

## Original Article

# High expression of long non-coding RNA SPRY4-IT1 predicts poor prognosis of clear cell renal cell carcinoma

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**Abstract:** Introduction: Long non-coding RNAs (lncRNAs) play a key role in cellular processes, such as cell growth, apoptosis, and carcinogenesis. lncRNAs SPRY4-IT1 has recently been identified to be involved in tumorigenesis of several cancers such as non-small cell lung cancer and esophageal squamous cell carcinoma. However, the role of SPRY4-IT1 in clear cell renal cell carcinoma (ccRCC) remains unclear. Methods: The expression of SPRY4-IT1 was examined in ccRCC patients and renal cancer cell lines by using quantitative real-time PCR (qRT-PCR). The relationship between SPRY4-IT1 level and clinicopathological parameters of ccRCC was analyzed with the Kaplan-Meier method and Cox proportional hazards model. Small interfering RNA (siRNA) was used to suppress SPRY4-IT1 expression in renal cancer cell line 786-O. In vitro assays were performed to further explore its role in renal cancer progression. Results: The relative level of SPRY4-IT1 was significantly higher in ccRCC tissues compared to the adjacent normal renal tissues. And higher expression of SPRY4-IT1 was found in renal cancer cell lines compared with the normal human proximal tubule epithelial cell line HK-2. The ccRCC patients with higher SPRY4-IT1 expression had an advanced clinical stage and poorer prognosis than those with lower SPRY4-IT1 expression. Multivariate analyses by Cox's proportional hazard model revealed that expression of SPRY4-IT1 was an independent prognostic factor in ccRCC. In vitro assays, our results indicated that knockdown of SPRY4-IT1 reduced renal cancer cell proliferation, migration, and invasion. Conclusions: Our data suggested that lncRNA SPRY4-IT1 might be considered as a potential prognostic indicator and a potential target for therapeutic intervention in RC.

**Keywords:** Long non-coding RNAs, SPRY4-IT1, clear cell renal cell carcinoma, prognosis

## Introduction

Renal cell carcinoma (RCC) is one of the most lethal genitourinary neoplasms with an incidence of approximately 5-10 per 100,000 and accounts for 2-3% of all cancers [1]. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC, responsible for approximately 75% of cases [2]. What is worse, the incidence and mortality rates of RCC all over the world are rising each decade. Apart from surgery, it is both chemotherapy and radiotherapy resistant [3]. The 5-year survival rate of RCC is estimated to be approximately 55%, and that of metastatic RCC is approximately 10% [4]. Although many genetic and epigenetic changes are found to be correlated with RCC, the carcinogenesis remains poorly understood.

Therefore, it is necessary to identify a new sensitive, reliable biomarkers enabling prediction of early metastasis and to develop a new targeted therapies for RC.

The rapid development of RNA genomics has highlighted the role of non-coding RNAs (ncRNAs) in post-transcriptional regulation human tumors. ncRNAs are collectively divided into three categories: housekeeping RNAs, small non-coding RNAs, and long non-coding RNAs [5]. Long non-coding RNAs (lncRNAs) is an RNA molecule that is longer than 200 nucleotides and is not translated into a protein [6]. Although these long non-coding transcripts were once considered to be simply transcriptional "noise" or cloning artifacts [7]. Recent evidences showed that lncRNAs play important

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**Table 1.** Correlation between SPRY4-IT1 expression and clinicopathologic features in patients with ccRCC

Parameters	Group	Total	SPRY4-IT1 expression		P value
			High	Low	
Gender	Male	54	29	25	0.888
	Female	44	23	21	
Age (years)	< 65	53	27	26	0.648
	≥ 65	45	25	20	
Tumor size (cm)	< 4 cm	61	32	29	0.878
	≥ 4 cm	37	20	17	
Histological grade	I-II	63	26	37	0.002
	III-IV	37	26	9	
Tumor stage	T <sub>1</sub> -T <sub>2</sub>	58	22	36	0.000
	T <sub>3</sub> -T <sub>4</sub>	40	30	10	
Lymph nodes metastasis	Absence	84	39	45	0.001
	Presence	14	13	1	
Distant metastasis	Absence	82	38	44	0.003
	Presence	16	14	2	

