAUCAUGCGAUAAG-3' and reverse (R), 5'-AUCUGCACAUAGUGACCUUAUU-3'); negative control (NC, F, 5'-UUCUCCGAACGUGACUUGUTT-3' and R, 5'-ACGUACAGCUCUGAGATAATT-3'); miR-18a-5p inhibitor (50 nM, 5'-CUAGCUAGCAUGAGACCUUAUU-3'); NC inhibitor (50 nM, 5'-UUCUCCGAACGUGACUUGUTT-3') (Shanghai GenePharma, Co., Ltd.), pcDNA3.1 (2.5 µg), and pcDNA3.1-PART1/FOXP2/SOX6 (2.5 µg) (TsingKe Biological Technology) were transfected into the Eca109 and EC9706 cell lines when cells reached approximately 80% confluence using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for about 20 min according to the manufacturer's instructions. The subsequent experiment was performed 48 h after transfection.

Cell counting Kit-8 (CCK-8) assay. Cell proliferation of the ESCC cell lines, with various treatments was performed in triplicate according to the manufacturer's protocol, then the ESCC cells (~2,000 cells/well) were seeded into a 96-well plate. At the time of measurement, CCK-8 reagent (Beyotime Institute of Biotechnology) was added to the corresponding wells, and the absorbance (450 nm) was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Matrigel assay. Cell invasion was investigated using a Transwell chamber harboring Matrigel (BD Biosciences). Briefly, the Eca109 and EC9706 cell lines (1x10$^5$) were added to the upper layer of the chamber and 20% FBS was added to the lower chamber. Afterwards, the invasive cells were fixed with methanol, followed by staining with 0.1% crystal violet at room temperature for 15 min, 48 h following transfection. Finally, the number of invasive cells was determined at x200 magnification under a light microscope (Leica, Germany). The results were from three independent repeated experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher
Table I. Sequences of the siRNA and si-NC used for synthesis.

| siRNAs       | Sense (5'-3’) | Antisense (5'-3’) |
|--------------|--------------|------------------|
| PART1 siRNA#1| GAGUUGACUUUUGUGUUAUACA | UUAACACAAAGUCGAUCUG |
| PART1 siRNA#2| GGGUGUGAAUAAGGUGUUAUG | UUAACCUUAUUCACCGUUA |
| PART1 siRNA#3| GAAAGUGUGUGUUAUAAACU | UUAUAAUUCAACUACUU |
| FOXP2 siRNA#1| CGACAGAGCAUAAGGCACA | UGGCUUAAUGCUUCGUGC |
| FOXP2 siRNA#2| GCCUGAGCUCUACUACUAAC | UUAGAUUGAGUUGCUAGC |
| FOXP2 siRNA#3| GACGCAACAAACCAACAACAA | UUGUGUUGUGUGUCUGCG |
| SOX6 siRNA#1| GGAUCUCGCGUGAAUAAUAG | UUGAUUUCAGGCGAAUUC |
| SOX6 siRNA#2| AGAACAGAUUGCGACACAA | UUGUCUCGCAAUUCGUUC |
| SOX6 siRNA#3| GAAUGGAAUCAGAGAUAUAA | UUAUUCUGAUUCAACUU |
| Si-NC        | UUCUCGAAGCGUGUCAGUUTT | ACGUGACACGUGUCAGAAATT |

Si, small interfering; PART1, prostate androgen regulated transcript 1; SOX6, SRY-box transcription factor 6; NC, negative control.

