LncRNA JPX Promotes Esophageal Squamous Cell Carcinoma Progression by Targeting miR-516b-5p/VEGFA Axis

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Research

Keywords: Esophageal squamous cell carcinoma, Proliferation, Angiogenesis, LncRNA JPX, VEGFA

Posted Date: August 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-798922/v1>

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Abstract

Background: Long non-coding RNAs (lncRNAs) are reported act as important regulators in various cancers. LncRNA JPX was identified as an oncogenic regulator in lung cancer. However, the function of lncRNA JPX in the progression of esophageal squamous cell carcinoma (ESCC) remains unclear.

Methods: The effects and molecular mechanism of JPX on the progression of ESCC were investigated using fluorescence in situ hybridization (FISH), cell proliferation, quantitative real-time PCR (qRT-PCR), western blot, dual luciferase, cell cycle, 5-Ethynyl-2'-Deoxyuridine (EdU) incorporation, transwell, RNA pull-down, tube formation and RNA immunoprecipitation (RIP) assays.

Results: In the present study, we found JPX was highly expressed in tissues of ESCC patients and different ESCC cell lines. Functional assays demonstrated that JPX promoted ESCC cell proliferation, migration and invasion *in vitro* and tumor growth *in vivo*. Moreover, we found JPX promoted ESCC mobility *in vitro*. Mechanistically, the results showed that JPX functions as a sponge of miR-516b-5p, which targets an oncogene vascular endothelial growth factor A (VEGFA) in ESCC cells. Interactions between miR-516b-5p and JPX or VEGFA were confirmed by luciferase reporter assays. Furthermore, inhibition of JPX significantly attenuated the cell growth and mobility ability of ESCC cells *in vitro*. In addition, miR-516b-5p overexpression abrogated JPX enhanced proliferation, migration, invasion, and angiogenesis of ESCC cells.

Conclusions: Our study demonstrated that JPX played an important role in promoting ESCC progression via the miR-516b-5p/VEGFA pathway and might serve as a promising novel therapeutic target for ESCC patients.

1. Background

Esophageal cancer is one of the major causes of cancer-related death worldwide, which ranks seventh in terms of incidence and sixth in mortality worldwide [1]. Esophageal cancer is a common malignant tumor of the digestive tract. Approximately 50% of the cancer mortality rate derives from digestive tract cancers in China, including gastric cancer, liver cancer and esophageal cancer [2]. Patients with esophageal cancer are mostly found and diagnosed due to dysphagia in the middle and late stages, and the 5-year survival rate after comprehensive treatment is still less than 20% [3]. Esophageal cancer is mainly classified into esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) [4]. Although the treatment of ESCC has been improved in recent years, the lower 5-year survival rate and worse prognosis are still disturb the patients with ESCC owing to the late discovery, early lymph node metastasis, difficult eradication surgery, and highly recurrence and metastasis rates [5–7]. At present, the mechanisms of ESCC proliferation, invasion and metastasis are still unclear. Therefore, studying the molecular mechanism of ESCC proliferation and metastasis is of great significance for improving treatment methods and strategies in clinic.

Long non-coding RNA (lncRNA) are a class of non-coding RNAs with a length of more than 200 nucleotides, which have highly conserved sequence elements, specific spatial secondary structure, tissue or cell specific expression, and clear subcellular localization [8]. LncRNAs has been proved to be closely related to the occurrence and development of tumors [9, 10]. LncRNAs are involved in the regulation of chromatin remodeling, transcription and post-transcriptional processing and other cellular biological processes, playing a corresponding role in the occurrence, development, proliferation, invasion, and metastasis of cancer cells [11, 12]. Previous studies have shown that lncRNAs functioned as oncogenic regulator or suppressor of human cancers *in vitro* and *in vivo* [13, 14]. A recent study has reported that lncRNA HERES promoted ESCC cell proliferation and metastasis by activating the Wnt signaling pathways via interaction with enhancer of zeste homolog 2 (EZH2) [15]. LncRNA CASC9 was revealed to be positively correlated with ESCC prognosis and metastasis, and might upregulate laminin gamma 2 (LAMC2) expression by binding with CREB-binding protein (CBP) and modifying histone acetylation [16]. However, a novel lncRNA KLF3-AS1 was found to be poorly expressed in ESCC and upregulation of KLF3-AS1 inhibited ESCC cell invasion and migration by sponging miR-185-5p to inhibit KLF3 expression [17]. Latest studies have shown that a critical oncogenic lncRNA JPX promoted the development and progression of lung cancer [18, 19]. The oncogenic role of JPX has also been reported in cervical, oral squamous cell carcinoma, and ovarian cancers [20–22]. The aim of this study is to investigate the function and molecular mechanisms of lncRNA JPX in ESCC. To clarify the functional role of JPX in ESCC cells, we investigated the effects of JPX knockdown or overexpression on malignant phenotypes of ESCC cells and the underlying mechanism.

