Int J Clin Exp Pathol 2015;8(2):1954-1960
www.ijcep.com /ISSN:1936-2625/IJCEP0004251

Original Article

Increased expression of SPRY4-IT1 predicts poor prognosis and promotes tumor growth and metastasis in bladder cancer

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Received November 29, 2014; Accepted January 28, 2015; Epub February 1, 2015; Published February 15, 2015

Abstract: Introduction: long non-coding RNAs (lncRNAs) are emerging as new regulators in the cancer paradigm, the involvement of lncRNAs in urothelial carcinoma of the bladder (UCB) is just beginning to be studied. In this study, we focused on lncRNA SPRY4-IT1 and investigated its expression pattern, clinical significance, and biological function in UCB. Methods: SPRY4-IT1 expression in UCB tissues was examined by quantitative Real-time PCR (qRT-PCR) and its correlation with clinicopathological features and patient prognosis was later analyzed. Moreover, in vitro assays were performed to explore its role in bladder cancer progression. Results: SPRY4-IT1 expression was elevated in UCB tissues, and SPRY4-IT1 levels were highly positively correlated with histological grade, tumor stage, and lymph node metastasis and reduced overall survival. A multivariate analysis showed that SPRY4-IT1 expression is an independent prognostic factor of overall survival in patients with UCB. Additionally, the results of in vitro assays showed that the suppression of SPRY4-IT1 expression in bladder cancer cells significantly inhibit cell proliferation, migration, and invasion. Conclusions: Our data suggested that lncRNA SPRY4-IT1 is a novel molecule involved in bladder cancer progression, which provide a potential prognostic biomarker and therapeutic target.

Keywords: SPRY4-IT1, urothelial carcinoma of the bladder, proliferation, migration, invasion

Introduction

Bladder cancer is the second most common genitourinary tumor in human populations, and it was estimated in 2013 that 72,570 new cases of cancer of the urinary bladder were diagnosed in the United States and 15,210 deaths were attributable to bladder cancer [1]. Urothelial carcinoma of the bladder (UCB) is the most common histological subtype of bladder cancer. Overall, 70% of bladder tumors present as noninvasive urothelial carcinoma, and the remainder present as muscle-invasive disease [2]. Clinically, radical cystectomy remains the most common treatment for patients with muscle-invasive UCB or for patients with superficial disease that is at high risk of recurrence and progression [3]. Despite advances in surgical technique and an improved understanding of the role of pelvic lymphadenectomy, the 5-year cancer-specific survival remains at only 50-60% [4]. In addition, while providing important prognostic information on UCBs, the currently clinical and pathological variables have a limited ability to predict tumor progression and patient survival. Therefore, it's necessary to identify new sensitive, reliable biomarkers enabling prediction of biological nature and to develop a new targeted therapies for UCB.

The rapid development of RNA genomics has highlighted the role of non-coding RNAs (ncRNAs) in post-transcriptional regulation human tumors. NcRNAs are collectivey divided into three categories: housekeeping RNAs, small non-coding RNAs, and long non-coding RNAs [5]. Long non-coding RNAs (lncRNAs) are 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 evidence showed that lncRNAs play important roles in diverse biological processes, such as transcriptional regulation, cell growth and tumorigenesis [8]. In this regard, highlighting the

potentially widespread functional roles of lncRNAs in human cancer is important. Geng et al showed that HOTAIR gene was significantly over-expressed in hepatocellular carcinoma tissues compared with adjacent non-tumor tissues and patients with high HOTAIR gene expression in their tumors had an increased risk of recurrence after hepatectomy [9]. Gutschner et al found that transient over-expression of MALAT1 enhanced cellular proliferation in lung cancer cells and tumor formation in nude mice, while depletion of MALAT1 in tumor cells reduced tumorigenicity [10]. Han et al revealed that lncRNA TUG1 was emerging as a novel player in the disease state of UCB and may have potential roles as a biomarker and a therapeutic target in UCB [11]. Unfortunately, the emerging functional role of lncRNAs in UCB remains largely unknown.