roles in diverse biological processes, such as transcriptional regulation, cell growth and tumorigenesis [8]. For example, The HOTAIR lncRNA which is known to regulate the expression of HOX gene clusters, is significantly up-regulated in non-small cell lung cancer tissues, and regulate non-small cell lung cancer cell invasion and metastasis, partially via the down-regulation of HOXA5. Thus HOTAIR may represent a new marker of poor prognosis and was a potential therapeutic target for non-small cell lung cancer intervention [9]. The MALAT-1 lncRNA is increased in prostate cancer and higher MALAT-1 expression is correlated with Gleason score, prostate specific antigen, tumor stage and castration resistant prostate cancer, moreover down-regulation of MALAT-1 can inhibit prostate cancer cell growth, invasion and migration [10]. The TUG1 lncRNA is emerging as a novel player in the disease state of bladder urothelial carcinoma and may have potential roles as a biomarker and a therapeutic target in bladder urothelial carcinoma [11]. Unfortunately, the emerging functional role of lncRNAs in RCC remains largely unknown.

lncRNA SPRY4-IT1 is a 687 nt unspliced polyadenylated transcript originally identified in adipose tissue and is transcribed from the second intron of the SPRY4 gene. SPRY4-IT1 is up-regulated and knock-down its expression resulted in defects in cell growth, invasion, and elevated rates of apoptosis in melanoma cells [12]. Xie et al. showed that esophageal squamous cell

carcinoma patients with higher SPRY4-IT1 expression had an advanced clinical stage and poorer prognosis than those with lower SPRY4-IT1 expression, and knockdown of SPRY4-IT1 reduced cell proliferation, invasiveness, and migration [13]. However, the lncRNA SPRY4-IT1 expression in RCC and underlying mechanism is still unclear.

In the present study, we aimed to investigate the expression of lncRNA SPRY4-IT1 in RCC and further explore the clinical significance and biological functions of SPRY4-IT1 in RCC. We first examined the expression level of SPRY4-IT1 in ccRCC tissues and cell lines by using qRT-PCR. Next, we analyzed its correlations with clinicopathological characters in order to determine the clinical significance of SPRY4-IT1 in ccRCC. Additionally, we conducted in vitro assays to demonstrate the biological functions of

SPRY4-IT1 in RCC development and progression. Our research revealed that lncRNA SPRY4-IT1 involved in the progression of RCC.

### Materials and methods

#### Patients and specimens

A total of 98 primary ccRCC tissues and adjacent normal renal tissues were obtained from patients who underwent radical nephrectomy in the Department of Urology, Shanghai Tenth People's Hospital of Tongji University between 2006 to 2008. None of the patients had received chemotherapy or radiotherapy before surgery. After surgical resection, tumor specimens and adjacent normal renal tissues were collected and stored in liquid nitrogen until use. 54 of these 98 patients were men and 44 were women. The median age of the patients was 61 years. Clinicopathological characteristics in our study are presented in **Table 1**. All patients were followed up until September 2011 with a median observation time of 35 months. For the use of these clinical materials for research purposes, prior patient's consent and approval from the Institute Research Ethics Committee were obtained.

#### Cell culture and treatment

Human RCC cell lines 786-O, ACHN, Caki-1, and Caki-2 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of

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Sciences (CCAS, China). Immortalized normal human proximal tubule epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC, USA). HK-2 cells were cultured in KSFM medium (Gibco), and other cells were cultured in RPMI-1640 medium (HyClone) with 10% fetal bovine serum (Gibco), 50 U/ml of penicillin and 50 µg/ml of streptomycin. All cells were cultured in a sterile incubator maintained at 37°C with 5% CO<sub>2</sub>.

Small interfering RNA that targeted SPRY4-IT1 RNA (si-SPRY4-IT1) and a scrambled negative control (si-NC) were generously provided by Life Technologies. The sequences of si-SPRY4-IT1 were 5'-CCCAGAATGTTGACAGCTGCCTCTT-3'. Human RCC 786-O cells were transfected with either 50 nmol si-SPRY4-IT1 or si-NC using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instruction. After 48 hours, cells transfected with siRNA were harvested for qRT-PCR to determine the transfection efficiency.

### *Cell proliferation assay*

The proliferation capacity of the renal cancer cells was assessed by MTT assay (Sigma) according to the manufacturer's instructions. The 786-O cells were plated into the 96-well plate after being transfected with si-SPRY4-IT1 or si-NC. These cells were left in the culture for 24 hours with 3 replicate wells at 10<sup>3</sup>/well. They were then treated with 100 µg MTT by adding it to the medium after the cells were incubated for 24, 48, 72, and 96 hours. The incubation was then continued for another 4 hours before the cell medium was removed, when DMSO (Sigma) was added for 15 minutes to lyse the cells. Finally, the absorbance was measured at 490 nm using an enzyme-labeled analyzer. Three independent experiments were performed.

