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Highly expressed long non-coding RNA FEZF1-AS1 promotes cells proliferation and metastasis through Notch signaling in prostate cancer

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Abstract. – OBJECTIVE: Growing studies indicated that long non-coding RNAs (lncRNAs) acted as imperative players in neoplasms initiation and progression. This research was designed to study the potential involvements of lncRNA FEZF1-AS1 (FEZF1-AS1) in the pathogenesis of prostate cancer (PCa).

PATIENTS AND METHODS: Real-time PCR was performed to detect the expressions of FEZF1-AS1 in PCa specimens and cell lines. Correlations between G- FEZF1-AS1 expressions and clinical characteristics and overall survivals were determined using statistical methods. The CCK-8 assays, colony formation assay, flow cytometry, transwell, and wound scratch assays were carried out to study cells viability, cells migration, and invasion. Western blot and RT-PCR were used for the determination of the influence of FEZF1-AS1 on Notch signaling pathway.

RESULTS: We found that FEZF1-AS1 expressions were distinctly reduced in human PCa tissues and cell lines compared with their non-tumor counterparts, and its higher levels were strongly associated with lymph node metastasis (p=0.012) and Angiolymphatic invasion (p=0.022). Then, Kaplan-Meier assays showed that patients with higher expressions of FEZF1-AS1 were shown to predict unfavorable overall survival. Cox proportional hazards risks assays revealed that FEZF1-AS1 acted as an independent prognostic factor for PCa. Functional investigations suggested that knockdown of FEZF1-AS1 could suppress cells proliferation, trigger late apoptosis, and inhibit cells invasion and migration. Mechanistic assays demonstrated that FEZF1-AS1 exhibited its tumor-promotive roles by activating the Notch signaling pathway.

CONCLUSIONS: We suggested that FEZF1-AS1 served as a tumor promoter in PCa and may develop a novel therapeutic target for PCa patients.

Key Words:
LncRNA FEZF1-AS1, Prognosis, Prostate cancer, Metastasis, Notch signaling pathway.

Introduction

Prostate cancer (PCa) is one of the most common cancers in men worldwide and is the fifth leading cause of cancer-associated death worldwide[1]. There were approximately 272,144 new cases and 28,370 deaths in the USA in 2016[2]. In recent years, the popularization of early prostate-specific antigen (PSA) testing has developed a positive influence on the control of the incidence rates in many countries[3]. However, approximately seventy-five percent of advanced PCa patients are found to develop bone metastases when they are firstly screened, which resulted in unfavorable prognosis in PCa patients[4,5]. Besides, over the past few years, the occurrences of PCa in Asia has increased more quickly than in developed countries[6]. Because of the effective treatments PCa with metastasis, there is an urgent need to explore the molecular mechanisms involved in the metastasis of PCa for the development of novel cancer biomarkers and therapeutic targets.

Most of the transcribed RNAs (95%) are not translated and consist of various classes of non-coding RNAs[7]. Long non-coding RNAs (lncRNAs) are a novel class of transcripts with a length of 210 nucleotides[8]. It has been confirmed that lncRNAs have gained increasing attention due to its potential as a novel and crucial player in the regulation of biological progress, such as cells replication, cytodifferentiation, growth, and apoptosis[9,10]. In addition, several cancer-specific lncRNAs are reported in recent years to be positively involved in the progression of diverse neoplasms progression by the modulation of recurrence and metastasis[11,12]. One case of such a lncRNA is lncRNA NEAT1, a well-studied tumor-related lncRNA which has been frequently reported to act as a new player in the proliferation and metastasis of various tumors by targeting

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several miRNA[13,14]. However, the expression pattern, clinical significance and molecular effects of the overwhelming majority of lncRNAs remain to be clarified.

FEZ family zinc finger 1 antisense RNA 1 (FEZF1-AS1) has been identified as a nuclear-restricted lncRNA and could act as an oncogene in several tumors progression[15-17]. However, the expressions pattern of FEZF1-AS1 and its functions in prostate cancer remains largely unclear. In this study, we attempted to explore the expression and potential effects of FEZF1-AS1 in PCa.

Patients and Methods

Patient Samples

109 paired PC specimens and matched normal samples were collected from PC patients treated at The People's Hospital of Zhu Ji from May 2008 to November 2012. All the procedures were approved by the Ethics Committee of The People's Hospital of Zhu Ji. These tissue samples were and preserved in liquid nitrogen immediately after surgery resection. Written informed consents were acquired from patients before the specimen collection. The patients did not receive preoperative treatment before surgery. The subjects' clinical features were shown in Table I.

Cell Transfection

The cells: WPMY-1 (control cells), 22RV1, PC-3, DU145, and LNCaP cells, were brought from HuiJia Biological company (Xiamen, Fujian, China), and cultured in RPMI-1640 media (containing 10% serum). The incubators containing 5% CO_2 and 37°C were used for cell culture. In addition, Lipofectamine 2000 reagents (HuiJia, Xiamen, Fujian, China) were employed to transfect small interfering RNAs (siRNAs) into cells. In brief, after the cells reached 60% cell confluent, 5 μl siRNAs solution was mixed with 10 μl Lipofectamine 2000 reagents (HuiJia, Xiamen, Fujian, China) in 200 μl binding buffer. After incubation for 25 min, these complexes were added into the cells, and the media was changed 5 h post-transfection. The siRNAs used in this study were brought from STAR Gene Biological company (Wuhan, Hubei, China).