SPRY4-IT1 (708 bp), 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 Divya Khaitan and colleagues to play an important role in melanoma pathogenesis in humans [12]. In their study they indicated that expression of the lncRNA SPRY4-IT1 is low in normal human melanocytes but high in melanoma cells. siRNA knockdown of SPRY4-IT1 blocked melanoma cell invasion and proliferation, and increases apoptosis. Zhang et al found that SPRY4-IT1 is up-regulated and associated with aggressive progression and poor prognosis in renal cancer [13]. Xie et al indicated that esophageal squamous cell carcinoma patients with higher SPRY4-IT1 expression had advanced clinical features and poorer prognosis than those with lower SPRY4-IT1 expression, knockdown SPRY4-IT1 expression reduced cell proliferation, migration and invasion [14]. Zou et al demonstrated that over-expression of SPRY4-IT1 modulates proliferation, migration, apoptosis, and network formation in trophoblast cells [15]. However, the lncRNA SPRY4-1T1 expression in UCB and underlying mechanism has not been reported yet.

In this study, we aimed to investigate the expression of lncRNA SPRY4-1T1 in UCB tissues and further explore the clinical significance and biological functions of SPRY4-1T1 in bladder cancer.

Materials and methods

Patients and specimens

Patient's data were accessed from the databank of the Department of Urology, Huaihe Hospital of Henan University (Kaifeng, China). 68 consecutive patients with UCB and who underwent surgery from 2005 to 2008 were included in the study. The cases selected were based on distinctive pathologic diagnosis of UCBs, undergoing transurethral resection, partial cystectomy and radical cystectomy without preoperative chemotherapy or radiotherapy. Tumor specimens and corresponding adjacent non-tumor tissues were collected and stored in liquid nitrogen until use. Clinical charts were reviewed, and follow-up data were collected. Patients were only included in the study if they had provided written consent to participate in the study after receiving oral and written information regarding its course and purpose. Approval for the study was received from the Ethics Committee of the host institution.

Cell culture

The human bladder cancer cell lines J82, T24, SW780 and SV-40 immortalized human uroepithelial cell line SV-HUC-1 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CCCAS, China). SV-HUC-1 cells were cultured in Ham's F12 medium (Sigma), and other cells were cultured in DMEM medium (Gibco) with 10% fetal bovine serum (FBS, 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_2.

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 CCCAGAATGTTGACAGCTGCCTCTT, Human J82 cells were transfected with either 50 nmol si-SPRY4-IT1 or si-NC using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instruction.

Quantitative real-time PCR assay

Total RNA was extracted from tissues using Trizol reagent (Invitrogen). A Reverse Transcription Kit (Takara) was used for the synthesis of cDNA by adding 1 mg total RNA to the RT Reaction Mix. The amplification of cDNA was