### *Cell cycle assays*

To determine cell cycle distribution, 786-O cells were plated in 6-well plates and transfected with si-SPRY4-IT1 or si-NC for 48 hours. After transfection, the cells were collected by trypsinization, fixed in 70% ice-cold ethanol overnight. The cells were then washed with PBS, and stained with propidium iodide (50 mg/ml) in PBS supplemented with RNase (50 mg/ml) in the dark at room temperature for 30 minutes. Tests were performed in triplicate for each

sample, and analyses of cell cycle distribution were performed by FACS Caliber in accordance with the manufacturer's guidelines (BD Bioscience).

### *Cell apoptosis assay*

To determine cell apoptosis, 786-O cells were plated into 6-well plates in antibiotic-free medium, after being transfected with si-SPRY4-IT1 or si-NC for 48 hours. Cells were collected and washed twice and stained with FITC-Annexin V and PI, using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's manual. Apoptotic cells were analyzed by using by FACS Caliber (BD Bioscience).

### *Wound healing assays*

To determine cell migration, 786-O cells transfected with si-SPRY4-IT1 or si-NC were seeded into 12-well plates, incubated in their respective complete culture medium and grown to confluence over-night. Wounds were made by scraping with a sterilized 200 µL pipette tip, and the debris was rinsed with PBS, photographs were obtained at 0, and 48 hours using a phase contrast microscope (Olympus). Three independent experiments were carried out.

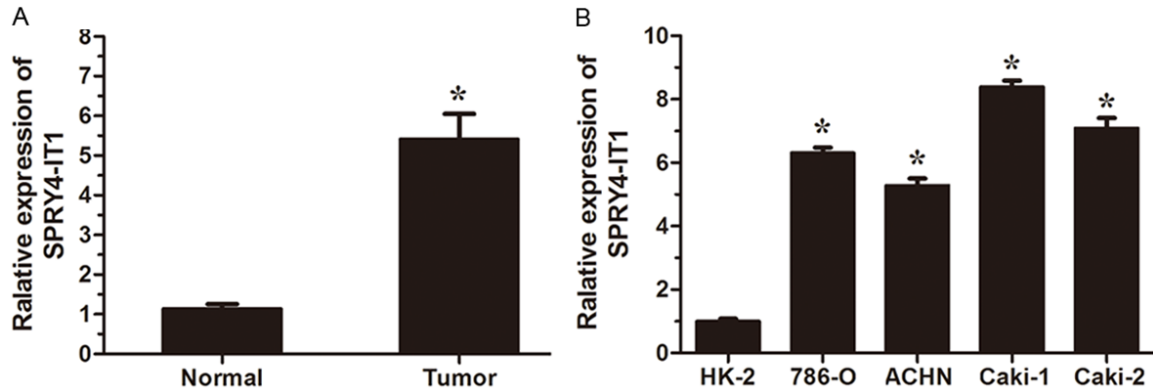
### *Transwell invasion assays*

The cell invasion assays were performed in a 24-well transwell chamber, which was pre-coated with 100 µg Matrigel. 786-O renal cancer cells transfected with si-SPRY4-IT1 or si-NC in each group were collected and resuspended in serum-free medium at a concentration of 1×10<sup>5</sup> cells/ml, respectively. Then, 200 µL cell suspensions were added into the upper chamber, and the bottom chamber was filled with 500 µL culture medium containing 10% FBS. After incubation for 48 hours at 37°C, 5% CO<sub>2</sub>. After incubation, the non-invaded cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed and stained using 0.1% crystal violet. The numbers of invaded cells were counted in five randomly selected high power fields under a microscope (Olympus).

### *RNA isolation and quantitative real-time PCR*

Total RNA was extracted from specimens or cell lines using Trizol reagent according to the man-

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**Figure 1.** Expression analysis of SPRY4-IT1 in ccRCC tissues and cell lines. The relative SPRY4-IT1 expression levels were determined using qRT-PCR and demonstrated using the comparative  $\Delta\Delta C_t$  method. A. Significant higher expression of SPRY4-IT1 was found in ccRCC tissues than in adjacent normal renal tissues. B. Significant higher expression of SPRY4-IT1 was found in four RCC cell lines than in normal human proximal tubule epithelial cell line HK-2. Results are expressed as mean  $\pm$  SD for three replicate determination. All data analyzed using Student's t test. \* $P < 0.05$ .