Real-Time PCR Assay

Trizol reagents (Bairui Biotech, Nanjing, Jiangsu, China) were applied to extract the total RNAs. The purity and concentration of RNAs were determined using spectrophotometer (NanoDrop ND-2000, Thermo Fisher Scientific, Waltham, MA, USA). To examine the levels of FEZF1-AS1, p21, Hes1, we first used cDNA synthesis kits (MaiGene, Binhai, Tianjin) according to the protocols in the kits. Then, qPCR SYBR Green kits (ZongHong Biotech, Changzhou, Jiangsu, China) were employed to carry out the qPCR reactions. Condition of experiments for FEZF1-AS1 amplifications was 30 s at 95°C of preliminary denaturation followed by 40 cycles of 95°C for 5 s. The expressing levels of FEZF1-AS1, p21, Hes1, were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data were presented as the means ± SD of three independent experiments. The changes in expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers sequences are as follows: FEZF1-AS1, 5'-TTAGGAGCCTTGTTCTGTGT-3' (forward) and 5'-GCGCATGTACTTAAGAAAGA-3' (reverse); GAPDH, 5'-ACAGTCAGCCGCATCTTCT-3' (forward) and 5'-GACAAGCTTCCCGTTCTCAG-3' (reverse).

Western Blot Analysis

PC-3 and DU145 cells, after treatment, were lysed by RIPA buffer (Junrui Biotech, Taicang, Jiangsu, China). The concentrations of proteins were demonstrated using the Bio-Rad protein assay kit (Limin, Pudong, Shanghai, China). Then, equal amounts of proteins were loaded into the wells of dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (8-10%), followed by transferring to polyvinylidene fluoride (PVDF) membranes. Prior to being probed with primary antibodies, the membranes were blocked in buffer (5% BSA in TBST) for 2.5 h. Next, the primary antibodies targeting Notch1, p21 or Hes1 were applied to incubate with the membranes, respectively. After incubation for 12-16 h, the membranes were washed using TBST buffer and probed with matched secondary antibodies for 2.5 h. Finally, the proteins were detected by ECL kits (YueChen Biotech, Shenzhen, Guangdong, China). The antibodies used in this study were all brought from Haling Biological company (Yangpu, Shanghai, China).

Cell Proliferation Analyses

Cell Counting Kit-8 analyses kits were used to estimate the cellular growth. In short, a total of about 1.5×10^3 PC cells (per well) were placed

Table I. Correlations of FEZF1-AS1 expression with the clinicopathological characteristics of 109 prostate cancer patients.

Variable	Cases	FEZF1-AS1 expression		*p*-value
		High	Low	
Age				0.627
≤ 60	58	30	28	
> 60	51	24	27	
Histological grade				0.582
G1 + G2	62	27	35	
G3	47	27	20	
Pathological stage				0.128
I + II	76	34	42	
III + IV	33	20	13	
Lymph node metastasis				0.012
Negative	89	39	48	
Positive	20	15	5	
Surgical margin status				0.366
Negative	77	36	41	
Positive	32	18	14	
Angiolymphatic invasion				0.022
Negative	72	30	42	
Positive	37	24	13	
Biochemical recurrence				0.186
Negative	77	35	42	
Positive	32	19	13	

into plates (96-well). After attachment, the cells were treated using CCK-8 solution (25 μl/well) at 24 h, 48 h, 72 h, and 96 h. After incubation for 1.5 h, the cellular proliferative curves were recorded at the 450 nm absorbance using a microplate reader apparatus.

Colony Formation Assays

PC-3 and DU145 cells (0.8×10^3 cells/well) were placed into plates (6-well). After culturing for appropriate two weeks, the colonies were visible. At that moment, paraformaldehyde (4%) and crystal violet (0.3%) were used to treat the colonies. After treatment for 25 min, the colonies were washed using PBS, followed by being photographed using a microscope.

Apoptosis Assays

PI/Annexin V-FITC detection kits (BioToo Biotech, Xiamen, Fujian, China) were applied to analyze the cellular apoptosis. In short, after the PC-3 and DU145 cells were treated using FEZF1-AS1 siRNAs for 48-72 h in plates (6-well), they were collected and placed into centrifuging tubes (with 350 μl binding buffer). Afterward, the cells were incubated with Annexin V-FITC (8 μl) and PI (10 μl) for 25 min in the light proof condition. Then, the cells were washed using PBS and analyzed with a flow cytometer.

Caspase 3/9 Activity Assays

For caspase 3/9 activity analyses, we used JiMei caspase 3 or 9 activity detection kits (JiMei, Haidian, Beijing, China). In short, the cells after treatment with FEZF1-AS1 siRNAs were collected by centrifuging (3,500 ×g, 5 min). After washing with PBS, the Lysis Buffer was added into the cells and incubated at 4°C for 20 min. Then, the cell lysates were centrifuged (12,000 ×g, 15 min) and the supernatants were collected. Subsequently, Ac-DEVD-pNA buffer (15 μl) was added into the supernatants. After incubation for 100 min, a microplate reader apparatus was applied to record the OD405 nm absorbance.