2. Materials And Methods

2.1. Patients and tissue samples

A total of 21 primary ESCC tissues and adjacent normal tissues specimens were collected from patients at Shanghai Chest Hospital (Shanghai, China). None of the patients with ESCC had received radiotherapy or chemotherapy before surgery. The clinical ESCC samples used in this study were histopathologically and clinically diagnosed in 2019. Written informed consent was obtained from all participants prior to sample collection. The clinical characteristics of patients were shown in Table S1. TNM staging of ESCC patients was according to the American Joint Committee on Cancer (AJCC). This study protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of the Shanghai Chest Hospital of the Shanghai Jiaotong University.

2.2. Cell culture

The human normal esophageal epithelial cell line Het-1A and human ESCC cell lines (KYSE150, KYSE450, Eca109, and EC9706) were obtained from the cell bank center of our institute. The cell line authentication was performed and the STR analysis of the cells was consistent with the STR data from China Infrastructure of Cell Line Resources database. Cells were cultured in RPMI-1640 (Gibco, NY, USA)

supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). at 37°C in a humidified atmosphere of 5 % CO₂.

2.3. Small interfering RNA (siRNA) synthesis, plasmid construction and transfection

The miR-516b-5p mimics, si-JPX, si-VEGFA and the respective negative control (NC) were obtained from GenScript (Nanjing, China). pcDNA3.1 vector was obtained from YouBio (Changsha, China). JPX sequences were cloned into pcDNA3.1 vector. The cell transfection was performed using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) or Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, cells were seeded in 6-well plate for 16 h. 4 µg DNA or 100 pmol RNA or 8 µl Lipofectamine 3000 was diluted by 250 µl serum-free medium. After incubation for 5 min, mixed them gently and incubated for 20 min at room temperature. After different time points of transfection, cells were harvested to further detection.

2.4. Subcellular fractionation

Cells were collected and used for nuclear and cytoplasmic protein extraction using the nuclear and cytoplasmic extraction reagents (Life Technologies, Carlsbad, CA, US). For nuclear and cytoplasmic RNA separation, cells were collected and extracted using PARIS™ kit (Life Technologies, Carlsbad, CA, US).

2.5. Fluorescence in situ hybridization (FISH)

The FISH assay was performed according to the instruction of the manufacturers. The Cy3-labeled JPX probes used in our study were designed and synthesized by GenePharma (Shanghai, China). Briefly, the prepared cells were fixed with 4% paraformaldehyde containing 0.5% Triton X-100 for 20 min. The cells were incubated with the probes at 37°C overnight. The cell nuclei were stained with DAPI (Beyotime). The staining results were observed using Zeiss confocal laser microscopy (Zeiss, Germany).

2.6. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was reversely transcribed to cDNA using a PrimeScript RT Master Mix Kit (Takara Bio). A miScript SYBR Green PCR Kit (Qiagen) was used to determine the gene expression using a 7900 real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). GAPDH or U6 was used as an internal control. Fold changes for the expression levels were calculated using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$). The results were performed in triplicate in real-time quantitative RT-PCR. The primers used in the study were shown in Table S2.

2.7. Western blot

After transfection, cells were treated in a lysis buffer, followed by the extraction and quantification of proteins. Subsequently, the protein was transferred into nitrocellulose membranes (NC membranes),

blocked, and incubated in specific primary antibody at 4°C overnight. The membranes were washed and incubated with second antibody for 2 h at room temperature. Finally, the protein bands were visualized using chemiluminescence detection system. The antibodies against VEGFA (ab51745, Abcam, Cambridge, UK), Fibronectin (ab2413, Abcam, Cambridge, UK), GAPDH (#14C10, Cell Signaling Technology, Danvers, MA, USA), N-cadherin (ab18203, Abcam, Cambridge, UK), Vimentin (ab3974, Abcam, Cambridge, UK), E-cadherin (sc-8426, Santa Cruz Biotechnology, CA, USA), and the secondary anti-IgG (ab150077, Abcam, Cambridge, UK) antibody were used to determine the protein expression levels.

