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LncRNA RP11-567G11.1 accelerates the proliferation and invasion of renal cell carcinoma through activating the Notch pathway

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Abstract. – OBJECTIVE: In recent years, the death number of renal cell carcinoma (RCC) has been enhanced annually. The crucial function of long non-coding RNA (lncRNA) in the occurrence and progression of cancer is of great significance. However, the specific role of lncRNAs in the pathogenesis and prognosis of RCC has not been fully elucidated. Therefore, the aim of this study was to uncover the role of lncRNA RP11-567G11.1 in regulating the progression of RCC.

PATIENTS AND METHODS: Relative expression level of RP11-567G11.1 in RCC tissues and cells was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The influences of RP11-567G11.1 on proliferative and invasive abilities of RCC cells were assessed. Subsequently, regulatory effects of RP11-567G11.1 on the viability and apoptosis of DDP-induced RCC cells were examined. Furthermore, the mRNA and protein levels of Notch pathway-related genes Jagged1/HES5/HEY1 in RCC were detected by qRT-PCR and Western blot, respectively.

RESULTS: RP11-567G11.1 expression was significantly up-regulated in RCC tissues and cells. Meanwhile, RP11-567G11.1 was highly expressed in RCC patients with advanced stage. Knockdown of RP11-567G11.1 significantly attenuated proliferative and invasive abilities of 786-O and 769-P cells. Silence of RP11-567G11.1 attenuated viability, while it induced apoptosis in DDP-induced RCC cells. In addition, knockdown of RP11-567G11.1 remarkably down-regulated both mRNA and protein levels of Jagged1, HES5, and HEY1 in RCC.

CONCLUSIONS: RP11-567G11.1 accelerates the proliferative and invasive abilities of RCC through activating the Notch pathway. Our findings suggest that it may be a new therapeutic target for RCC.

Key Words:
Renal cell carcinoma (RCC), RP11-567G11.1, Notch pathway.

Introduction

Kidney cancer, also called renal cell carcinoma (RCC), mainly originates from renal tubular epithelial cells. In adults, kidney cancer originates from renal parenchyma and renal pelvis, and most of them are adenocarcinoma from renal parenchyma[1]. At present, the incidence of RCC accounts for 2%-3% in all malignant tumors. As a common cancer in the urinary system, RCC can eventually lead to death if not effectively controlled. The pathogenesis of RCC is complex, involving genetic mutations, genomic instability, epigenetic changes, as well as abnormally expressed encoding and non-coding genes. In recent years, the incidence of RCC is still on the rise. Frequent recurrence and numerous side effects of RCC are the difficulties in its treatment[2]. Hence, it is urgent to uncover the molecular mechanism and to develop new therapeutic targets for RCC.

Long non-coding RNA (lncRNA) is a type of non-coding RNAs with more than 200 nucleotides in length, which is widely distributed in the genome[3]. With no protein-coding function, lncRNA is capable of regulating gene expressions at epigenetic and transcriptional levels by binding to miRNAs, chromatin modifications or genomic imprinting[4]. Melissari and Grote[5] have shown that lncRNA is involved in regulating multiple cellular behaviors and disease progression. LncRNA has been considered[6] to be the initiator for tumor cell differentiation, proliferation, and metastasis. Differentially expressed lncRNAs in tumors/non-tumor tissues are well concerned[7]. Huang et al[8] have reported that lncRNA RP11-567G11.1 is up-regulated in pancreatic cancer, which mediates the proliferative and invasive abilities of tumor cells. However, the potential function of RP11-567G11.1 in RCC has not been fully elucidated.

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In this paper, we first examined the expression level of RP11-567G11.1 in RCC tissues and cells. The potential role of RP11-567G11.1 in regulating cellular behaviors of RCC and DDP-resistance was further explored. Our findings identified that RP11-567G11.1 could regulate Notch pathway in RCC, providing a novel direction in the pathogenesis of RCC.