Cellular Metastatic Potentials Analyses

The cell invasion and migration were estimated using transwell assays and wound-healing assays, respectively. For transwell assays, we first placed Matrigel-pretreated-Corning transwell inserts (Jindu Biotech, Shenzhen, Guangdong, China) into plates (24-well). Subsequently, we planted 250 μl serum-free media (containing 2.5×10^5 cells/well) into the upper chambers, followed by adding 650 μl media (containing 15% FBS) into the bottom chambers. 36 h later, paraformaldehyde (4%) and crystal violet (0.3%) were used to treat the invasive cells on the bottom surfaces of the inserts. After washing using PBS, the cells were photographed by a microscope. For wound-healing assays, cells

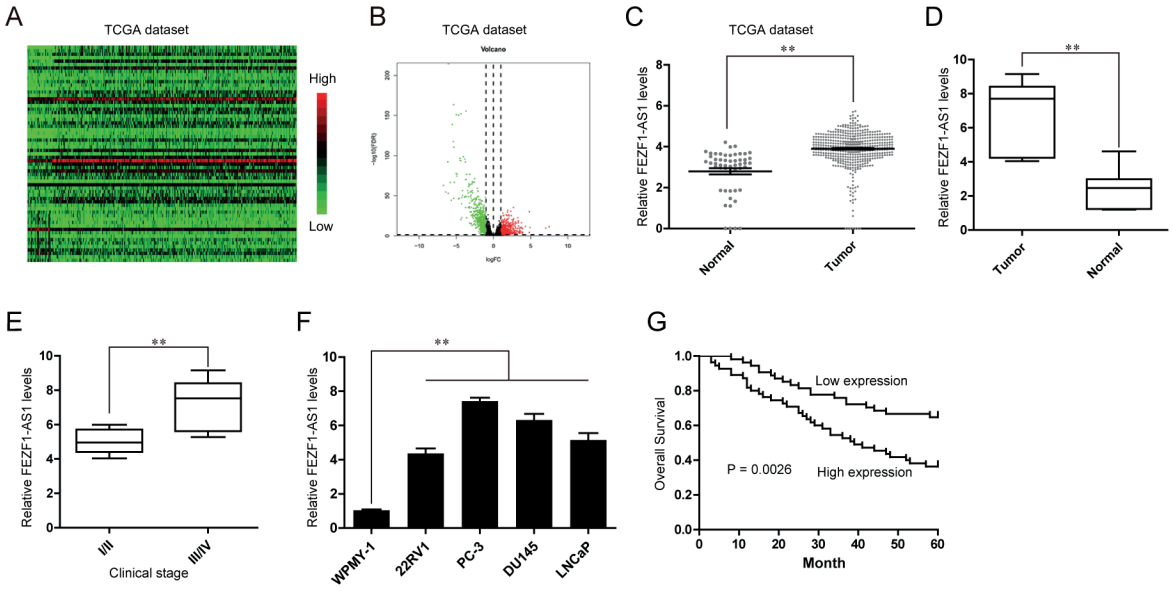

Figure 1. Highly expressed FEZF1-AS1 in PCa were associated with poor prognosis. **A,** Clustering of lncRNAs expression profiling of PCa cases from TCGA datasets. **B,** Aberrant expressions of lncRNAs in PCa tissues from TCGA datasets were shown using the volcano plot. **C,** TCGA cohort assays of the expressions of FEZF1-AS1 in PCa samples and normal prostate tissues. **D,** FEZF1-AS1 was regulated in 109 PCa samples compared with paired normal samples. **E,** Higher levels of FEZF1-AS1 predicted advanced clinical stages. **F,** Expressions of FEZF1-AS1 were determined in four PCa cell lines and normal prostate cell line (WPMY-1). *p<0.05, **p<0.01.

cultured in plates (12-well) reached near 100% confluent, and a 200 µl pipette tip was used to scratching across the cell monolayers. The cell debris was then removed by PBS washing, and the width of the wounds was photographed at 0 h and 48 h after the wounds were generated.

Statistical Analysis

Statistical analyses were conducted by SPSS software (vision, 20.0; SPSS, Inc., Chicago, IL, USA). Survival curves were generated using the Kaplan-Meier methods with the log-rank test). Student's *t*-test was employed for comparisons between groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Univariate and multivariate Cox models were performed for the identification of prognostic factors. A *p*-value of < 0.05 was considered to be statistically significant.