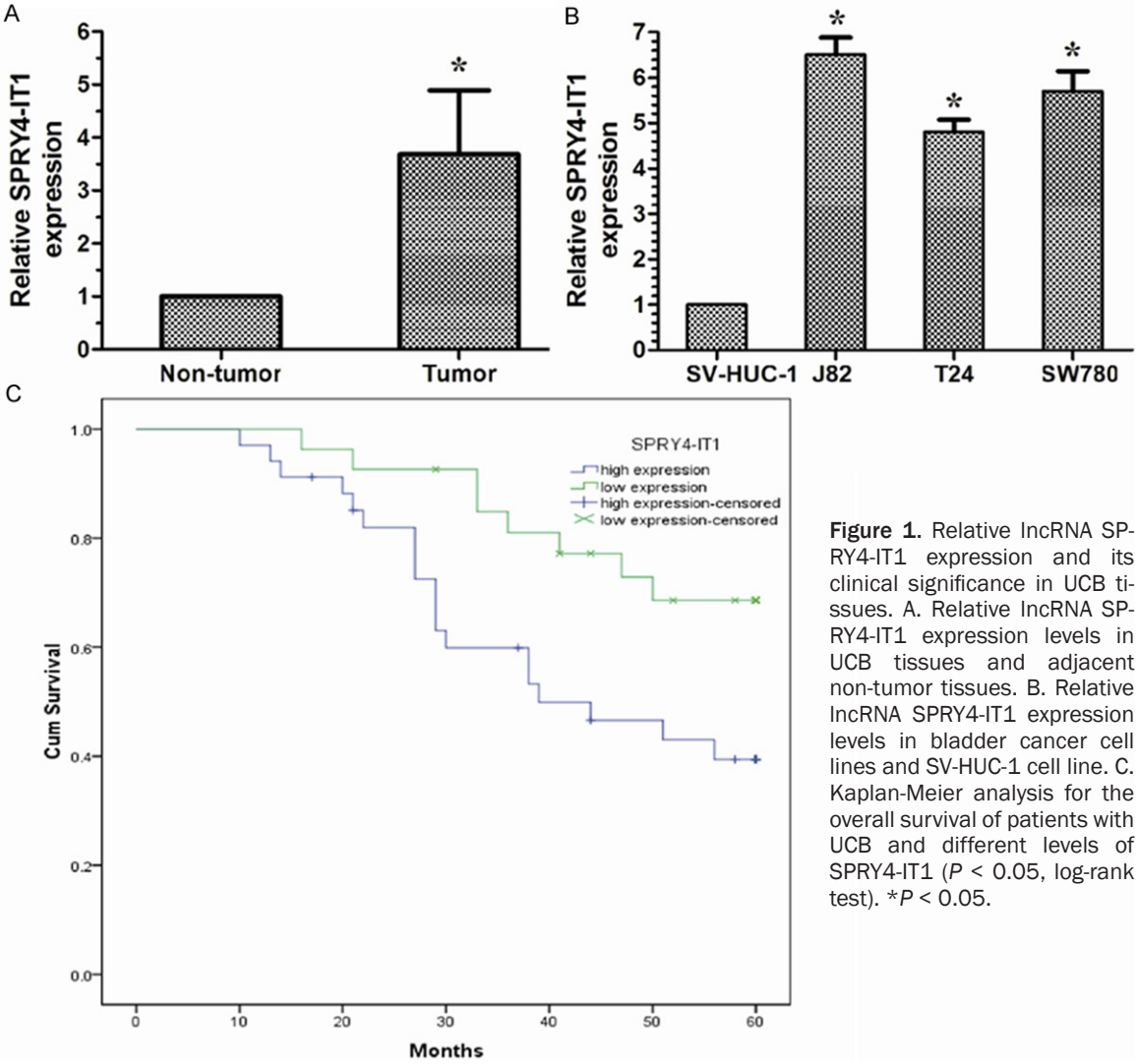

Figure 1. Relative lncRNA SPRY4-IT1 expression and its clinical significance in UCB tissues. A. Relative lncRNA SPRY4-IT1 expression levels in UCB tissues and adjacent non-tumor tissues. B. Relative lncRNA SPRY4-IT1 expression levels in bladder cancer cell lines and SV-HUC-1 cell line. C. Kaplan-Meier analysis for the overall survival of patients with UCB and different levels of SPRY4-IT1 ($P < 0.05$, log-rank test). *$P < 0.05$.

done by Power SYBR Green (Takara) in a total volume of 20 μL reaction mix for qPCR. According to the manufacturer's instruction, the reverse transcription was performed at 37°C for 15 min, 85°C for 5 second. In order to normalize the results for the qPCR, the GAPDH expressions were used. The sequence of the primers were as following: SPRY4-IT1 (Forward: 5'-AGCCACATAAATTCAGCAGA-3', Reverse: 5'-CGATGTAGTAGGATTCCTTTCA-3') and GAPDH (Forward: 5'-GACTCATGACCACAGTCCATGC-3', Reverse: 5'-AGAGGCAGGGATGATGTTCTG-3'). An ABI 7500 was used to carry out the qPCR and data collections.

Cell proliferation assay

The proliferation capacity of the bladder cancer cells was assessed by MTT assay (Sigma) according to the manufacturer's instructions. The J82 cells were plated into the 96-well plate after being transfected with si-RNAs targeting SPRY4-IT1. These cells were left in the culture for 24 h with five replicate wells at 10^4 cells per 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 h. The incubation was then continued for another 4 h before the cell medium was removed, when DMSO was added for 10 minutes to lyse the cells. Finally, the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader. Each experiment was performed in triplicate.

Migration and invasion assay

The invasion assay was performed using a Biocoat Matrigel Invasion Chamber from Becton Dickson (8 μm pore size), and the migra-

Table 1. Correlation between SPRY4-IT1 expression and clinicopathological features of UCB patients

Parameters	Group	Total	SPRY4-IT1 expression		*P* value
			High	Low	
Gender	Male	38	22	16	0.707
	Female	30	16	14	
Age (years)	< 59	42	24	18	0.790
	≥ 59	26	14	12	
Tumor size (cm)	< 3 cm	37	21	16	0.874
	≥ 3 cm	31	17	14	
Multiplicity	Single	42	23	19	0.813
	Multiple	26	15	11	
Histological grade	G_1	27	7	20	0.000
	G_2, G_3	41	31	10	
Tumor stage	T_a, T_1	32	13	19	0.017
	T_2, T_3, T_4	36	25	11	
Lymph nodes metastasis	No	49	20	29	0.000
	Yes	19	18	1	