Scientific, Inc.) according to the manufacturer’s instructions. For PART1, SOX6 and FOXP2 expression analysis, RT-qPCR was performed using the Quant One-Step RT-qPCR kit (SYBR Green, FP303) (Tiangen Biotech Co., Ltd.) and an ABI 7500 series PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.), with the following specific primers: PART1 forward, 5'-CCAGAGCCACGAAAACTCATTT-3' and reverse 5'-CTGTCTTTTCCCCCTCGAC-3'; SOX6 forward, 5'-AAG ATGCAAGGGGAGGTGC-3' and reverse, 5'-GGTGGCTTTC TCTAGGTGTGGGA-3'; FOXP2 forward, 5'-GAGCACCAATTAG ATGCTGCCG-3' and reverse 5'-ATCTAGGGCCTAGCACACAA GG-3'; GAPDH forward, 5'-CGGAGTCTACAAGGATTTGG TGCATAT-3'; and reverse 5'-AGCCTTCTCCATGGTGGT GAAGAG-3'). PCR reaction conditions were as follows: 95°C for 30 sec, 30 cycles (95°C for 5 sec, 50°C for 30 sec, and 72°C for 30 sec). For the miR-18a-5p assay, total RNA was reverse transcribed using the miRcute Plus miRNA First-Strand cDNA kit (cat. no. KR211; Tiangen Biotech Co., Ltd.). RT-qPCR for miR-18a-5p was performed using the miRcute Plus miRNA qPCR kit (SYBR Green) (FP411; Tiangen Biotech Co., Ltd.) using the following specific forward primers, with the reverse primers from the kit: miR-18a-5p forward, 5'-TAAGGTGCA TCTAGTGCGAGATAG-3' and U6 forward, 5'-CTGTCTTTCC AGAGACA-3'). PCR reaction conditions were as follows: 95°C for 15 min in one cycle, 40 cycles (95°C for 5 sec, 60°C for 30 sec). The experiments were normalized to U6. Relative gene expression was analyzed using the 2-∆∆Cq method (31). The results were from three independent repeated experiments.

Subcellular fractionation. A cell nucleus and cytoplasm RNA isolation kit (Beibei, Biotech, Co., Ltd.) was used to extract the nuclear and cytoplasmic RNA, respectively, according to the manufacturer’s instructions. Then, RT-qPCR was performed to detect the levels of PART1, U6 and GAPDH. The results were from three independent repeated experiments.

Promoter activity assay. The fragments, containing wild-type (WT) or mutant (MUT) binding sites of FOXP2 in the PART1 transcript were constructed using the pGL3-basic vector (Promega Corp.). The pGL3-PART1-promoter-WT and pGL3-PART1-promoter-MUT vectors along with pcDNA3.1 and pcDNA3.1-FOXP2 were transfected into the Eca109 and EC9706 cell lines. Luciferase activity was determined 48 h following transfection using a Luciferase Reporter Assay System (Promega, Corp.). The results were from three independent repeated experiments.

Dual-luciferase reporter assay. A dual-luciferase reporter assay system was performed to investigate the interaction between miR-18a-5p and PART1 or SOX6 in the Eca109 and EC9706 cell lines. Recombinant vectors, pmirGLO-PART1-WT and pmirGLO-PART1-MUT, as well as pmirGLO-SOX6-WT and pmirGLO-SOX6-MUT (TsingKe Biological Technology) along with the miR-18a-5p mimic and NC mimic were transfected into the Eca109 and EC9706 cell lines using Lipofectamine™ 2000, respectively. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Corp.), 48 h following transfection, according to the manufacturer’s protocol. The results were from three independent repeated experiments.

RNA immunoprecipitation (RIP). A RIP assay was performed in the Eca109 and EC9706 cell lines using a RNA-binding protein IP kit (EMD Millipore) according to the manufacturer’s protocol. Briefly, RIP lysates were prepared from the Eca109 and EC9706 cell lines transfected with miR-18a-5p mimic or NC mimic, and then subjected to IP using either 5 µl normal mouse IgG or 5 µl anti-ago2 antibody and the Mana RIP™ RNA-binding Protein IP kit. PART1 and miR-18a-5p enriched on the beads was determined using RT-qPCR and corresponding specific primers. The results were from three independent repeated experiments.

Western blot analysis. Total proteins were extracted from the ESCC cell lines using RIPA lysis (Beijing Solarbio Science and Technology Co., Ltd.), while the concentration was measured using the Bradford method. The proteins were separated using 10% SDS-PAGE, transferred to PVDF membranes (EMD Millipore), and then blocked with skimmed milk and incubated with primary antibodies against SOX6 (cat. no. ab84880),
β-catenin (cat. no. ab22656), c-myc (cat. no. ab17355) and β-actin (cat. no. ab8226) (1:200 dilution, Abcam) overnight at room temperature. Subsequently, the membrane was incubated with the secondary antibody (cat. no. TA130005 (1:5,000 dilution, OriGene Technologies, Inc.). Finally, enhanced chemiluminescence reagents (Beyotime Institutes of Biotechnology) were used to develop the protein signal. The quantification of the blots was analyzed using ImageJ software (v.1.8.0) (National Institutes of Health, USA). The data were from three independent repeated experiments.