Results

FEZF1-AS1 Is Upregulated in PCa Tissues and Human Cell Lines

To study the expressions pattern of lncRNAs in PCa, microarray data were downloaded from TCGA datasets. Using R software, we showed the most distinctly differentially expressed lncRNAs in PCa tissues which were reflected using heat map (Figure 1A). In addition, our group also displayed aberrant expressions lncRNAs in PCa tissues by performing volcano plot (Figure 1B). Intriguingly, for the data from TCGA datasets, FEZF1-AS1 was observed to be distinctly upregulated in PCa tissues. For the demonstration of above results, we collected tissues samples form 109 PCa patients and performed RT-PCR, finding that the expressions of FEZF1-AS1 in PCa samples were notably increased compared with the matched normal tissues (p<0.01). In addition, the positive associations between FEZF1-AS1 levels and the advanced stages of PCa patients were also confirmed in Figure 1E. Furthermore, the expressions of FEZF1-AS1 were examined in five PCa cell lines by RT-PCR which showed that FEZF1-AS1 expressions were notably reduced in all five PCa cells (p < 0.01, Figure 1F). Overall, our data suggested that FEZF1-AS1 could be involved in PCa progression.

FEZF1-AS1 Expressions Are Conversely Associated With Overall Survival in PCa

For the exploration of the correlations of FEZF1-AS1 levels with clinicopathological fac-

Table II. Univariate and multivariate analyses of prognostic factors in prostate cancer patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
Age	1.214	0.754-1.885	0.246	–	–	–
Histological grade	1.762	0.892-2.432	0.179	–	–	–
Pathological stage	1.545	0.952-2.412	0.134	-	–	–
Lymph node metastasis	3.142	1.325-4.552	0.013	2.809	1.129-4.183	0.017
Surgical margin status	1.424	0.835-1.994	0.155	–	-	–
Angiolymphatic invasion	2.896	1.349-4.892	0.016	2.669	1.249-4.337	0.021
Biochemical recurrence	1.472	0.486-2.217	0.189	–	–	–
FEZF1-AS1 expression	3.104	1.482-4.447	0.009	2.985	1.238-4.015	0.016

tors, FEZF1-AS1 expressions in PCa samples were categorized as high or low according to the median value of FEZF1-AS1 expressions. Then, the results of chi-square test indicated that higher FEZF1-AS1 levels were inversely correlated with lymph node metastasis (p=0.012), angiolymphatic invasion (p=0.022) (Table I). However, no significant correlations between FEZF1-AS1 expressions and other factors were observed (p>0.01). Subsequently, using Kaplan-Meier analysis, we explored the influence of FEZF1-AS1 expressions on overall survival (OS), finding that patients with high level of FEZF1-AS1 exhibited decreased survival times compared with patients with low level of FEZF1-AS1 (p=0.0026). Next, our group carried out Cox proportional-hazards regression to determine the prognostic values of FEZF1-AS1 in PCa patients. In univariate assays, lymph node metastasis, angiolymphatic invasion, and FEZF1-AS1 were detected to be associated with the overall survival of patients (p<0.05). Further findings from multivariate study suggested that that FEZF1-AS1(HR=2.985, 95% CI: 1.238-4.015, p =0.016) was an independent prognostic marker in predicting the overall survival of PCa patients (Table II).

Depletion of FEZF1-AS1 Suppressed Cellular Growth and Promoted Apoptosis of PCA Cells

Considering the levels of FEZF1-AS1 were associated with PCa patients' survival, we next thought to discover the biological roles of FEZF1-AS1 in PCa cellular growth. The levels of FEZF1-AS1 were transiently silenced in PC-3 and DU145 cells with specific siRNAs targeting FEZF1-AS1 (Figure 2A). Subsequently, CCK-8 assays revealed that FEZF1-AS1 depletion remarkably inhibited the proliferation of PC-3 and DU145 cells (Figure 2B). Moreover, clonogenic assays demonstrated that suppression of FEZF1-AS1 markedly depressed the clonogenic capacities of PCa cells (Figure 2C and D). Then, the flow cytometry analyses indicated that the percentage of apoptotic PCa cells were prominently elevated by FEZF1-AS1 silence (Figure 2E). Furthermore, repressing the levels of FEZF1-AS1 significantly resulted in increasing the activities of caspase 3/9 in PC-3 and DU145 cells (Figure 2F). These data revealed that silence of FEZF1-AS1 repressed the proliferation and induced apoptosis of PCa cells *in vitro*.

FEZF1-AS1 Deficiency Impaired the Metastasis of PCa Cells

The influences of FEZF1-AS1 on metastatic potentials of PCa cells were investigated using transwell and wound-healing assays. Transwell assays confirmed that the invaded cell number was markedly reduced after down-regulation of FEZF1-AS1 (Figure 3A). In addition, wound-healing assays demonstrated that repressing the levels of FEZF1-AS1 notably depressed the cellular migratory capabilities of PC-3 and DU145 cells (Figures 3B and C). Therefore, these results indicated that FEZF1-AS1 could suppress the mobility of PC cells.