tion assay was performed in a similar fashion without matrigel. The cells ($4x10^4$ for migration and invasion) were plated in the upper chamber with 200 μL serum-free medium. The bottom chamber contained 400 μL DMEM with 10% FBS. After 48 hours, the chambers were fixed using cold methanol and stained with crystal violet. The cells that migrated or invaded to the lower surface were counted in every five high power fields under a microscope. Each migration and invasion assay was performed in three times.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data were analyzed with an independent t-test, and categorical data were analyzed with the chi-square test. Overall survival curves were plotted following the Kaplan-Meier method, and the log-rank test was used to compare the survival in the different groups. A Cox proportional hazards model was used to examine the significance of the effects of different variables on survival in a multivariate analysis. Differences were considered statistically significant when *P* was less than 0.05.

Results

LncRNA SPRY4-IT1 up-regulation in UCB

The relative expression levels of lncRNA SPRY4-IT1 was examined by qRT-PCR in 3 bladder cancer cell lines and a SV-40 immortalized human uroepithelial cell line SV-HUC-1. Our results showed that SPRY4-IT1 expression was higher in bladder cancer cell lines than that in SV-HUC-1 cells ($P < 0.05$, **Figure 1A**). Expression was also examined by qRT-PCR in a total of 68 patients with UCB. SPRY4-IT1 expression was significantly increased in UCB tissues compared to adjacent non-tumor tissues ($P < 0.05$, **Figure 1B**).

LncRNA SPRY4-IT1 up-regulation associates with aggressive clinicopatholigcal features of UCB

The UCB patients were divided into two groups based on the mean value (3.68) of relative SPRY4-IT1 expression. As shown in **Table 1**, the high level of SPRY4-IT1 expression was closely correlated with advanced tumor stage, higher histological grade, and positive lymph node metastasis ($P < 0.05$). But not correlated with patient's age, gender, tumor size and tumor multiplicity ($P > 0.05$). Taken together, these observations suggested that increased SPRY4-IT1 expression is associated with the progression of UCBs.

LncRNA SPRY4-IT1 up-regulation associates with poor prognosis in patients with UCB

The association between SPRY4-1T1 expression and survival of UCB patients was investigated by Kaplan-Meier analysis and log-rank test. As shown in **Figure 1C**, UCB patients with high SPRY4-1T1 expression tend to have shorter overall survival than those with low SPRY4-1T1 expression (log-rank test: $P < 0.05$). Univariate analysis indicated that histological grade, tumor stage, lymph node metastasis, and SPRY4-1T1 expression were significantly associated with overall survival of UCB patients ($P < 0.05$, **Table 2**). Multivariate analysis using the Cox proportional hazards model for all variables that were significant in the univariate analysis showed that histological grade, tumor stage, lymph node metastasis and SPRY4-1T1

Table 2. Prognostic factors in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Gender Male vs Female	0.897	0.646-2.172	0.429			
Age (years) ≥ 59 vs < 59	1.722	0.619-3.178	0.311			
Tumor size ≥ 3 cm vs < 3 cm	2.319	1.448-5.179	0.117			
Multiplicity Multiple vs Single	1.176	0.713-2.521	0.449			
Tumor stage ≥ T2 vs < T2	2.893	2.1149-5.189	0.013	2.525	1.974-4.883	0.007
Histological grade G_2, G_3 vs G_1	3.419	2.237-6.346	< 0.001	2.971	1.811-5.737	< 0.001
Lymph node Yes vs No	4.339	3.017-8.116	< 0.001	3.514	2.128-6.145	0.011
SPRY4-IT1 High vs Low	4.117	2.241-8.137	0.009	3.716	2.084-6.719	< 0.001

expression were independent prognostic factors for patients with UCB ($P < 0.05$, **Table 2**).

Knockdown of lncRNA SPRY4-IT1 inhibited cell proliferation, migration and invasion

In order to study the functional role of lncRNA SPRY4-IT1 in bladder cancer tumorigenesis, J82 cells were transfected with si-SPRY4-IT1 or si-NC. 48 hours after transfection, qRT-PCR was performed to examine the expression of si-SPRY4-IT1. As shown in **Figure 2A**, compared with si-NC group, the expression of SPRY4-IT1 showed a significant down-regulation in the transfected group with si-SPRY4-IT1 ($P < 0.05$), Next, the cell viability was determined using the MTT assay. As shown in **Figure 2B**, in J82 cells transfected with si-SPRY4-IT1 cell viability significantly decreased compared with the si-NC transfected cells. Then we performed Transwell migration and invasion assays to investigate the role of SPRY4-IT1 in the regulation of cellular migration and invasion on J82 cells. As shown in **Figure 2C**, the migratory rate of J82 cells transfected with si-SPRY4-IT1 was significantly suppressed compared to the si-NC group ($P < 0.05$). Transwell invasion assay showed that J82 cells transfected with si-SPRY4-IT1 was notably down-regulated when compared to the si-NC group ($P < 0.05$, **Figure 2D**). These data showed that down-regulated expression of SPRY4-IT1 could inhibit bladder cancer cell growth and metastasis.