**Table 2.** Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	<i>P</i>	Risk ratio	95% CI	<i>P</i>
Gender	1.378	0.649-2.178	0.337			
Male vs. Female						
Age (years)	2.289	0.214-3.208	0.298			
$\geq 65$ vs. $< 65$						
Tumor size	2.912	0.271-5.196	0.143			
$\geq 4$ cm vs. $< 4$ cm						
Histological grade	3.927	2.379-6.733	$< 0.001$	3.187	2.018-5.649	0.009
III-IV vs. I-II						
Tumor stage	2.946	2.162-5.272	0.031	2.614	2.079-4.848	0.019
$T_{3-4}$ vs. $T_{1-2}$						
Lymph node	4.413	2.934-7.619	0.008	3.866	2.112-6.718	0.014
Presence vs. Absence						
Distant metastasis	5.529	3.275-9.684	0.004	4.826	3.117-9.519	0.007
Presence vs. Absence						
SPRY4-IT1	3.829	2.274-8.178	0.011	3.375	1.824-7.391	$< 0.001$
high vs. low						

ufacturer's protocol (Invitrogen). RNA was reverse transcribed using SuperScript First Strand cDNA System (Invitrogen) according to the manufacturer's instructions. The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with 1.0  $\mu$ l of cDNA and SYBR Green Real-time PCR Master Mix (Takara). Data was collected and analyzed by SDS2.3 Software (Applied Biosystems). The expression level of each candidate gene was internally normalized against that of the GAPDH. The relative quantitative value was expressed by the  $2^{-\Delta\Delta C_t}$

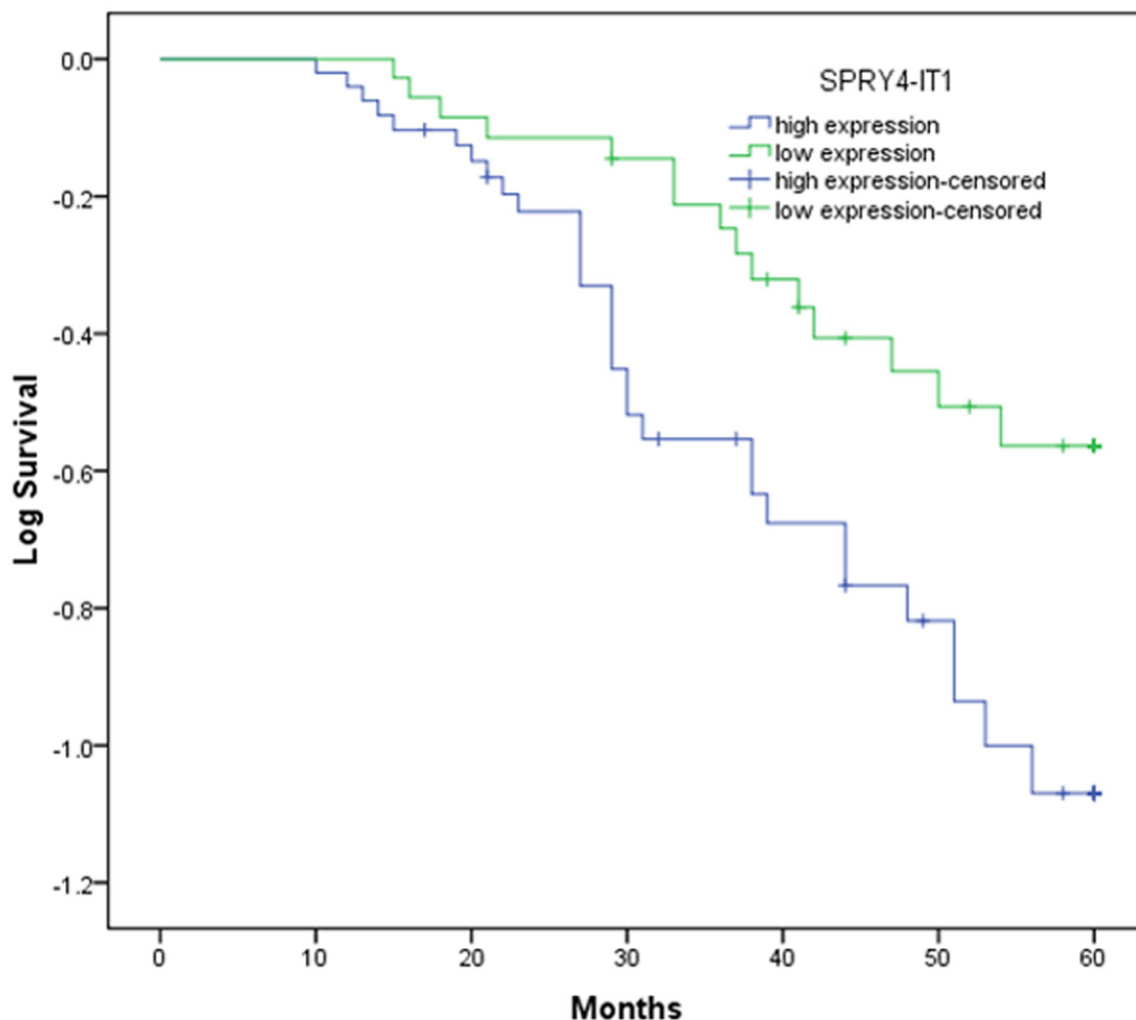
method. Each experiment was performed in triplicates and repeated three times.