Depression of FEZF1-AS1 Inhibited the Activity of Notch1 Signaling in PCa Cells

It was well defined that lncRNA exerted its functions by sponging miRNAs or modulating relevant signaling pathways[18]. Hence, to discover the possible molecular mechanisms by which FEZF1-AS1 regulated the development and progression of PCa, we next performed real-time PCR and Western blot analyses to evaluate the expressing changes of relevant signaling pathways. Among these signaling pathways involved in regulating multiple sides of malignant behav-

iors, we focused on Notch1 signaling, because it was closely related to cancerous development and progression. Real-time PCR analyses validated that the mRNA expressing levels of Notch1, p21 and Hes1 were significantly decreased in PC-3 and DU145 cells when their endogenous FEZF1-AS1 was knocked down (Figure 4A and B). Similar results that FEZF1-AS1 siRNAs remarkably recued the levels of Notch1, p21, and Hes1 in PCa cells were also observed when using Western blot analyses (Figure 4C). Hence, these data suggested that FEZF1-AS1 was capable to modulate the activity of Notch1 signaling in PCa cells.

Discussion

Growing studies have suggested the critical effects of epigenetics regulation on tumor growth and neoplasia and lncRNAs attracted increasing attention due to its extensive modulation among various epigenetics mechanisms[19]. In recent years, increasing lncRNAs such as lncRNA AC009014.3, lncRNA HOTAIR, and lncRNA LINP1 have been functionally characterized in the progression of PCa[20-22]. In this research, we explored the expression pattern of FEZF1-AS1 in PCa by analyzing the online data and performing

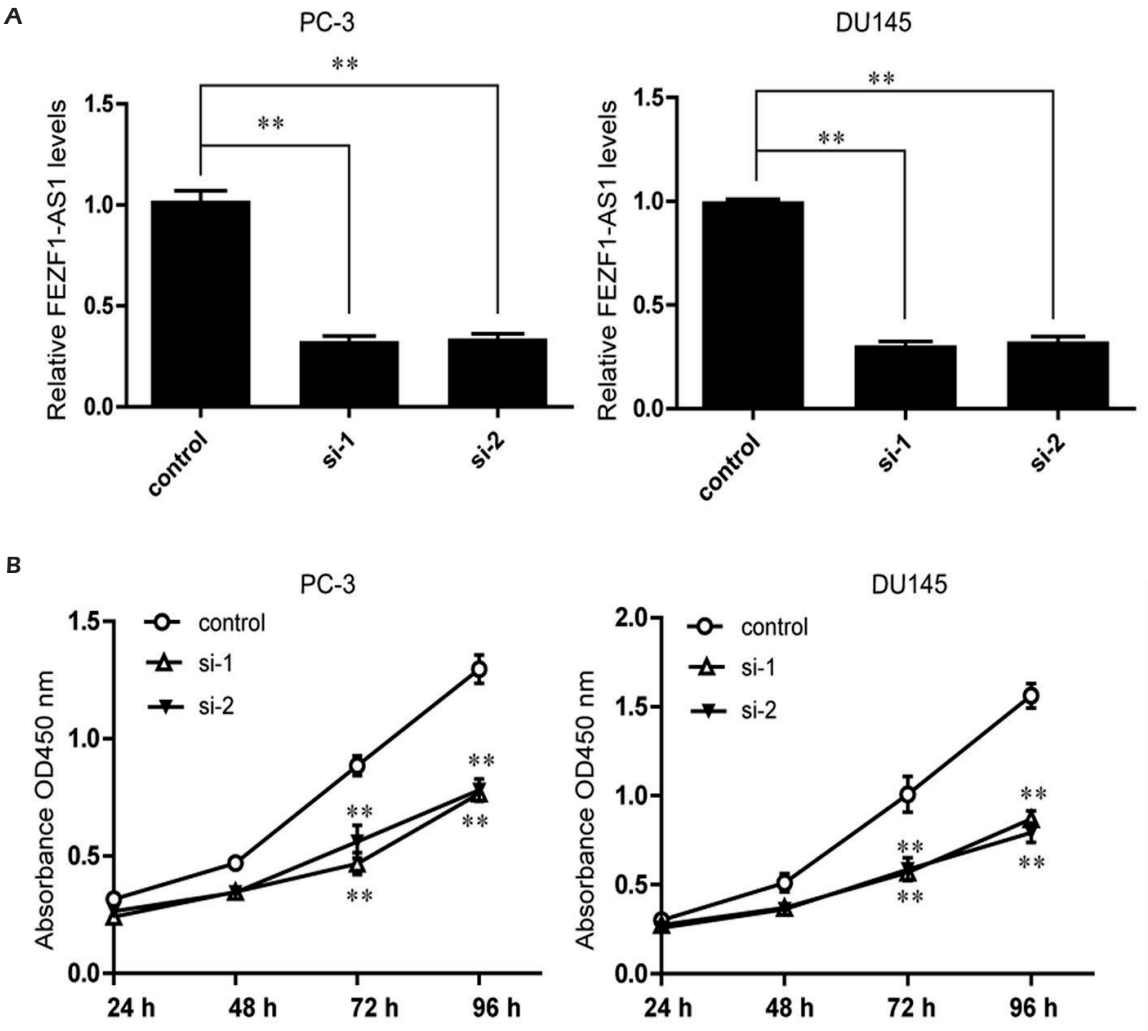

Figure 2. The impact of FEZF1-AS1 on cellular growth and apoptosis. **A,** The knockdown efficiency of FEZF1-AS1 siRNAs (si-1, si-2) was determined by real-time PCR analyses. **B,** The viability of PC-3 and DU145 cells with different treatments were evaluated by CCK-8 assays.