Patients and Methods

Sample Collection

RCC tissues and para-cancerous tissues were harvested from 48 RCC patients during the surgery. None of these patients received pre-operative treatment, and collected samples were post-operatively diagnosed. This investigation was approved by the Medical Ethics Committee. Informed consent was obtained from each subject before the study. This research was conducted in accordance with the Declaration of Helsinki.

Cell Culture

RCC cell lines (786-O and 769-P) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), and renal tubular epithelial cell line (HK-2) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. The cell growth was regularly observed.

Cell Transfection

Cells were pre-seeded into 6-well plates at 60% of confluence. Cell transfection was conducted according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 6 h, the medium containing 10% FBS was replaced. Transfected cells for 24 h were harvested for subsequent functional experiments. The shRNA-RP11-567G11.1 sequence was 5′-AAGAGGCTTGCCTAAAGTTA-3′, which was constructed by GenePharma (Shanghai, China).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells and tissues was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into cDNA according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer. QRT-PCR was then performed in accordance with SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) with the following conditions: at 94°C for 5 min, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. Relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates at a density of 2.0×10^3 cells per well. At appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well, followed by incubation in the dark for 2 h Absorbance at 450 nm of each sample was measured by a micro-plate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were first inoculated into 96-well plates with 1×10^5 cells per well. Then, the cells were labeled with 100 μL of EdU reagent (50 μM) per well for 2 h. After washing with phosphate-buffered saline (PBS), the cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. Next, the cells were washed with PBS once, and stained with 1× Hoechst33342 in the dark for 30 min. EdU-positive ratio was finally determined under a fluorescent microscope.

Table I. The sequences related to the study.

Gene	Sequences
RP11-567G11.1 F:	TGGAAAACCAGGATAGTTGAGT
RP11-567G11.1 R	ATCCCTTACAGACCCTTTATTG
Jagged1 F	AATAGGACGGGCTGACATGC
Jagged1 R	GTCTCCGTACATGCACTGCT
HES5 F	GCACAGCAAAGCCTTCGTC
HES5 R	GTCCGGGGTGATCACTGTTT
U6 F	CTCGCTTCGGCAGCAGCACATATA
U6 R	AAATATGGAACGCTTCACGA
HEY1 F	GCTCTCTGCGGATTGAGCTA
HEY1 R	GATAACGCGCAACTTCTGCC
β-actin F	CGGTATCGTTCTGGACTCCG
β-actin R	GCGGTGGTGGTGAAAGAGTA

Transwell Assay

Cell density was first adjusted to 3×10^5 cells/ml. 100 μL of cell suspension was applied in the upper side of transwell chamber (Corning, Corning, NY, USA) coated with Matrigel. Meanwhile, 600 μL of medium containing 20% FBS was applied in the bottom side. After 48 h of incubation, cells penetrated to the bottom side were fixed in 4% paraformaldehyde for 20 min and stained with crystal violet for 20 min. Migratory cells were observed under a microscope, and the number of migratory cells was counted (magnification 200×). 5 fields of view were randomly selected for each sample.

Flow Cytometry

Cells were washed with PBS twice and suspended in binding buffer. Subsequently, the cells were incubated with 5 μL of AnnexinV-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) at room temperature in the dark. Apoptotic rate was finally examined by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA). Protein concentration was quantified by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 2 h, the membranes were incubated with primary and secondary antibodies. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA, USA) was used for statistical analysis. Experimental data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). Inter-group data were compared using the *t*-test. $p<0.05$ was considered statistically significant.

Results

RP11-567G11.1 Was Upregulated in RCC

A total of 48 pairs of RCC tissues and para-cancerous tissues were collected for detecting the expression level of RP11-567G11.1. QRT-PCR results revealed that RP11-567G11.1 expression was significantly up-regulated in RCC tissues (Figure 1A). Identically, RP11-567G11.1 was highly expressed in RCC cell lines relative to renal tubular epithelial cell line (Figure 1B). According to the TNM staging, RCC patients were divided into two groups, including T1+T2 group (n=16) and T3+T4 group (n=16). RP11-567G11.1 level increased gradually with the worsening of TNM staging of RCC (Figure 1C). The above data demonstrated the involvement of RP11-567G11.1 in the progression of RCC.