The primers of SPRY4-IT1 and GAPDH used for qRT-PCR are listed as follows: SPRY4-IT1 Forward 5'-AGCCACATAAATTCAGCAGA-3', SPRY4-IT1 Reverse: 5'-CGATGTAGTAGGATTCCTTTC-3', GAPDH Forward: 5'-GACTCATGACCACAGTCCATGC-3', GAPDH Reverse: 5'-AGAGGCA-GGGATGATGTTCTG-3'.

### Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data were ana-

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**Figure 2.** Kaplan-Meier overall survival curves by SPRY4-IT1 level. Patients with higher SPRY4-IT1 expression in tumor tissue were closely correlated with poorer overall survival than patients with lower SPRY4-IT1 expression ( $P < 0.05$ ).

lyzed using independent two-tailed t test. Categorical data were analyzed using the two-side chi-square test. Overall survival was estimated by using Kaplan-Meier method, and univariate analysis was conducted by log-rank test. The Cox proportional hazards model was used in the multivariate analysis. Values of  $P < 0.05$  were considered statistically significant.

### Results

#### *SPRY4-IT1 is over-expressed in ccRCC tissues and RCC cell lines*

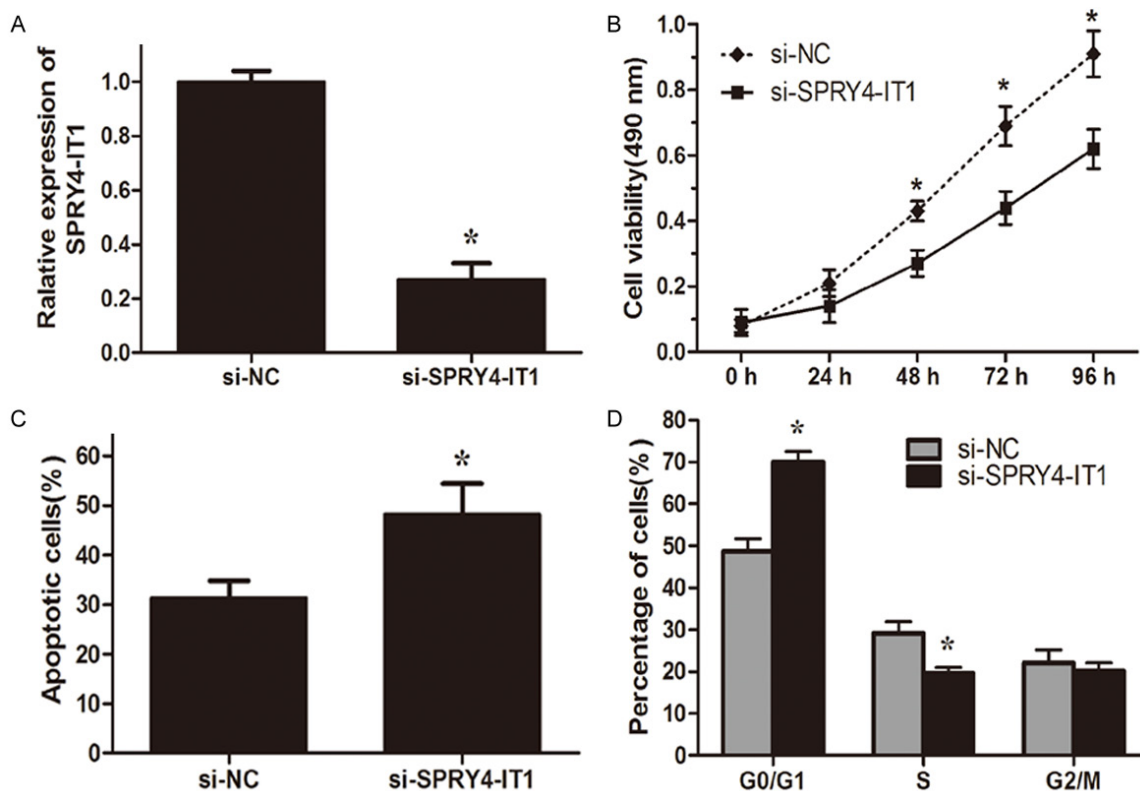
qRT-PCR was used to measure SPRY4-IT1 expression levels in a total of 98 patients with ccRCC. SPRY4-IT1 expression was significantly increased in clinical ccRCC tissues compared to adjacent normal renal tissues ( $P < 0.05$ , **Figure 1A**). Expression was also examined by