Figure continued

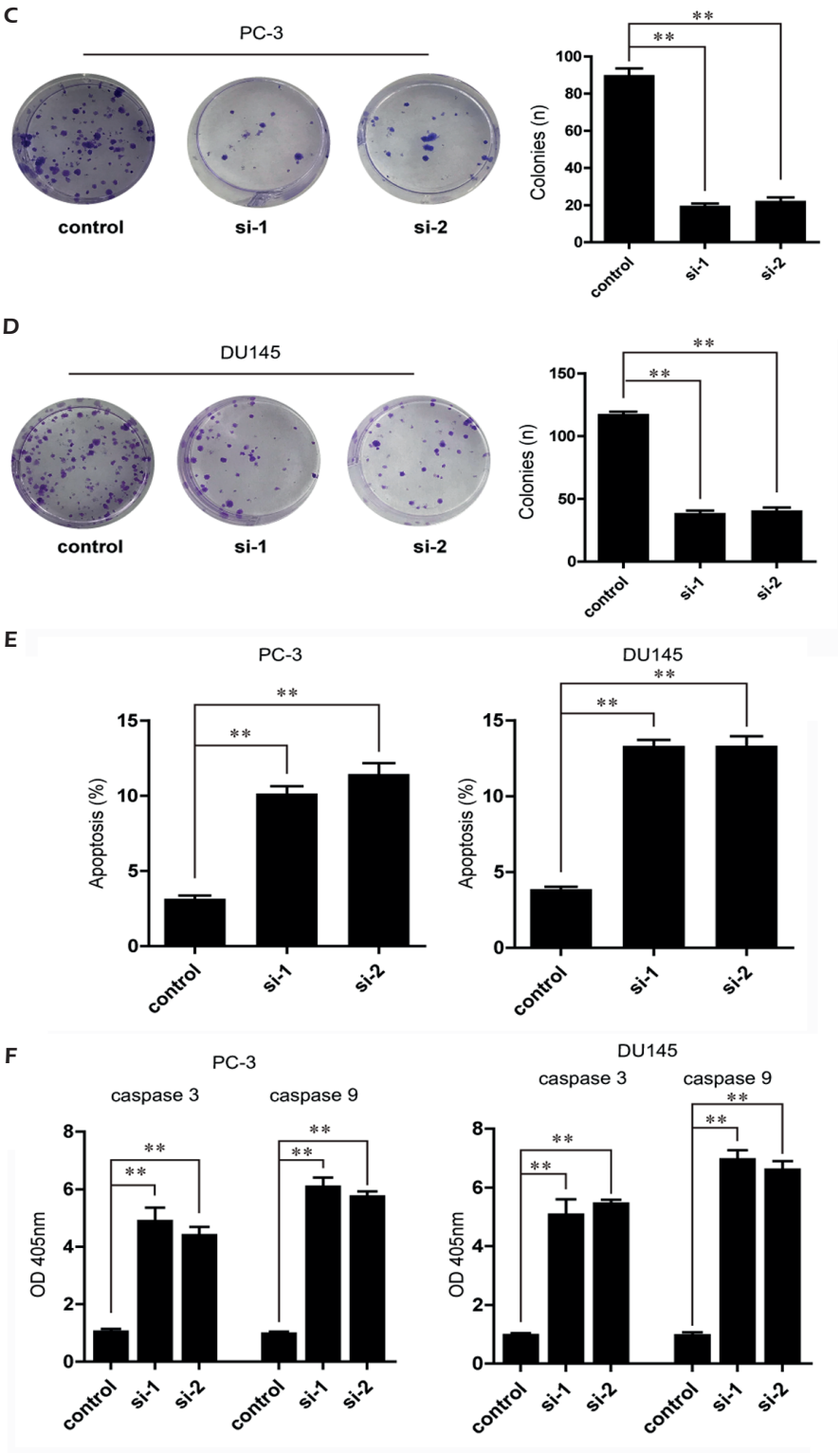

Figure 2. *(Continued).* **C-D,** Clonogenic assays displayed the less colony numbers after the silence of FEZF1-AS1 (Magnification: 10×). **E,** The apoptosis of PC-3 and DU145 cells with different treatment was examined by flow cytometry. **F,** Caspase 3/9 activities were assessed in PC-3 and DU145 cells with different treatment. *$p<0.05$, **$p<0.01$.