Silence of RP11-567G11.1 Suppressed the Proliferative and Invasive Abilities of RCC

Transfection of shRNA-RP11-567G11.1 in 786-O and 769-P cells sufficiently down-regulated RP11-567G11.1 level (Figure 2A). Knockdown of RP11-567G11.1 markedly reduced the viability of RCC cells (Figure 2B). EdU assay showed significantly lower EdU-positive ratio in 786-O and 769-P cells transfected with shRNA-RP11-567G11.1 relative to controls, suggesting inhibited proliferative ability (Figure 2C). Besides, transwell assay indicated a significantly decreased number of invasive cells after transfection of shRNA-RP11-567G11.1 (Figure 2D). As a result, knockdown of RP11-567G11.1 attenuated the proliferative and invasive abilities of RCC.

Silence of RP11-567G11.1 Enhanced DDP-Induced Apoptosis in RCC

Cisplatin (DDP) is commonly applied in the chemotherapy for RCC. In DDP-induced RCC cells, transfection of shRNA-RP11-567G11.1 down-regulated RP11-567G11.1 expression level (Figure 3A). CCK-8 assay revealed significantly declined viability of DDP-induced RCC cells transfected with shRNA-RP11-567G11.1 relative to those transfected with scramble (Figure 3B). Moreover, silence of RP11-567G11.1 remarkably elevated the apoptotic rate in DDP-induced 786-O and 769-P cells (Figure 3C).

RP11-567G11.1 Regulated the Notch Pathway in RCC

The Notch pathway has been indicated of great importance in influencing tumor progression. To uncover the potential role of the Notch pathway in the progression of RCC mediated by RP11-567G11.1, relative expression levels of Jag-