qRT-PCR in 4 RCC cell lines and a normal human proximal tubule epithelial cell line HK-2. The result showed that SPRY4-IT1 expression was higher in RCC cell lines than in HK-2 ( $P < 0.05$ , **Figure 1B**).

#### *Correlation between SPRY4-IT1 expression and clinicopathologic features in patients with ccRCC*

The patients with ccRCC were classified into two groups based on the mean value (3.38) of relative SPRY4-IT1 expression. The association between SPRY4-IT1 expression and clinicopathologic features in patients with ccRCC was analyzed using chi-square test. As shown in **Table 1**, SPRY4-IT1 expression showed association with histological grade, tumor stage, lymphnode metastasis and distant metastasis ( $P < 0.05$ ) but not association with patient's age,

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**Figure 3.** SPRY4-IT1 is essential for renal cancer cell proliferation. A. The relative expression level of SPRY4-IT1 in 786-O cells is significantly decreased by si-SPRY4-IT1 compared with the si-NC detected by qRT-PCR. B. The proliferation was decreased in si-SPRY4-IT1 group as compared to that of the si-NC group as identified by MTT assays. C. Inhibition of the SPRY4-IT1 promotes apoptosis in 786-O cells. D. Inhibition of the SPRY4-IT1 promotes cell cycle arrest in G0/G1 phase in 786-O cells. Results are expressed as mean  $\pm$  SD for three replicate experiments. All data analyzed using Student's t test. \* $P < 0.05$ .

gender, and tumor size ( $P > 0.05$ ). Taken together, these results indicated that overexpressed SPRY4-IT1 was correlated with the progression and development of ccRCC.

### Correlation between SPRY4-IT1 expression and prognosis in patients with ccRCC

Kaplan-Meier survival analysis data showed that in the SPRY4-IT1 high-expression group had significantly shorter overall survival time than those with low-expression (log-rank test,  $P < 0.05$ ) (Figure 2). As in Table 2, SPRY4-IT1 expression level, histological grade, tumor stage, lymphnode metastasis and distant metastasis were significantly correlated with overall survival rate of patients with ccRCC ( $P < 0.05$ ). A multivariate analysis showed that relative expression of SPRY4-IT1, histological grade, tumor stage, lymphnode metastasis and distant metastasis were independent prognostic factors for the overall survival of patients with ccRCC (Table 2). These results suggested that SPRY4-IT1 expression can be developed

as a powerful independent prognostic factor in patients with ccRCC.