**Statistical analysis.** GraphPad Prism v8.0 software (GraphPad Software, Inc.) was used to analyze all the experimental data. Data are presented as the mean with standard deviation. The statistical analysis was performed using the log-rank test, and survival curves were drawn using Kaplan-Meier. For the matched samples, the data were analyzed using Wilcoxon signed rank, and for non-matched samples, the data were compared by Mann-Whitney test. Comparison between two groups was determined using a Student's t-test, and the comparison of >3 groups was determined using one-way ANOVA, and then Bonferroni test was selected for further statistical assay when data sets contained >3 groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Low expression level of PART1 and SOX6 in ESCC tissues and cells is associated with prognosis in the patients with ESCC. To investigate the level of PART1 and SOX6 in ESCC tissues and cells, as well as their clinical value, The Cancer Genome Atlas (TCGA) database, and the GEO dataset were analyzed. The data from TCGA database and GEO dataset revealed that the level of PART1 in ESCC tissues was significantly lower compared with that in normal tissues (P<0.05) (Fig. 1A and B), which was further verified using RT-qPCR in 75 ESCC samples and matched adjacent normal samples (P<0.0001) (Fig. 1C). In addition, the GEO GSE111011 dataset revealed that ESCC tissues exhibited lower SOX6 mRNA expression levels compared with that in paired adjacent normal tissues (P<0.0001) (Fig. 1D), and similar results were found using RT-qPCR in 75 ESCC samples and matched adjacent normal samples (P<0.0001) (Fig. 1E). Furthermore, RT-qPCR demonstrated that the ESCC cell lines (Eca109, EC9706, TE1, KYSE70 and KYSE450) had significantly lower levels of PART1 and SOX6 compared with those in the Het-1A normal esophageal epithelial cell line (P<0.01) (Fig. 1F and G). Correlation analysis showed that PART1 expression level was positively correlated with SOX6 expression level in ESCA tissues (Fig. 1H and I). To further determine the association between PART1 and SOX6 levels with prognosis in patients with ESCC, a log-rank test was used. The results revealed that patients with low levels of PART1 and SOX6 exhibited shorter survival times compared with those with high expression levels of PART1 and SOX6 (Fig. 1J-L). The data suggest that PART1 and SOX6 may participate in the development and progression of ESCC and may be novel prognostic factors in patients with ESCC.

**Associations between PART1 and SOX6 levels and the clinicopathological features and prognosis in ESCC.** To further investigate the roles of PART1 and SOX6 in ESCC development and progression, GraphPad Prism v8.0 software was used to investigate the association between PART1 and SOX6 level and the clinicopathological features in the patients with ESCC. It was found that low expression levels of PART1 and SOX6 were both associated with higher TNM stage and positive lymph node metastasis (all P<0.05); however, there was no association with sex, age, tumor diameter and differentiation degree (all P>0.05) (Tables II and III). The data suggest that PART1 and SOX6 may be novel predictive factors for TNM stage and lymph node metastasis in ESCC.

**lncRNA PART1 is a direct transcriptional target of FOXP2 in the ESCC cell lines.** To further investigate which factor affects PART1 expression in ESCC, direct targets of PART1 were determined. The upstream promoter sequence (~2 kb upstream) of PART1 was detected using promoter analysis tools (UCSC and JASPAR) and the binding site of FOXP2 (ATGTAAACAAT) was found to be within the upstream promoter region of PART1 (Fig. 2A). To preliminarily investigate the expression level of FOXP2 in ESCC, RT-qPCR was used and the results revealed that the FOXP2 mRNA expression level in 75 ESCC tissues was significantly lower compared with that in the matched adjacent normal tissues (Fig. 2B). Correlation analysis demonstrated that PART1 level was positively correlated with FOXP2 mRNA expression level in the ESCC tissues (Fig. 2C). Further investigation revealed that the expression level of FOXP2 was significantly reduced by FOXP2 siRNAs (si-FOXP2#1 and #2), and si-FOXP2#2 was more effective at reducing FOXP2 expression level in the ESCC cell lines (Fig. 2D), whereas pcDNA3.1-FOXP2 significantly increased the expression level of FOXP2 in the ESCC cell lines (Fig. 2E). Based on these results, si-FOXP2#2 significantly suppressed the expression level of PART1 in the ESCC cell lines; however, FOXP2 upregulation significantly increased the expression level of PART1 (Fig. 2F and G). Then, it was investigated whether FOXP2 directly binds to the predicted promoter region in PART1. The fragments containing WT or MUT binding sites were constructed using the pGL3-basic vector. As expected, the luciferase activity was notably enhanced following co-transfection with pcDNA3.1-FOXP2 and pGL3-PART1-promoter-WT in the Eca109 and EC9706 cell lines; however, there was no effect following co-transfection with pcDNA3.1-FOXP2 and pGL3-PART1-promoter-MUT (Fig. 2H and I). These data suggest that the PART1 expression level may be affected by FOXP2 in ESCC cells.