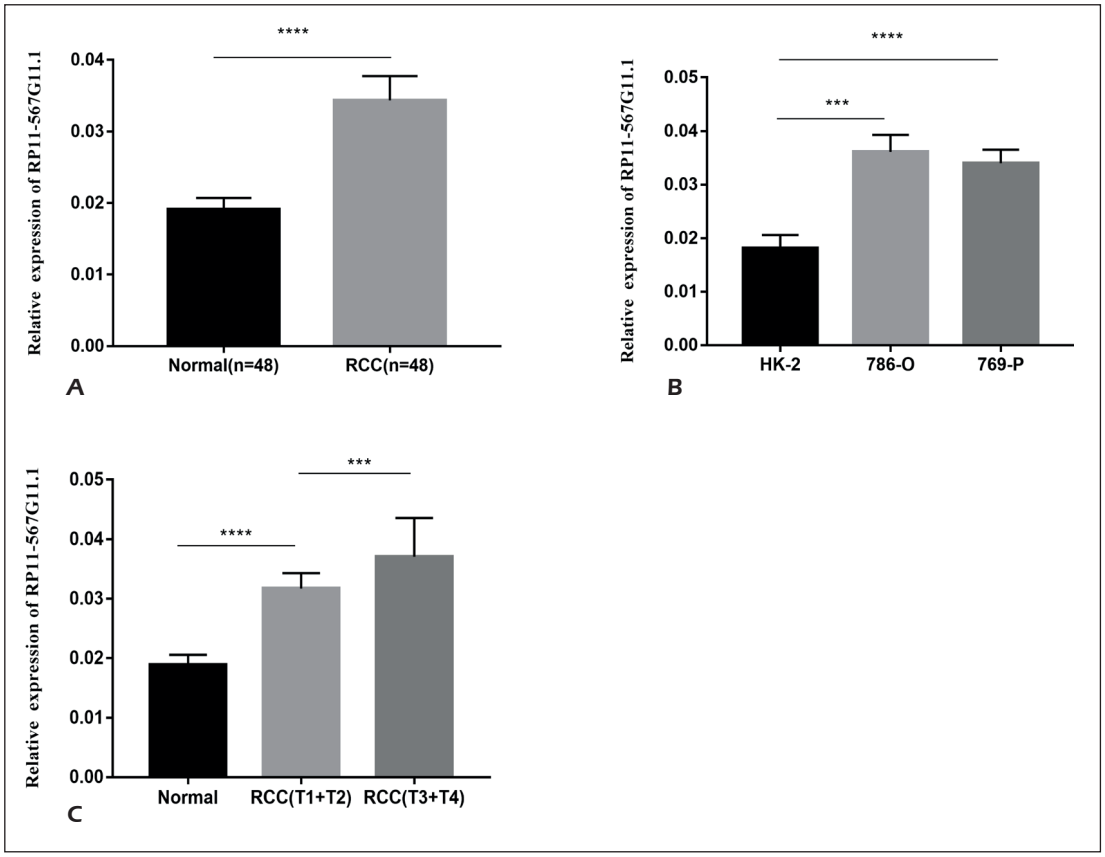

Figure 1. RP11-567G11.1 was up-regulated in RCC tissues and cells. **A**, Relative level of RP11-567G11.1 in RCC tissues and para-cancerous tissues. **B**, Relative level of RP11-567G11.1 in HK-2, 786-O and 769-P cells. **C**, Relative level of RP11-567G11.1 in para-cancerous tissues, RCC tissues in T1+T2 stage and those in T3+T4 stage.

ged1, HES5, and HEY1 were then determined. In 786-O and 769-P cells transfected with shRNA-RP11-567G11.1, the mRNA levels of Jagged1, HES5, and HEY1 were markedly down-regulated (Figure 4A). Similarly, the protein levels of these molecules also decreased significantly after silence of RP11-567G11.1 (Figure 4B). All the above findings suggested that RP11-567G11.1 accelerated the progression of RCC by activating the Notch pathway.

Discussion

RCC is one of the most common malignant tumors of the genitourinary system, accounting for about 3% of adult systemic malignancies. Globally, the incidence of RCC continues to grow at a rate of 2% to 3% every ten years[9]. RCC is the number one killer for urinary tumor-related deaths. There are many risk factors for RCC, such as smoking, obesity, and underlying diseases (i.e., kidney cysts)[10]. With an insidious onset, there is still a lack of evident clinical signs of RCC in the early stage. Fever and elevated body temperature only occur in a small number of RCC patients. In general, these patients have already aggravated into middle or advanced stage at the initial diagnosis. Treatments for RCC, including surgery, radiotherapy, chemotherapy, and dialysis, pose a great pain. Meanwhile, their therapeutic outcomes are still far from satisfactory. Currently, the pathogenesis of RCC has not been fully elucidated. Therefore, it is necessary to develop effective prevention, diagnosis, and treatment approaches for RCC.

LncRNA was initially considered as a transcriptional byproduct of RNA polymerase II that could not encode proteins and did not have biological functions. Therefore, it has been called "dark matter" by scholars[11]. Recent investigations have shown that most lncRNAs exhibit significant spatial and temporal expression specificities during the proliferation and differentiation of cells and tissues.