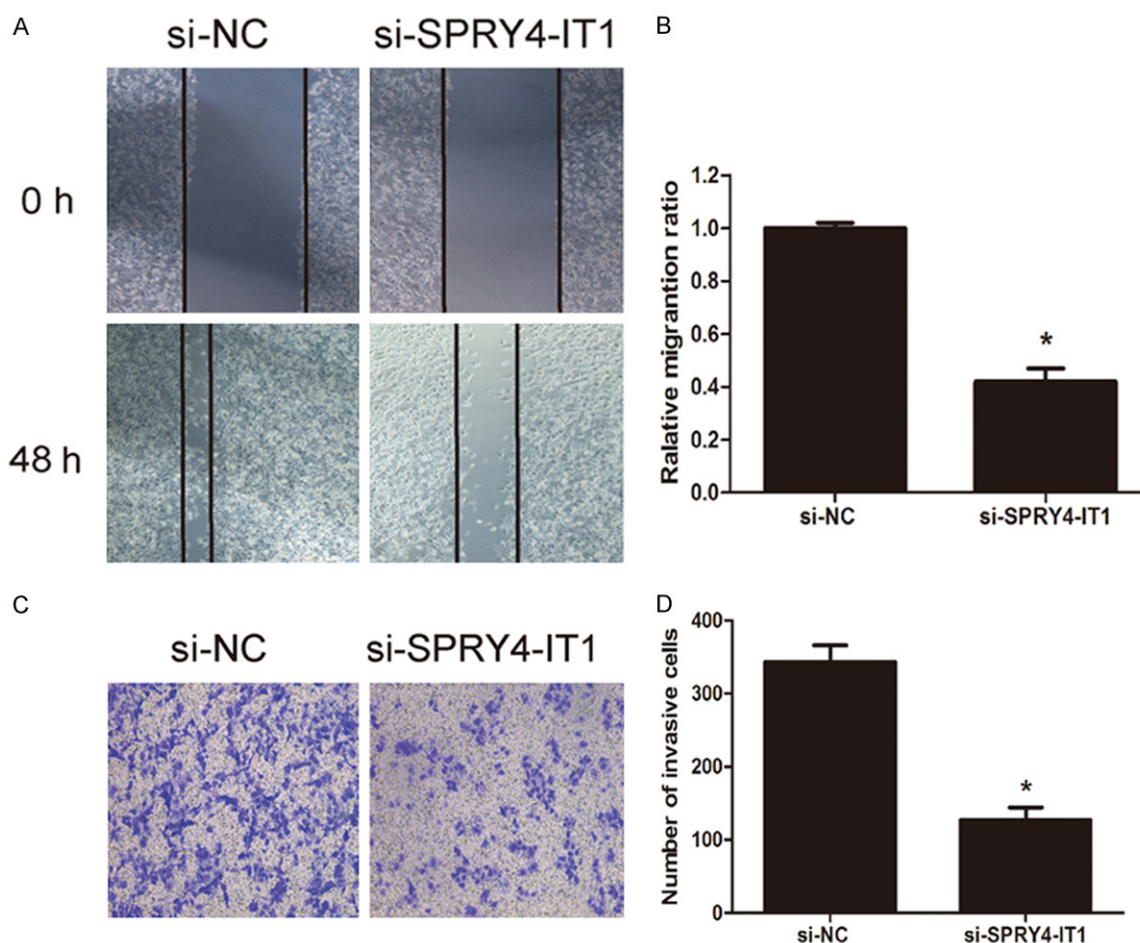
### si-SPRY4-IT1 significantly downregulated the expression of SPRY4-IT1 in 786-O cells

To further investigate the role of SPRY4-IT1 in human renal cancer cells, SPRY4-IT1 specific siRNA (si-SPRY4-IT1) was transfected into 786-O cells, respectively. Nonspecific siRNA was used as a negative control (si-NC). As shown in Figure 3A, after transfection with si-SPRY4-IT1 cells showed a significant decreased mRNA expression of si-SPRY4-IT1 compared to the si-NC group ( $P < 0.05$ ). The result suggested that we successfully downregulated the SPRY4-IT1 expression in human renal cancer 786-O cells.

### Knockdown of SPRY4-IT1 inhibited cell proliferation

To further study the potential role of SPRY4-IT1 in cell proliferation of renal cancer cells, MTT

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**Figure 4.** The effects of SPRY4-IT1 on 786-O cells migration and invasion. A, B. Wound healing assays showed that 786-O cells transfected with si-SPRY4-IT1 displayed significantly lower migration capacity compared with those transfected with si-NC. C, D. Transwell invasion assays indicated that 786-O cells transfected with si-SPRY4-IT1 displayed significantly lower invasion capacity compared with those transfected with si-NC. Five areas were randomly selected in each chamber. The number of cells in these areas was counted, and results are expressed as means  $\pm$  SD for three replicate experiments. All data analyzed using Student's t test. \* $P < 0.05$ .

assay was performed after the transfection of si-SPRY4-IT1 into the 786-O cells, respectively. As demonstrated in **Figure 3B**, the si-SPRY4-IT1 group showed a downregulated proliferative rate compared with the si-NC group ( $P < 0.05$ ).

Next we explored the potential mechanisms of cell proliferation inhibition by SPRY4-IT1 knock-down, flow cytometry was used. 48 hours after transfection of 786-O cells with si-SPRY4-IT1 resulted in a increase in apoptosis compared with the si-NC group ( $P < 0.05$ , **Figure 3C**). This increase in the number of 786-O cells in the G1/G0 phase and a decreased number of cells in S phase ( $P < 0.05$ , **Figure 3D**). These data suggest that forced downregulation of SPRY4-IT1 has an inhibitory effect on the proliferation of renal cancer cells.