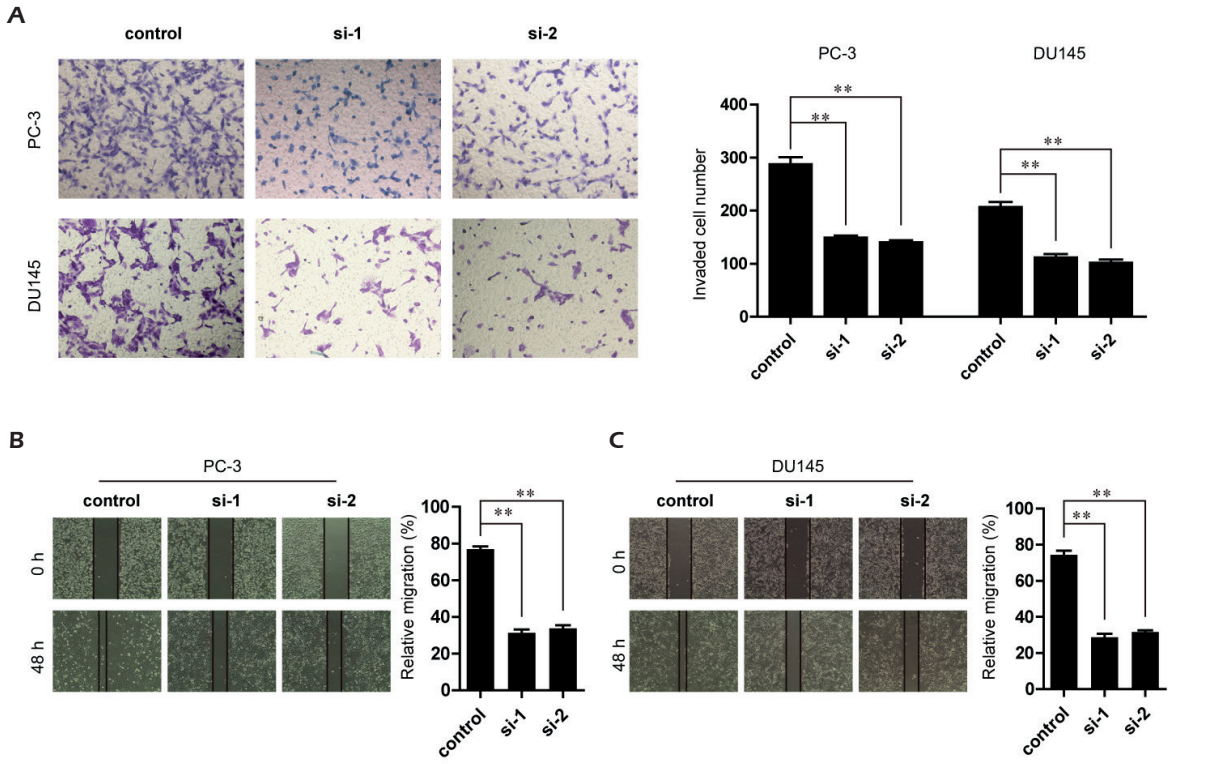

Figure 3. The influence of FEZF1-AS1 on the mobility of PCa cells. **A,** Tumor cell invasion was determined by transwell assays (Magnification: 40×). **B, C,** The wound closures of PC-3 and DU145 cells with different treatment were measured by wound-healing assays (Magnification: 10×). *p<0.05, **p<0.01.

RT-PCR. In addition, validation and functional experiments were also carried out to study the effects of FEZF1-AS1 in the progression of prostate cancer.

Previously, it has been reported that FEZF1-AS1 expression was dysregulated in several different tumors and play a positive regulator in tumor growth and metastasis[16,17]. In this study, with RT-PCR, together with the data from GCTA, we observed frequent upregulation of FEZF1-AS1 in PCa. Higher levels of FEZF1-AS1 were also found in PCa tissues with advanced stages. Clinical research indicated that high FEZF1-AS1 expressions were correlated with lymph node metastasis, angiolymphatic invasion and shorter overall survival, suggesting that FEZF1-AS1 acted as a contributor in clinical progress of PCa. For better understanding of prognostic values of FEZF1-AS1 in PCa patients, multivariate analysis was performed and we found that FEZF1-AS1 played a notable role of independent markers in predication of prognosis of overall survival in PCa patients. Previously, in other studies on the prognostic significance of FEZF1-AS1 were also reported. In general, higher expressions of FEZF1-AS1 predicated positive metastasis, advanced clinical stages and shorter overall survival in several tumors, such as pancreatic cancer, nasopharyngeal carcinoma and breast cancer[15,23,24]. Thus, our data, together with previous findings, suggested that FEZF1-AS1 may be a common marker for tumor patients.

With the system development of surgical operation and adjuvant therapies, some primary malignancies have been well cured. However, metastatic tumors remain to be incurable due to the resistances of circulated cancer cells to existing traditional treatment methods. Recently, lncRNAs have been confirmed to be involved in the progress of tumor metastasis. FEZF1-AS1 which palyed a functional effect in several tumor metastasis was also reported. For instance, FEZF1-AS1 was found to suppress hepatocellular carcinoma cells invasion via JAK2/STAT3 signaling pathways when its expressions were

suppressed[25]. In breast cancer, it was shown that overexpression of FEZF1-AS1 resulted in the suppression of the ability of migration and invasion by modulating miR-30a[24]. In this study, we also performed numerous cells experiments for the examination of FEZF1-AS1 in tumor progress. Our group found that downregulation of FEZF1-AS1 distinctly suppressed PCa cells proliferation, migration, and invasion, and triggered apoptosis by suppressing the levels of caspase 3/9. Our data suggested that FEZF1-AS1 may act as a tumor promoter in PCa.