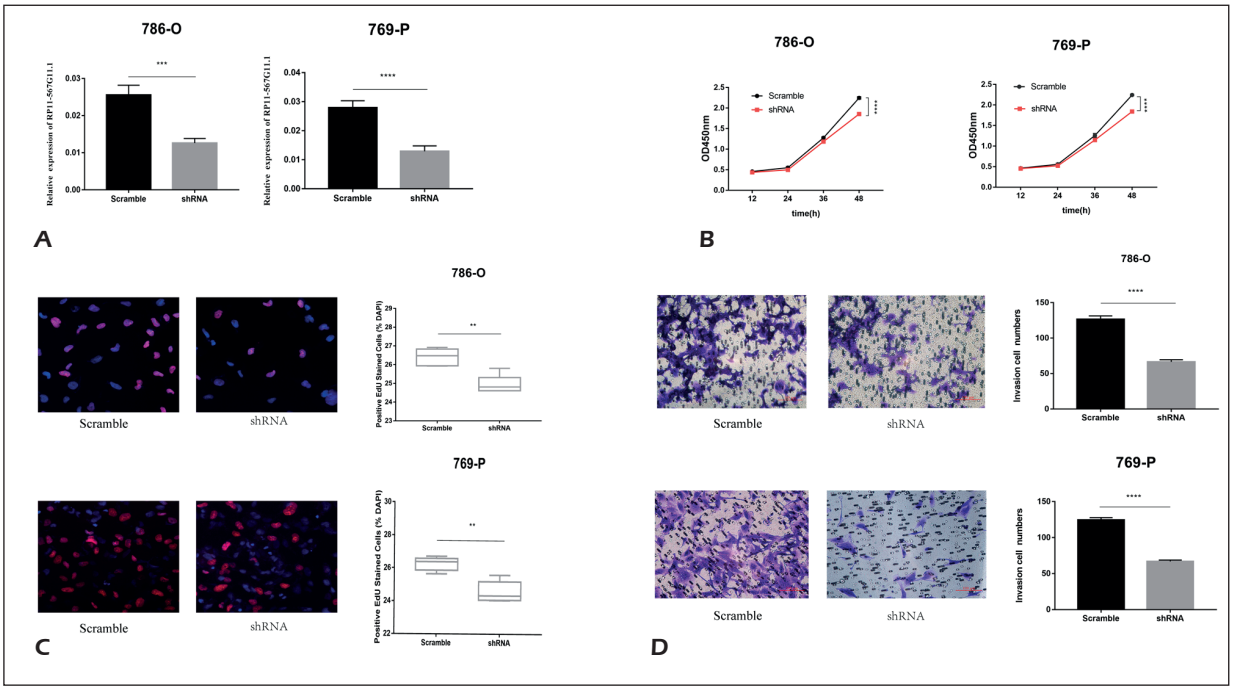

Figure 2. Silence of RP11-567G11.1 suppressed the proliferative and invasive abilities of RCC. **A**, Transfection efficacy of shRNA-RP11-567G11.1 in 786-O and 769-P cells. **B**, Viability in 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1. **C**, EdU-positive ratio in 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1 (magnification:400×). **D**, Number of invasive 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1 (magnification: 40×).