2.8. Cell proliferation assay

Cell proliferation was examined using a CCK-8 assay kit (Dojindo, Japan). Briefly, cells were transfected with various vectors using Lipofectamine 3000 Reagent. After different time point of transfection, cells were added CCK-8 and incubated for 2h. The absorbance at 450 nm was measured with a microplate reader (ELX800, BioTek, USA).

2.9. 5-Ethynyl-2'-Deoxyuridine (EdU) incorporation assay

The EdU incorporation assay kit (RiboBio, Guangzhou, China) was used to evaluate cell proliferation. After transfection, cells were seeded into 96-well plates at a density of 1×10^4 cells/well. 24 h after seeded, 50 μ M EdU was added into each well and cells were incubated for 2 h at 37°C, fixed with 4% paraformaldehyde, then stained with Hoechst. The images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan).

2.10. Transwell migration and invasion assays

For the transwell migration assay, the above-transfected cells were plated to the upper chambers of non-matrigel-coated, 8- μ m pore polyethylene membranes transwell plates (Corning, MA, USA). For the matrigel-coated transwell invasion assay, pre-coated matrigel (BD Biosciences, USA) and transfected cells were placed in the upper chambers of transwell plates. cells were seeded on the upper chambers with serum-free medium. The lower chambers were added the medium contained 10% FBS as a chemoattractant. After culturing at 37°C for 48 h, cells that appeared on the undersurface of the filter were fixed with methanol, stained with 0.1% crystal violet and cells on the undersides of the filters were observed and counted was counted in three randomly selected areas under a light microscope.

2.11. Cell cycle analysis

Cells were seeded and transfected with si-JPX or si-NC for 48h at 37°C. The treated cells were washed by PBS and fixed in cold 70% ethanol for 30 min. After washed by PBS, the cells were incubated with 400 μ L PI for 30min at room temperature. Finally, 400IL PI was added and incubated for 30min in the dark at room temperature. The DNA content was detected using a flow cytometer (BD Biosciences, CA, USA) and cell cycle was analyzed using FlowJo.7.6.1 software (FlowJo LLC, Ashland, OR).

2.12. RNA pull-down assay

The biotin (bio)-labeled JPX and control oligo probe (GenePharma, Shanghai) were incubated with magnetic beads (Life Technologies, Carlsbad, CA, USA) at 37°C for 4 h. The RNA pull-down assay was carried out as previously described [23]. JPX overexpressing were lysed and incubated with the beads at 4°C overnight. After washing with wash buffer, the microRNA (miRNA) in the pull-down was determined using RT-qPCR.

2.13. RNA immunoprecipitation (RIP) assay

RNA-binding protein immunoprecipitation assay was performed by a Magna RIP Kit (Millipore, USA) according to the manufacturer's methods. Cells were lysed by RIP lysis buffer, and magnetic beads with AGO2 (ab32381, Abcam, Cambridge, UK) or IgG (ab2410, Abcam, Cambridge, UK) antibody were prepared. The immunoprecipitation reactions were carried out by incubating the RIP lysate and the beads-antibody complex together with rotation overnight at 4°C. Immunoprecipitated RNAs were purified and analyzed with qRT-PCR and normalized to the input control.

2.14. Luciferase report assay

The fragments contain the wild type (wt) and the mutant type (mut) of JPX or 3'UTR of VEGFA was respectively cloned into pmirGLO vector separately. EC9706 cells were co-transfected with miR-516b-5p mimics or mimics control and luciferase reporter. 24 h post transfection, cells were lysed using passive lysis buffer (promega, Madison, WI, USA) and the luciferase activity was measured using the dual-Luciferase reporter Assay System (Promega, Madison, WI, USA) and normalized to renilla luciferase activity respectively.

2.15. Tube formation assay

75 μ L Matrigel (BD Biosciences, USA) was added into each well of a 48-well plate and allowed to solidify for more than 1h at 37°C. HUVECs were suspended in the indicated conditioned medium and seeded onto the gel. After 4h of incubation, a light microscope was used to observe the tubular structures and acquire images. Tube formation was quantified by measuring the total length of the tubes using ImageJ software.