**lncRNA PART1 suppresses cell proliferation and invasion in the ESCC cell lines.** To investigate the role of PART1 in the
proliferation and invasion of the ESCC cell lines, CCK-8 and Matrigel assays were used. It was found that pcDNA3.1-PART1 significantly upregulated PART1 expression level in the Eca109 and EC9706 cell lines (P<0.0001) (Fig. 3A). In addition, PART1 upregulation markedly suppressed the cell proliferation of the Eca109 and EC9706 cell lines compared with that in the pcDNA3.1 group (P<0.05) (Fig. 3B). The Matrigel assay, using a Transwell chamber, revealed that PART1 overexpression significantly inhibited cell invasion in the Eca109 and EC9706 cell lines (Fig. 3C and D). Conversely, three siRNAs against PART1 were used to downregulate the expression level of PART1 in the Eca109 and EC9706 cell lines, as expected, all three PART1 siRNAs significantly reduced the expression level of PART1, in which PART1 siRNA#1 was the most
PART1 downregulation significantly promoted cell proliferation in the Eca109 and EC9706 cell lines (P<0.05) (Fig. 3F). Meanwhile, PART1 knockdown accelerated cell invasion in the Eca109 and EC9706 cell lines (Fig. 3G and H). These data suggest that PART1 plays a pivotal role in the regulation of cell proliferation and invasion of the ESCC cell lines.

miR-18a-5p is the direct target of PART1 in the ESCC cell lines. To investigate the underlying molecular mechanism of PART1 in ESCC progression, lncATLAS online software was used to predict the subcellular localization of PART1. The data revealed that PART1 was primarily localized in the cytoplasm in most of the indicated cell types (Fig. 4A). To validate the predicted result, RT-qPCR was used in the ESCC cell lines. The results demonstrated that PART1 was principally localized in the cytoplasm of the Eca109 and EC9706 cell lines (Fig. 4B). According to the localization of PART1, a proposed ceRNA function of PART1 was suggested. The possible binding sites of miRNA in PART1 were predicted using the LncBase Predicted v.2 software, integrated in DIANA Tools, in which miR-18a-5p was found to contain a target site and was investigated further in the present study (Fig. 4C). To preliminarily elucidate whether PART1 could bind to miR-18a-5p in the ESCC cell lines, an Ago2-RIP experiment was used to verify the direct interaction between PART1 and miR-18a-5p. The results showed that PART1 and miR-18a-5p expression levels in the anti-Ago2 antibody group were significantly higher compared with that in the IgG group (P<0.0001) (Fig. 4D and E). Notably, PART1 enrichment in the miR-18a-5p mimic group was significantly higher compared with that in the NC mimic group (P<0.0001) (Fig. 4F). These findings suggested that PART1 and miR-18a-5p exhibited a direct interaction in the ESCC cell lines via the Ago2-dependent pathway. In addition, a dual-luciferase reporter assay revealed that the relative luciferase intensity was significantly reduced following co-transfection with PART1-WT and miR-18a-5p mimic, but not in the PART1-MUT group, in the Eca109 and EC9706 cell lines (Fig. 4G). Simultaneously, PART1 overexpression significantly reduced the expression level of miR-18a-5p (Fig. 4H), whereas PART1 silencing significantly promoted miR-18a-5p expression in the Eca109 and EC9706 cell lines (Fig. 4I). These findings indicated that PART1 directly regulated the expression level of miR-18a-5p in the ESCC cell lines.