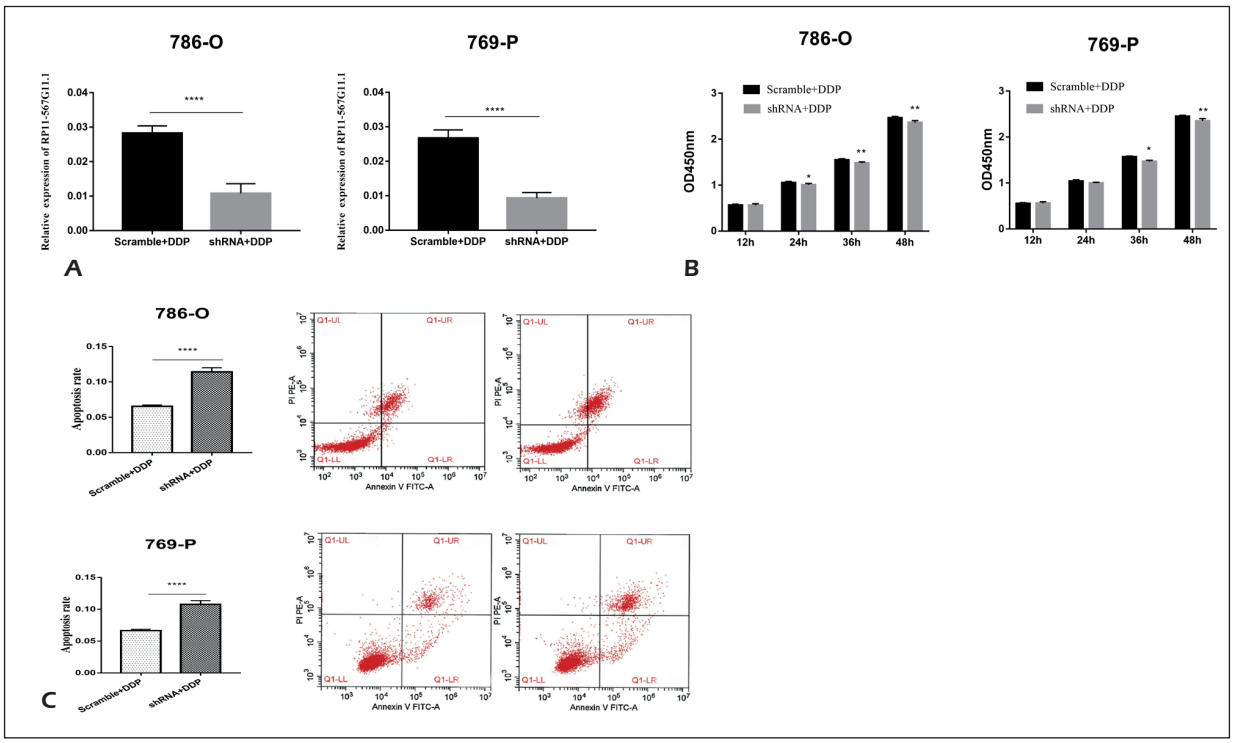

Figure 3. Silence of RP11-567G11.1 enhanced DDP-induced apoptosis in RCC. **A**, Relative level of RP11-567G11.1 in 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1. **B**, Viability in DDP-induced 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1. **C**, Apoptotic rate in DDP-induced 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1.