2.16. In vivo experiments

4-6-week-old male BALB/c nude mice were obtained from Slac Laboratory Animal (Shanghai, China) and maintained under specific pathogen-free conditions. 8 mice were divided randomly into two groups, and 1×10^6 of EC9706 cells with or without JPX overexpression were injected into the tail vein of nude mice. The mice were sacrificed after 6 weeks, and the maximum and minimum length and weight of the tumors were measured. Tumor volume (mm^3) was calculated with the formula: tumor volume (mm^3) = longer diameter $\times$ shorter diameter²/2. The animal studies were approved by the Institutional Animal Care and Use Committee of Shanghai Chest Hospital, School of Medicine, Shanghai Jiaotong University.

2.17. Statistical analysis

The data were analyzed using Graphad Prism 5 software or SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as the mean $\pm$ SEM of at least three independent experiments. The statistical differences were calculated by the Student's t-test or one-way ANOVA analysis of variance with Dunnett's test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. LncRNA JPX was upregulated in ESCC

To explore whether JPX exerted an impact on the progression of ESCC, we first investigated the expression levels of JPX in ESCC tissues and adjacent normal tissues. The results showed that JPX was more highly expressed in ESCC tissues than in adjacent normal tissues (Fig. 1A). The correlation analysis of JPX expression with clinical characteristics revealed that JPX expression was increased when the ESCC advanced (Table S1). These results indicated that JPX might exert an oncogenic lncRNA in ESCC. We then investigated JPX expression in four esophageal cancer cell lines and normal esophageal epithelial cell line. The results showed that the expression levels of JPX was significantly higher in esophageal cancer cells, including EC9706, Eca109, and KYSE150, than normal esophageal epithelial cell line, Het-1A (Fig. 1B). These results suggested that JPX upregulation may promote the progression of ESCC. To further investigate the role of JPX in ESCC, the subcellular distribution of JPX was detected in EC9706 cells. The results showed that JPX was mainly located in the cytoplasm of EC9706 cells (Fig. 1C), which was further confirmed by FISH analysis (Fig. 1D).

3.2. JPX promoted cell proliferation and tumor growth in ESCC

To investigate the biological function of JPX in esophageal cancer cells, JPX was knockdown by JPX siRNA (si-JPX). The knockdown efficiency was confirmed in EC9706 and KYSE150 cells (Fig. 2A). After JPX knockdown for 48h, the EC9706 and KYSE150 cell growth was significantly decreased compared with the control (Fig. 2B). Next, we investigated the effects of JPX knockdown on cell proliferation and cell cycle, which may contribute to inhibit the cell growth. The results showed that the proliferative cells labeled by Edu were significantly decreased 48 h after knockdown of JPX in EC9706 and KYSE150 cells (Figs. 2C and D). The cell cycle analysis demonstrated that inhibiting JPX expression led to cell cycle arrest in G1 phase and reduced the cell proportion in S phase (Fig. 2E), which was consistent with the result of Edu proliferation assays. These results suggested that inhibition of JPX might inhibit ESCC growth by reducing cell proliferating rate and arresting cell cycle. Subsequently, a xenograft model using EC9706 cells injection was used to validate the biological function of JPX *in vivo*. Consistent with the results *in vitro*, JPX overexpression significantly increased tumor weight and tumor volume compared with those in the control group (Fig. 2F). These results indicated that JPX could promote esophageal cancer cell proliferation and and tumor growth in ESCC.

3.3. JPX promoted ESCC mobility *in vitro*

To explore the role of JPX in the motility of esophageal cancer cells, the cell migration and invasion were determined after JPX knockdown or overexpression. The results showed that JPX knockdown significantly impaired the migration and invasion of esophageal cancer cells, whereas JPX overexpression led to a significant increase in cell mobility (Figs. 3A and B). Moreover, the effect of JPX on EMT-related markers was assessed after JPX knockdown or overexpression. The results showed that JPX knockdown could increase the expression of epithelial marker E-cadherin and decrease the expression of mesenchymal markers, including Fibronectin, N-cadherin, and Vimentin (Fig. 3C), whereas overexpression of JPX obviously decreased E-cadherin levels and increased Fibronectin, N-cadherin, and Vimentin levels (Fig. 3D). In addition, the esophageal cancer cells conditioned medium was used to stimulate angiogenesis in HUVECs to evaluate angiogenesis activity *in vitro* [24]. The results showed that the relative length of tubes was decreased following JPX knockdown compared with the control, whereas JPX overexpression led to a significantly elevated tube length (Fig. 3E).