### *Knockdown of SPRY4-IT1 inhibited cell migration and invasion*

We then performed cell wound healing assay and transwell invasion assays to investigate the role of SPRY4-IT1 in the regulation of cell migration and invasion in human renal cancer cells. Wound healing assays showed that the migratory rate of 786-O cells transfected with si-SPRY4-IT1 was significantly downregulated compared with si-NC group ( $P < 0.05$ , **Figure 4A, 4B**). Transwell invasion assays showed that the invasion of 786-O cells transfected with si-SPRY4-IT1 was notably downregulated compared with si-NC group ( $P < 0.05$ , **Figure 4C, 4D**). These data indicate that SPRY4-IT1 may promote the migration and invasion of renal cancer cells.

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## Discussion

The prognosis of renal cell carcinoma is quite poor, because most patients are diagnosed at an advanced stage, when treatments are less effective [14], so finding a new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategies and outcomes of this disease. It is now recognized that only 2% of the human genome encodes for protein-coding RNAs, whereas 60-70% of our DNA is transcribed into non-protein coding RNAs [15]. Among these, microRNAs (miRNA) have been certified as important biological RNAs in the post-transcriptional regulation of the target genes [16]. Besides, long non-coding RNAs (lncRNAs) as a newly discovered class of non-coding genes, are another crucial member of these biological RNAs [17]. Early studies showed that long non-coding RNAs were considered to be simply transcriptional "noise" or cloning artifacts [18]. However, in recent years, more and more studies showed that dysregulation in lncRNAs are proved to contribute in tumor development in many cancer types and can be used to develop as biomarkers and prognosis factors [19]. SPRY4-IT1 which was localized in 5q31.3, was derived from an intronic region within the SPRY4 gene and was predicted to contain several long hairpins in its secondary structure, which was originally reported by Khaitan and colleagues to play an important role in melanoma pathogenesis in humans [12].

In the study, we investigated the clinical significance of SPRY4-IT1 in ccRCC patients for the first time. By using qRT-PCR, we found that lncRNA SPRY4-IT1 was up-regulated in ccRCC tissues and renal cancer cell lines to a greater extent than in corresponding normal renal tissues and normal human proximal tubule epithelial cell line HK-2. We also found that the relative expression level of SPRY4-IT1 was associated with histological grade, tumor stage, lymphnode metastasis and distant metastasis of ccRCC patients. However, lncRNA SPRY4-IT1 expression was not correlated with patient's age, gender, and tumor size. In addition, SPRY4-IT1 overexpression was associated with lower overall survival rates and could be an independent prognostic factor in patients with ccRCC. These results indicated that lncRNA SPRY4-IT1 expression was an

independent prognostic factor for patients with ccRCC, and play an important role in development, tumorigenesis, and progression of RCC.

Khaitan et al. also showed the knockdown of SPRY4-IT1 expression resulted in cell growth defects, decreased invasion and migration, and increased rates of apoptosis in melanoma cells [12]. Xie et al. found that SPRY4-IT1 regulates the cell proliferation, migration and invasion in human esophageal squamous cell carcinoma (ESCC). siRNA-mediated SPRY4-IT1 knockdown in human esophageal squamous cells result in defects in cell growth, decreased migration and invasion in vitro [13]. From our clinical pathological data, we found that high SPRY4-IT1 expression is closely associated with histological grade, tumor stage, lymphnode metastasis and distant metastasis, thus we suppose SPRY4-IT1 may also regulate the growth and metastasis of renal cells. So, we identify the biological function of SPRY4-IT1 in renal cancer cells.

To further understand the mechanism of SPRY4-IT1 in renal cancer cell process, in vitro experiments were conducted. siRNA-mediated knockdown of SPRY4-IT1 significantly decreased proliferation, migration and invasion capability of 786-O cells compared with control group, which indicated that downregulation of SPRY4-IT1 can suppress the development of RCC. Our data showed that overexpression of SPRY4-IT1 can promote the malignant phenotypes of renal cancer cells.

In summary, we showed that lncRNA SPRY4-IT1 expression is increased in ccRCC tissues and renal cancer cell lines for the first time and SPRY4-IT1 is significantly associated with advanced tumor progression. Furthermore, SPRY4-IT1 expression was demonstrated to be an independent marker for predicting the clinical outcome of patients with ccRCC. The up-regulation of SPRY4-IT1 plays an important role in RCC progression. The data suggested that SPRY4-IT1 is a promising biomarker and a therapeutic target for the treatment of renal cancer.

## Disclosure of conflict of interest

None.

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