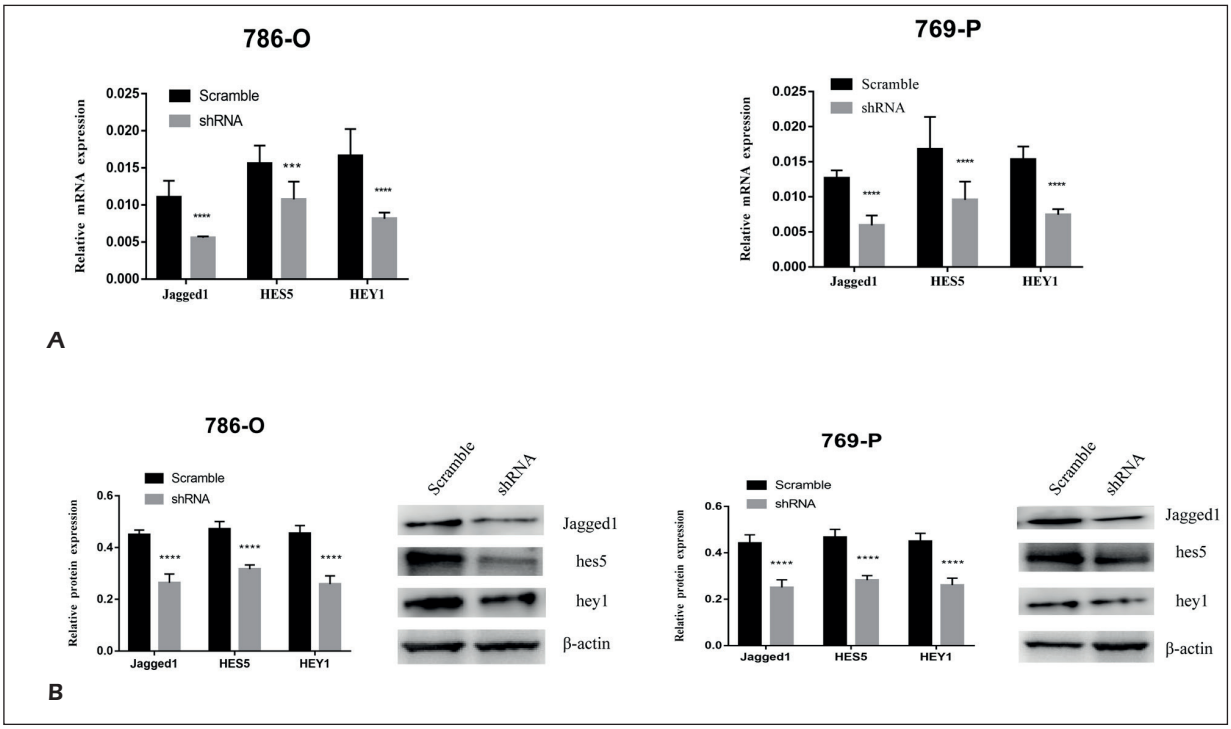

Figure 4. RP11-567G11.1 regulated the Notch pathway in RCC. **A**, MRNA levels of Jagged1, HES5, and HEY1 in 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1. **B**, Protein levels of Jagged1, HES5 and HEY1 in 786-O and 769-P cells transfected with scramble or shRNA-RP11-567G11.1.

LncRNA participates in multiple biological processes by chromatin modification, chromosome silence, transcription intervene, and intracellular transportation[12]. Several researches have identified the important role of lncRNAs in malignant tumors. These certain lncRNAs serve as oncogenes or tumor suppressors. Consistently, lncRNA HOTAIR[13] up-regulates histone demethylase JM-JD3 and down-regulates histone methyltransferase EZH2, thereby exerting a carcinogenic effect by regulating the methylation of histones at different loci. LncRNA TP73-AS1[14] sponges miR-449a to promote EZH2 expression, eventually accelerating the invasion and metastasis of lung cancer cells. In a variety of tumor cells, lncRNA MEG3[15] serves as a tumor suppressor gene by up-regulating p53.

Huang et al[8] have reported the carcinogenic role of RP11-567G11.1 in pancreatic cancer. In this study, we mainly explored the biological function of RP11-567G11.1 in RCC and the underlying mechanism. First of all, RP11-567G11.1 level was found significantly up-regulated in both RCC tissues and cell lines. This indicated the potential involvement of RP11-567G11.1 in the progression of RCC. *In vitro* experiments illustrated that RP11-567G11.1 promoted the proliferative and invasive abilities of RCC cells. In addition, silence of RP11-567G11.1 greatly enhanced the sensitivity of apoptosis in DDP-induced RCC cells.

Tumor growth, recurrence, and metastasis are closely correlated with tumor angiogenesis. Multiple factors are involved in the regulation of tumor angiogenesis, including mTOR pathway, VEGF, and fibroblast growth factor[16]. Ran et al[17] have reported that the Notch pathway is of great significance in tumor angiogenesis. Abnormally activation of the Notch pathway can be observed in many types of tumors[18-21]. Thus, we speculated that the Notch pathway was involved in the progression of RCC influenced by RP11-567G11.1. Here, we examined the expressions of key genes in the Notch pathway, including Jagged1, HES5, and HEY1. Both mRNA and protein levels of these molecules were significantly down-regulated in RCC cells with RP11-567G11.1 knockdown. These findings suggested that RP11-567G11.1 activated the Notch pathway in RCC.

Conclusions

RP11-567G11.1 is up-regulated in RCC tissues and cells, which accelerates the proliferative and invasive abilities of RCC by activating the Notch pathway. Our findings suggest that it may be a new therapeutic target for RCC.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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