3.4. JPX functions as a sponge of miR-516b-5p in ESCC

To further investigate the mechanism of JPX regulated esophageal cancer cell proliferation and mobility, the potential function of JPX as competing endogenous RNAs and act as a miRNA sponge was speculated for the predominant distribution of JPX in the cell cytoplasm. Subsequently, the tentative miRNAs were predicted by the target prediction program (RegRNA, miRDB, and RNA22) analysis (Fig. 4A). We identified ten overlapped miRNAs as potential direct downstream targets of JPX (Fig. 4A). To determine the functional miRNA targets of JPX, a JPX-specific probe was constructed and used to JPX-overexpressing EC9706 cells. We then purified the JPX-associated RNAs by the specific probe and investigated the ten potential miRNA targets. As a result, miR-516b-5p was significantly enriched by the JPX-specific probe (Fig. 4B), indicating that miR-516b-5p is a potential target of JPX in EC9706 cells. Moreover, the putative binding site of miR-516b-5p and JPX was shown in Fig. 4C. To validate the binding potential, a luciferase reporter assay was performed and the results showed that overexpression of miR-516b-5p with 10 nM concentration significantly reduced the luciferase activity of the JPX wild-type vector but failed to decrease that of the mutant vector (Fig. 4D). The AGO2 immunoprecipitation assay showed that the AGO2 antibody was able to pull down both endogenous JPX and miR-516b-5p (Figs. 4E and F), further validating their binding potential. Moreover, JPX knockdown promoted miR-516b-5p expression in EC9706 and KYSE150 cells (Fig. 4G). In addition, the miR-516b-5p levels were lower in ESCC tissues than in adjacent normal tissues (Fig. 4H), indicating that miR-516b-5p may be negatively associated with JPX in ESCC.

To examine the role of miR-516b-5p in esophageal cancer cell growth and mobility, miR-516b-5p was upregulated by transfection with miR-516b-5p mimics. The transfection efficiency of miR-516b-5p mimics was determined in EC9706 and KYSE150 cells (Fig. 5A). Consistent with the effect of JPX knockdown on cell proliferation, miR-516b-5p overexpression led to a decreased proliferation rate in EC9706 and KYSE150 cells (Fig. 5B). Moreover, miR-516b-5p overexpression reduced cell migration and invasion in

EC9706 and KYSE150 cells (Fig. 5C). More importantly, JPX enhanced cell proliferation, migration and tube length, whereas cotransfection of JPX and miR-516b-5p abolished JPX promoted cell proliferation, migration and tube length in EC9706 cells (Figs. 5D and E), indicating that JPX promoted esophageal cancer cell growth and mobility by sponging miR-516b-5p.

3.5. JPX promoted esophageal cancer cell growth and mobility via miR-516b-5p/VEGFA signaling pathway

To further explore the downstream signaling of miR-516b-5p involved esophageal cancer cell growth and mobility, the target genes was predicted by miRDB and TargetScan databases and we found that VEGFA was a potential target of miR-516b-5p. Upregulated expression of VEGFA was correlated with the progression of ESCC [25], indicating that VEGFA acted as an oncogene in ESCC. Consistently, the VEGFA levels were highly expressed in ESCC tissues compared those in adjacent normal tissues (Fig. 6G). The putative binding site of miR-516b-5p and 3'UTR of VEGFA was shown in Fig. 6A. We found overexpression of miR-516b-5p with 10 nM concentration significantly reduced the luciferase activity of the wild-type 3'UTR of VEGFA vector but failed to decrease that of the mutant 3'UTR of VEGFA vector (Fig. 6B). Moreover, overexpression of miR-516b-5p or JPX knockdown significantly decreased VEGFA expression in EC9706 and KYSE150 cells (Figs. 6C and D). To validate the role of VEGFA in esophageal cancer cells function, VEGFA was downregulated using si-VEGFA transfection. The transfection efficiency of si-VEGFA was determined in EC9706 and KYSE150 cells (Fig. 6E). As a result, downregulation of VEGFA led to a decreased proliferation rate in EC9706 and KYSE150 cells (Fig. 6F). Furthermore, downregulation of VEGFA significantly decreased cell migration and invasion in EC9706 and KYSE150 cells (Fig. 6H). In addition, we found JPX enhanced VEGFA protein expression, whereas cotransfection of JPX and miR-516b-5p suppressed JPX promoted VEGFA protein expression in EC9706 cells (Fig. 6I), indicating that JPX upregulated VEGFA expression via inhibition of miR-516b-5p. Collectively, these results suggested that JPX promoted esophageal cancer cell growth and mobility via miR-516b-5p/VEGFA signaling pathway.