SOX6 is the direct target of miR-18a-5p in the ESCC cell lines. It is well-known that miRNA functions as either an oncogene or tumor suppressor, depending on its downstream target gene. To further investigate the role of miR-18a-5p in ESCC progression, its expression level and downstream target genes were determined. The StarBase online software was used to investigate...
the expression level of miR-18a-5p in 162 ESCA tissues and 11 normal samples, and it was found that miR-18a-5p expression level was markedly higher compared with that in normal samples (Fig. 5A), which was further verified using the GEO GSE43732 dataset in 119 ESCC samples and matched adjacent normal samples. (D) A total of 3 siRNAs targeting FOXP2 were used to downregulate FOXP2 expression level in the ESCC cell lines and validated using RT-qPCR; si-FOXp2#2 was the most effective. "P<0.01 and ""P<0.0001 vs. the si-NC group. (E) pcDNA3.1-FOXp2 significantly promoted the expression level of FOXP2 in the ESCC cell lines as determined by RT-qPCR. "P<0.01 vs. the pcDNA3.1 group; the data were examined by one-way ANOVA and Bonferroni test. (F) si-FOXp2#2 significantly downregulated the expression level of PART1 in the ESCC Eca109 and EC9706 cell lines as determined by RT-qPCR. """"P<0.0001 vs. the si-NC group. PC3-PART1-promoter-WT or pcGL3-PART1-promoter-MUT in the FOXP2-overexpressing (H) Eca109 and (I) EC9706 cell lines. """"P<0.0001 vs. MUT. RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type; MUT, mutant; si, small interfering; NC, negative control; ESCC, esophageal squamous cell carcinoma; PART1, prostate androgen regulated transcript 1. (E-I) All data were examined by independent-sample t-test.

Figure 2. Transcriptional factor, FOXP2, regulates PART1 expression level in the ESCC cell lines. (A) A predicted FOXP2 binding site in the PART1 promoter region. (B) RT-qPCR assay was used to analyze the expression level of FOXP2 in 75 ESCC and matched normal tissues. P<0.0001, as investigated by paired t-test. (C) Spearman's correlation result between PART1 and FOXP2 mRNA expression levels in 75 ESCC and matched normal samples. (D) A total of 3 siRNAs targeting FOXP2 were used to downregulate FOXP2 expression level in the ESCC cell lines and validated using RT-qPCR; si-FOXp2#2 was the most effective. ""P<0.01 and """"P<0.0001 vs. the si-NC group. (E) pcDNA3.1-FOXp2 significantly promoted the expression level of FOXP2 in the ESCC cell lines as determined by RT-qPCR. """"P<0.0001 vs. the si-NC group. (G) pcDNA3.1-FOXp2 significantly promoted the expression level of PART1 in the ESCC Eca109 and EC9706 cell lines as determined by RT-qPCR. """"""""P<0.0001 vs. the pcDNA3.1 group. (H and I) Luciferase reporter assay was used to determine the luciferase activity in pcGL3-PART1-promoter-WT or pcGL3-PART1-promoter-MUT in the FOXP2-overexpressing (H) Eca109 and (I) EC9706 cell lines. """"""""P<0.0001 vs. MUT. RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type; MUT, mutant; si, small interfering; NC, negative control; ESCC, esophageal squamous cell carcinoma; PART1, prostate androgen regulated transcript 1. (E-I) All data were examined by independent-sample t-test.
Role of the PART1/miR-18a-5p/SOX6 signaling axis in cell proliferation and invasion of the ESCC cell lines. To investigate the potential role of the PART1/miR-18a-5p/SOX6 signaling axis in cell proliferation and invasion of the ESCC cell lines, miR-18a-5p inhibitor or mimic alone, combined with PART1 siRNA and SOX6 siRNA or pcDNA3.1-PART1 and pcDNA3.1-SOX6 were used to transfect the Eca109 and EC9706 cell lines, and then CCK-8 and Matrigel assays were performed. It was found that miR-18a-5p inhibitor significantly blocked cell proliferation and invasion in the Eca109 cell lines.
and EC9706 cell lines, which was partly reversed by PART1 and SOX6 siRNA (Fig. 6A-C). Notably, miR-18a-5p inhibitor triggered the upregulation of SOX6 proteins and the downregulation of β-catenin and c-myc proteins, which was also partly reversed by pcDNA3.1-PART1 and pcDNA3.1-SOX6 (Fig. 6H). These data suggest that the PART1/miR-18a-5p/SOX6/β-catenin signaling axis may be a novel therapeutic strategy in patients with ESCC.