Notch genes encode several cells exterior receptors to regulate biological progress[26]. Notch signaling has been demonstrated to act as an extensive regulator in the modulation of cells growth, apoptosis, adhesion and transition between epithelia and mesenchymal cells[27,28]. Recently, numerous studies showed the regulator effects of lncRNAs on the progress of metastasis of various tumors. For instance, Liu et al[29] reported that overexpression of lncRNA HNF1A-AS1 which was modulated by STAT3 promoted_the proliferation and migration of oral squamous cell carcinoma cells through the regulation of Notch signaling pathways. Cui et al[30] indicated that suppressing the levels of lncRNA SNHG1 inhibited pancreatic cancer cells growth and migration through the inhibition of the Notch-1 signaling pathways. However, the studies on the influence of lncRNAs in PCa are limited. In this study, our group tried to explore whether FEZF1-AS1 promoted PCa progress by activating Notch signaling. Using si-FEZF1-AS1, we built FEZF1-AS1-knockdown PCa cells. Then the results of RT-PCR and Western blot assays suggested that knockdown of FEZF1-AS1 in PCa cells resulted in the inhibition of Nothch1, p21 and Hes1 levels in both mRNA and protein level. These findings revealed that FEZF1-AS1 displayed its roles of tumor promoter in PCa by modulating Notch signaling.

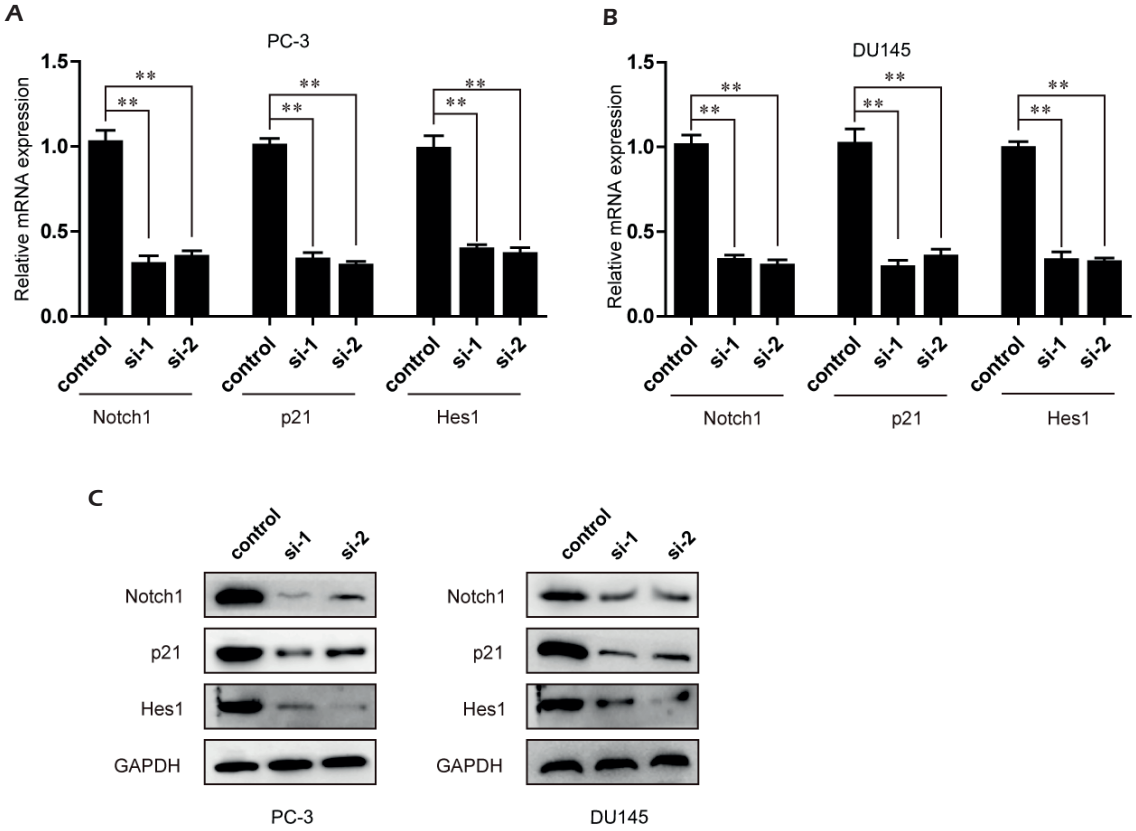

Figure 4. FEZF1-AS1 knockdown suppressed the activity of Notch1 signaling pathway PCa cells. **A-B,** The real-time PCR analyses were conducted to measure the mRNA levels of Notch1, p21 and Hes1 in PC-3 and DU145 cells with different treatment. **C,** Western blot analyses detected the protein levels of Notch1, p21 and Hes1. *$p<0.05$, **$p<0.01$.

Conclusions

We firstly showed that FEZF1-AS1 expressions were distinctly downregulated in PCa and correlated with several clinical pathologies. Besides, detection of FEZF1-AS1 levels could be used for the prediction of the clinical outcome of PCa patients. Further *in vitro* assays showed that FEZF1-AS1 promotes cells proliferation and metastasis, and reduces apoptosis of PCa cells, probably via the modulation of Notch signaling.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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