4. Discussion

ESCC is a malignant cancer with poor prognosis and 5-year survival rate owing to the eradication of unresectable, highly recurrence and metastasis rates [26]. Although tumor suppressors and oncogenes have been found to play a vital role in the occurrence and development of esophageal cancer [27, 28], the fundamental molecular mechanism of ESCC carcinogenesis and development has not been fully elucidated. Therefore, understanding the molecular basis of ESCC progression and metastasis will provide effective therapeutic targets, screening and early diagnosis biomarkers of ESCC. Here, we demonstrated that highly expressed JPX promoted the proliferation and mobility of ESCC cells through sponging miR-516b-5p via upregulation of VEGFA expression. Moreover, miR-516b-5p overexpression abrogated JPX enhanced proliferation, migration, invasion and angiogenesis in ESCC cells. Collectively,

our results demonstrate that JPX promotes ESCC progression via the miR-516b-5p/VEGFA pathway, which may serve as a promising novel therapeutic strategy for the treatment of ESCC.

JPX is a nonprotein-coding lncRNA transcribed from a gene within the X-inactivation center with a length of 1696 bp, located on Xq13.2 [29]. A large number of studies have shown that JPX is abnormally expressed in a variety of malignancies, and it acts oncogenic regulator or suppressor relying on the cancer type (18, 20, 30). However, the function role of aberrant JPX expression in ESCC is still unknown. A previous study showed downregulation of JPX was associated with the poor prognosis and overall survival of hepatocellular carcinoma [30]. In contrast, our study demonstrated that a highly expressed JPX level in tissues of ESCC patients and JPX overexpression promoted cell proliferation and mobility in ESCC cells *in vitro* and tumor growth *in vivo*, suggesting that JPX exerts critical function in the progression of ESCC by conferring more aggressive phenotypes on cancer cells. Consistently, upregulation of JPX has been demonstrated to expedite cancer cell proliferation, invasion and metastasis in a variety of cancer types [18–22]. In accordance with the results in ESCC patients, we further found that the expression levels of JPX were significantly increased in various ESCC cell lines compared with the normal esophageal epithelial cell.

To explore the effect of JPX knockdown or overexpression on ESCC cell functions, we testified the JPX role in ESCC cell proliferation, migration, invasion, and angiogenesis. We found upregulation of JPX promoted ESCC cell proliferation, migration and invasion *in vitro* and tumor growth *in vivo*. Inhibition of JPX led to cell cycle arrest in G1 phase, suggested that JPX might promote ESCC growth by facilitating cell cycle. Moreover, inhibition of JPX downregulated EMT-related markers, including Fibronectin, N-cadherin, and Vimentin, whereas overexpression of JPX reversed their expression in ESCC cells, suggested that JPX promoted EMT progression in ESCC cells. In line with these results, Pan J et al. demonstrated that JPX/miR-33a-5p/Twist1 axis activated EMT pathway, which correlated the invasion and metastasis of lung cancer [18]. In addition, overexpression of JPX enhanced ESCC conditioned medium stimulated angiogenesis activity of HUVECs, indicating that JPX promote cell angiogenesis *in vitro*.

A number of studies have proposed that lncRNAs function as ceRNAs to affect tumor occurrence and development by regulating the corresponding gene expression through miRNA mediated pathway [31, 32]. lncRNAs can sponge miRNAs by the same miRNA response elements (MREs), thereby regulating the target genes and ultimately affecting tumor progression [33]. Thus, we analyzed the tentative miRNAs by the RegRNA, miRDB, and RNA22 target prediction program. Ten overlapped miRNAs were identified as potential targets of JPX and miR-516b-5p was a direct target confirmed by RIP and luciferase reporter assays. On the other hand, miR-516b-5p was lowly expressed in ESCC patients, further demonstrated that miR-516b-5p might be negatively associated with JPX in ESCC. Interestingly, overexpression of miR-516b-5p suppressed cell proliferation, invasion, migration, and angiogenesis, while overexpression of miR-516b-5p and JPX abolished JPX enhanced cell proliferation, migration, and angiogenesis *in vitro*. These findings revealed that downregulation of miR-516b-5p was also involved in the occurrence and progression of ESCC, suggested that miR-516b-5p could be used as an indicator for the diagnosis and therapeutic target of ESCC. In addition, VEGFA, a target of miR-516b-5p, was found to be coordinately