**Discussion**

An increasing number of studies have demonstrated that lncRNAs play a pivotal role in tumor progression and may be novel diagnostic and therapeutic opportunities for
Figure 5. SOX6 is a direct molecular target of miR-18a-5p in the ESCC cell lines. (A) StarBase online software was used to analyze the expression level of miR-18a-5p in 162 ESCA and 11 normal samples. (B) The GEO GSE43732 dataset was used to analyze the expression level of miR-18a-5p in 119 ESCC and matched adjacent normal specimens. (C) RT-qPCR assay was used to analyze the expression level of miR-18a-5p in 75 ESCC and paired adjacent normal tissues. (D) RT-qPCR was used to analyze the expression level of miR-18a-5p in the ESCC cell lines (Eca109, EC9706, TE1, KYSE70 and KYSE450) and the Het-1A normal esophageal epithelial cell line. ****P<0.0001 vs. the Het-1A cell line. Data were examined by one-way ANOVA and Bonferroni test. (E) TargetScan and miRDB databases were used to predict the potential binding sites between miR-18a-5p and SOX6. (F) A dual-luciferase reporter assay was performed to investigate the interaction between miR-18a-5p and SOX6. **P<0.01 vs. NC mimic. (G) Pearson correlation test analysis between miR-18a-5p and SOX6 mRNA expression levels in 75 ESCC and matched adjacent normal samples. (H) miR-18a-5p inhibitor significantly suppressed the miR-18a-5p expression level in the Eca109 and EC9706 cell lines. ****P<0.0001 vs. the NC inhibitor. (I) miR-18a-5p mimic significantly enhanced the miR-18a-5p expression level in the Eca109 and EC9706 cell lines. ****P<0.0001 vs. the NC mimic. (J) miR-18a-5p downregulation significantly promoted the expression level of SOX6 in the Eca109 and EC9706 cell lines. ****P<0.0001 vs. the NC inhibitor. (K) miR-18a-5p overexpression significantly suppressed the expression level of SOX6 in the Eca109 and EC9706 cell lines. ****P<0.0001 vs. the NC mimic; si, small interfering; NC, negative control; WT, wild-type; MUT, mutant; miR, microRNA; ESCC, esophageal squamous cell carcinoma; SOX6, SRY-box transcription factor 6. (B and C) Data were analyzed using paired t-test; (F and H-K) All data were examined by independent-sample t-test.
Table IV. Associations of miR-18a-5p expression with clinicopathological features in the ESCC cases (N=75).

| Features                        | miR-18a-5p expression | n  | High | Low | χ²  | P-value |
|---------------------------------|-----------------------|----|------|-----|-----|---------|
| Sex                             |                       |    |      |     |     |         |
| Male                            |                       | 49 | 36   | 13  | 1.943 | 0.163  |
| Female                          |                       | 26 | 15   | 11  |       |         |
| Age (years)                     |                       |    |      |     |     |         |
| <60                             |                       | 35 | 20   | 15  |       |         |
| ≥60                             |                       | 40 | 31   | 9   | 3.555 | 0.059  |
| Tumor diameter (cm)             |                       |    |      |     |     |         |
| <4                              |                       | 50 | 32   | 18  |       |         |
| ≥4                              |                       | 25 | 19   | 6   | 1.103 | 0.294  |
| Differentiation degree          |                       |    |      |     |     |         |
| High/moderate                   |                       | 44 | 28   | 16  | 0.932 | 0.334  |
| Poor                            |                       | 31 | 23   | 8   |       |         |
| TNM staging                     |                       |    |      |     |     |         |
| I-II                            |                       | 40 | 23   | 17  |       |         |
| III-IV                          |                       | 35 | 28   | 7   | 4.343 | 0.037  |
| Lymph node metastasis           |                       |    |      |     |     |         |
| Yes                             |                       | 23 | 20   | 3   |       |         |
| No                              |                       | 52 | 31   | 21  | 5.478 | 0.019  |

ESCC, esophageal squamous cell carcinoma.