highly expressed with JPX in ESCC cells. Previous studies have found that VEGFA was upregulated in various cancers, including non-small cell lung cancer, colorectal cancer, glioma, and breast cancer [34–37]. More importantly, upregulated expression of VEGFA was correlated with the progression of ESCC [25], indicating that VEGFA acted as an oncogene in ESCC. In the present study, we demonstrated that VEGFA levels were highly expressed in ESCC tissues and downregulation of VEGFA could inhibit cell proliferation, migration and invasion in ESCC cells. Moreover, JPX enhanced VEGFA protein expression, whereas cotransfection of JPX and miR-516b-5p suppressed JPX promoted VEGFA protein expression in ESCC cells, indicating that JPX upregulated VEGFA expression via inhibition of miR-516b-5p. These findings indicated that JPX plays an oncogenic role via its interaction with miR-516b-5p/VEGFA pathway.

5. Conclusions

In summary, our results demonstrated that the JPX/miR-516b-5p/VEGFA axis may act as a new ceRNA regulatory network, participating in the angiogenesis and EMT pathway, thereby facilitating the progression of ESCC. These findings suggested that JPX and miR-516b-5p may serve as the potential therapeutic target and the novel biomarkers for the treatment and diagnosis of ESCC.

Abbreviations

LncRNAs: Long non-coding RNAs

ESCC: Esophageal squamous cell carcinoma

FISH: Fluorescence in situ hybridization

qRT-PCR: Quantitative real-time PCR

EdU: 5-Ethynyl-2'-Deoxyuridine

RIP: RNA immunoprecipitation

VEGFA: Vascular endothelial growth factor A

EAC: Esophageal adenocarcinoma

EZH2: Enhancer of zeste homolog 2

LAMC2: Laminin gamma 2

CBP: CREB-binding protein

AJCC: American Joint Committee on Cancer

FBS: Fetal bovine serum

siRNA: Small interfering RNA

NC: Negative control

CT: Cycle threshold

NC membranes: Nitrocellulose membranes

MiRNA: MicroRNA

Bio-labeled: Biotin-labeled

wt: Wild type

mut: Mutant type

Declarations

Funding

This work was supported by grants from Shanghai Municipal Education Commission-Gaofeng Clinical Medicine (No.20181816).

Competing interests

The authors declare no conflict of interest.

Availability of data and material

The datasets used and/or analyzed in the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of the Shanghai Chest Hospital of the Shanghai Jiaotong University, and written informed consent was obtained from each patient included in the study.

Consent for publication

Not applicable.

Authors' contributions

YH and ZL conceived and designed the experiments; YH, BL, YY, and RH performed the experiments and acquired the data; BL and RH analyzed the data; YH drafted the manuscript and ZL revised the

manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

None.

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Figures

Figure 1

LncRNA JPX was upregulated in ESCC. A Expression levels of JPX in 21 ESCC tissues and adjacent normal tissues. B Expression of JPX in normal esophageal epithelial cell line Het-1A, ESCC cell lines (KYSE150, KYSE450, EC109, EC9706). C Expression level of JPX in the subcellular fractions of EC9706 cells. GAPDH and U6 were used as cytoplasmic and nuclear markers, respectively. D The location of JPX (red) in EC9706 cells was determined by FISH assay. Nuclei was stained by DAPI (blue). Scale bar, 50 μ m. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. ** $P < 0.01$, *** $P < 0.001$.

Figure 2

Knockdown of JPX inhibited esophageal cancer cell growth in vitro and in vivo. A Knockdown of JPX expression in EC9706 and KYSE150 using si-JPX. si-JPX#2 was used in the further study for its stable efficiency. B Knockdown of JPX inhibited EC9706 and KYSE150 cell growth in vitro. C-D EdU assays were used to detect the proliferation rate of EC9706 and KYSE150 cells after JPX knockdown. Columns are the average of three independent experiments. Scale bar, 50 μ m. E Cell cycle was determined by flow cytometry analysis. Columns are the percentage of cells in G1, S, and G2 phases, respectively. F Upregulation of JPX promoted esophageal tumor growth in vivo. Tumors developed from JPX transfected EC9706 cells showed significantly increased volume and weights compared with pcDNA3.1 control. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. * P < 0.05, ** P < 0.01.