numerous patients with cancer. For example, HOTAIR, as a chromatin-regulating IncRNA, was found to function as an oncogene, and its overexpression has been associated with tumor progression, invasion and metastasis (32,33). ANRIL overexpression was associated with rapid tumor progression and shortened survival times in patients with nasopharyngeal carcinoma (34) and hepatocellular carcinoma (35). In addition, prostate androgen regulated transcript 1 (PART1) was found to be significantly upregulated in non-small cell lung cancer (NSCLC), and was associated with shorter overall survival times; therefore, it could serve as an independent prognostic marker in patients with NSCLC with stage I-III (36). Jin et al (24) found that there was a low expression level of PART1 in glioma tissues, which was confirmed using TCGA database and RT-qPCR. PART1, at high expression levels, was associated with advanced stage and predicated poor survival time in patients with prostate cancer (37). These data highlighted the clinical value of IncRNAs in tumors, particularly in diagnosis and for prognosis. Currently, the expression level and the potential clinical value of PART1 in ESCC remain elusive. In the present study, PART1 was found to be expressed at low levels in ESCC tissues and cells, and a low expression level was associated with TNM stage and lymph node metastasis. Notably, patients with ESCC and low PART1 expression level had poorer survival times compared with those with high PART1 expression levels. Combined with previous reports, it is hypothesized that differential expression patterns of PART1 in different types of tumor are dependent on the various tumor types. Overall, the data from the present study supports PART1, as a potential molecular marker for prognosis in patients with ESCC.

Transcriptional regulation of IncRNAs is important for IncRNA expression level in numerous types of tumor. A number of IncRNAs that have been identified to be abnormally expressed were found to be regulated by either a tumor suppressor, oncogenic pathways or other transcriptional factors, such as myc, p53 and NF-κB (38-40). In pancreatic cancer, hypoxia-inducible factor-1α induced the transcription of IncRNA-BX111 in the presence of hypoxia, which contributed to the hypoxia-induced epithelial-mesenchymal transition progression by regulating the expression level of ZEB1 (12). In addition, the E2F transcription factor was found to bind to the core promoter region of TINCR to induce the expression of TINCR in gastric carcinoma, which was verified using in vivo chromatin IP (41). To address the possible reason for the low expression level of PART1 in ESCC tissues and cells, the UCSC and JASPAR online software programs were used to predict the binding site of FOXP2, within the upstream promoter region of PART1, and was further confirmed using a reporter assay in the current study. Increasing evidence has demonstrated that FOXP2 plays important roles in cell proliferation, invasion and metastasis in a variety of different tumors (42,43), which suggest that FOXP2 is associated with tumor development and progression. To preliminarily investigate the function of FOXP2 in ESCC, the low expression level of FOXP2 was verified using TCGA database, and was further confirmed using RT-qPCR in 75 ESCC tissues and matched adjacent normal tissues. Notably, the PART1 level was positively correlated with FOXP2 mRNA expression level in ESCA tissues, and FOXP2 downregulation markedly suppressed the expression level of PART1; however, FOXP2 upregulation significantly increased the expression level of PART1. These data indicated that the transcription factor, FOXP2 is a direct inducer of PART1 in ESCC, and the FOXP2/PART1 signaling axis may be a novel therapeutic target; however, the functions and molecular mechanisms involved require further investigation.

It is well known that IncRNAs widely participate in the regulation of cell proliferation, invasion and metastasis in a large number of tumors. Sun et al (37) found that PART1 downregulation repressed cell proliferation and promoted cell apoptosis in prostate cancer. Furthermore, PART1 functions as a tumor suppressor by sponging miR-190a-3p to inactivate the PTEN/AKT signaling pathway in glioma (24). To further uncover the function of PART1 in ESCC cell proliferation and invasion, PART1 siRNA knockdown and pcDNA3.1-PART1 overexpression was used to investigate the role of PART1 in cell proliferation and invasion in the ESCC cell lines. It was found that PART1 downregulation significantly promoted cell proliferation and invasion, whereas PART1 upregulation markedly suppressed cell proliferation and invasion in the ESCC cell lines. These findings suggest that PART1 exerts a tumor-suppressive role in ESCC cell lines and may be a promising therapeutic target in patients with ESCC.

Determining the regulatory role of IncRNAs as key players is important to understand the etiology of several types of tumor (44). To further investigate the regulatory
mechanisms of PART1 in ESCC, a localization assay was performed using the lncATLAS online predictive software and RT-qPCR. It was found that PART1 was primarily localized in the cytoplasm of the ESCC cell lines, which further identified the possible function of PART1 as a ceRNA. Further investigation revealed that miR-18a-5p and
PART1 were both enriched using an anti-Ago2 antibody, and miR-18a-5p mimic significantly increased the accumulation of PART1 in the ESCC cell lines, suggesting that miR-18a-5p and PART1 both appear in the RNA-induced silencing complex. In addition, the binding of miR-18a-5p and PART1 was confirmed using a dual-luciferase reporter assay, in which, PART1 downregulation promoted the miR-18a-5p expression level and PART1 upregulation suppressed the miR-18a-5p expression level in the ESCC cell lines. These findings suggest that PART1 could affect the expression level of miR-18a-5p using a ceRNA mechanism.

Recently, miR-18a-5p has been reported to participate in tumor progression, and may be a potential biomarker for the diagnosis and prognosis in a variety of different tumors (45-47). To further investigate the function of miR-18a-5p expression in ESCC development and progression, it was found that miR-18a-5p was highly expressed in ESCC tissues and cells, and its high expression level was associated with TNM stage and lymph node metastasis in patients with ESCC, suggesting that miR-18a-5p functions as a tumor oncogene in ESCC. Further investigation revealed that SOX6 was confirmed as the downstream target gene of miR-18a-5p in the ESCC cell lines, and its expression level was negatively correlated with the miR-18a-5p expression level in 75 ESCC samples. In addition, miR-18a-5p inhibitor significantly promoted the expression level of SOX6, whereas miR-18a-5p mimic notably downregulated the expression level of SOX6. These findings suggest that SOX6 is a direct target gene of miR-18a-5p in the ESCC cell lines. To further elucidate the underlying function of miR-18a-5p in cell proliferation and invasion, CCK-8 and Matrigel assays were used and the results demonstrated that miR-18a-5p inhibitor notably blocked cell proliferation and invasion in the Eca109 and EC9706 cell lines, which was partly reversed by PART1 siRNA and SOX6 siRNA, while converse data were found when miR-18a-5p mimic was combined with pcDNA3.1-PART1 or pcDNA3.1-SOX6. These data highlight the important role of the PART1/miR-18a-5p/SOX6 signaling axis in ESCC development and progression.

Several reports have demonstrated that SOX6 downregulation is found in ESCC, hepatocellular carcinoma and pancreatic cancer, and is associated with the promotion of tumor progression (48-50). Notably, SOX6 was found to be able to bind to the armadillo repeats 1-4 of β-catenin to intercept its oncogenic activities (51), and c-myc was found to be a pivotal downstream target of β-catenin and to regulate thousands of genes (52). Based on the aforementioned research, we hypothesized that PART1 absorbs miR-18a-5p to enhance the expression level of SOX6, which further results in the inhibition of the β-catenin/c-myc signaling axis in ESCC. To further investigate the aforementioned hypothesis, the alterations of β-catenin and its downstream gene, c-myc, were investigated in this signaling axis. It was found that miR-18a-5p inhibitor triggered the upregulation of SOX6 proteins and the downregulation of β-catenin and c-myc proteins, which was also in part reversed by PART1 siRNA and SOX6 siRNA, and the inverse effect was exhibited following the utilization of miR-18a-5p mimic combined with pcDNA3.1-PART1 or pcDNA3.1-SOX6. The data in the present study suggest that the PART1/miR-18a-5p/SOX6/β-catenin signaling axis may be involved in ESCC development and progression, and may be a promising therapeutic target in patients with ESCC.

In conclusion, the data in the present study revealed that PART1 was expressed at low levels in ESCC tissues and cells, and low expression levels were associated with TNM stage, lymph node metastasis and poor prognosis in patients with ESCC. PART1 was found to be a direct transcriptional target of the transcriptional factor, FOXP2, in the ESCC cell lines. PART1 overexpression and knockdown suppressed and promoted cell proliferation and invasion, respectively, in the Eca109 and EC9706 cell lines. PART1 was found to function as a ceRNA to sponge miR-18a-5p further resulting in the elevation of SOX6 expression levels, which triggered the downregulation of β-catenin and c-myc proteins, and thus, suppressed ESCC progression. The findings from the present study suggest that targeting the PART1/miR-18a-5p/SOX6/β-catenin signaling axis may be a novel therapeutic strategy for patients with ESCC.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
SY conceived the research idea and supervised this study. YZ performed the majority of experiments and interpreted the data. QZ collected the clinical samples and performed data analysis. HL, NW and XZ assisted with all the experiments and revised the manuscript. HL carried out the bioinformatics assay. YZ prepared the manuscript. All authors discussed the study, read and approved the final manuscript. All contributing authors agreed to the submission of this manuscript for publication.

Ethics approval and consent to participate
This study was reviewed and approved by the Research and Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) (approval no. 2019016). The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. All subjects provided informed consent to participate in the study.

Patient consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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