Figure 3

Knockdown of JPX inhibited esophageal cancer cell mobility in vitro. A Knockdown of JPX inhibited EC9706 cell migration and invasion. Columns are the average of three independent experiments. B Upregulation of JPX promoted EC9706 cell migration and invasion. Columns are the average of three independent experiments. C Knockdown of JPX inhibited expression of EMT-related proteins in EC9706 and KYSE150 cells. D Upregulation of JPX enhanced expression of EMT-related proteins in EC9706 and KYSE150 cells. E Tube formation in HUVECs was inhibited by conditioned medium from EC9706 cells after JPX knockdown and was promoted by that from EC9706 cells after JPX overexpression. Columns are the average of three independent experiments. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. ** $P < 0.01$.

Figure 4

JPX functions as a sponge of miR-516b-5p in ESCC. A Schematic illustration showing the overlapping interaction miRNAs of JPX predicted by RegRNA, miRDB, and RNA22 program analysis. B RIP assays were performed using JPX-overexpressing EC9706 cells with a JPX-specific probe and control oligo probe. Q-PCR assays were performed to analyze potential miRNAs associated with JPX. The enrichment of JPX and potential miRNAs were normalized to that of the control probe. C Wild type (wt) and mutated type (mut) sequences of the putative binding sites between JPX and miR-516b-5p. D Dual-luciferase reporter assays were performed to validate the association of JPX and miR-516b-5p. The luciferase activity in EC9706 cells with or without miR-516b-5p overexpression and transfected with the wt or mut luciferase plasmids. E-F RIP and Q-PCR assays were performed to validate the binding of JPX or miR-516b-5p to the AGO2 protein. G Expression levels of miR-516b-5p in EC9706 and KYSE150 cells after JPX

knockdown. H Expression levels of miR-516b-5p in 21 ESCC tissues and adjacent normal tissues. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

JPX promoted esophageal cancer cell growth and mobility by sponging miR-516b-5p. A Expression levels of miR-516b-5p in EC9706 and KYSE150 cells after miR-516b-5p mimics treatment. B Upregulation of miR-516b-5p inhibited EC9706 and KYSE150 cell proliferation in vitro. C Upregulation of miR-516b-5p

inhibited EC9706 and KYSE150 cell migration and invasion. Columns are the average of three independent experiments. D Cotransfection of JPX and miR-516b-5p inhibited JPX promoted EC9706 cell proliferation. E Cotransfection of JPX and miR-516b-5p reversed JPX promoted EC9706 cell migration and tube formation of HUVECs. Columns are the average of three independent experiments. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. * P < 0.05, ** P < 0.01.

Figure 6

JPX promoted esophageal cancer cell progression by miR-516b-5p/VEGFA axis. A The predicted and the mutated binding sites of miR-516b-5p in the 3'UTR of VEGFA. B Dual-luciferase reporter assays were performed to validate the association of miR-516b-5p and VEGFA. The luciferase activity in EC9706 cells with or without miR-516b-5p overexpression and transfected with the VEGFA wt or mut luciferase plasmids. C-D Expression levels of VEGFA in EC9706 and KYSE150 cells after miR-516b-5p overexpression or JPX knockdown. E Expression levels of VEGFA in EC9706 and KYSE150 using si-VEGFA. si-VEGFA#1 was used in the further study. F Knockdown of VEGFA inhibited EC9706 and KYSE150 cell proliferation in vitro. G Expression levels of miR-516b-5p in 21 ESCC tissues and adjacent normal tissues. H Knockdown of VEGFA inhibited EC9706 and KYSE150 cell migration and invasion. Columns are the average of three independent experiments. I Cotransfection of JPX and miR-516b-5p reversed JPX enhanced VEGFA protein expression in EC9706 and KYSE150 cells. Data were mean $\pm$ SEM. Statistical analyses were performed with Student's t-test or one-way ANOVA followed by Dunnett's test. *P< 0.05, **P< 0.01, ***P< 0.001.

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