Discussion

Urothelial carcinoma of the bladder remains to be one of the leading causes of death, so finding new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategies and outcomes of this disease. Now, there are several clinicopathologic features have been the standard for determining the clinical outcome of UCB patients, this classification scheme is probably an imprecise predictor of the prognosis of an individual patient [16, 17]. With the advance of high resolution microarray and genome wide sequencing technology, lncRNAs have recently caught increasing attention [18]. Dysregulated expression of lncRNAs in cancer marks the spectrum of disease progression and may serve as an independent predictor for patient outcomes. Some well defined lncRNAs, including GAS5, MEG3, and LET have been reported to be strongly associated to survival of cancer patients, thus they have been determined as prognostic indicators for a certain type of cancers [19-21]. However, to our knowledge, no published studies have investigated the clinical significance or biological function of SPRY4-IT1 in UCB.

In the present study, we investigated the expression pattern of SPRY4-IT1 in UCB tissues and analyzed the clinical significance of SPRY4-

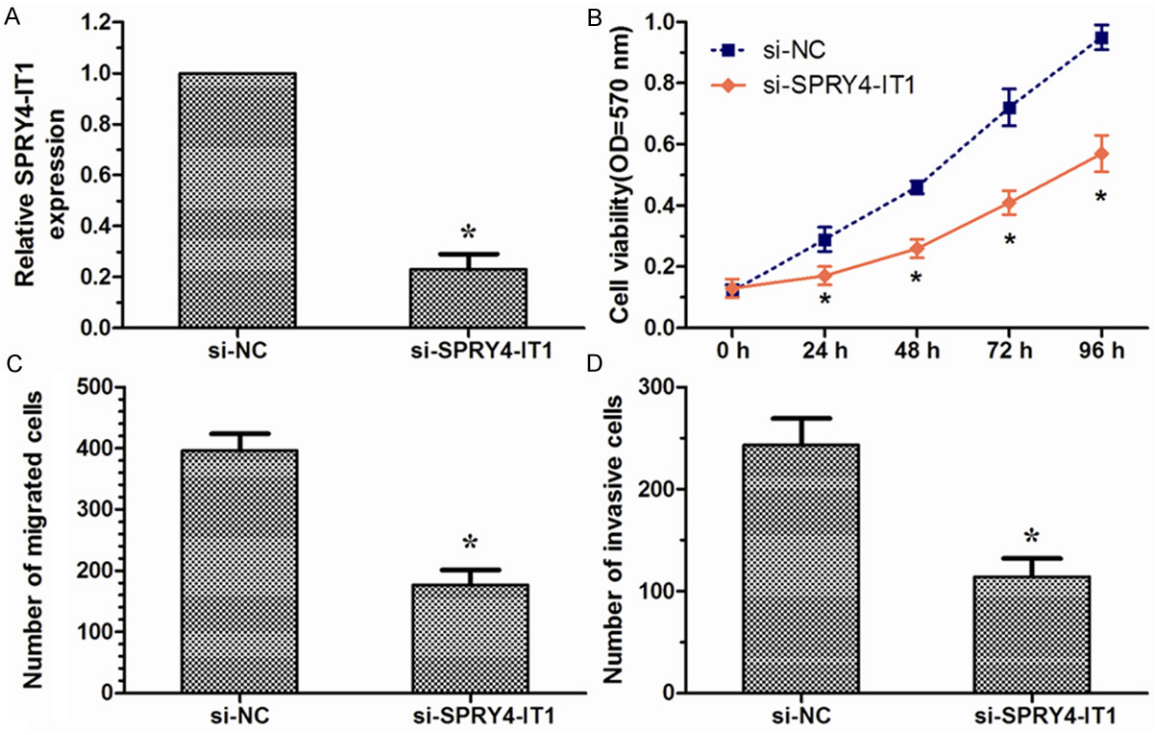

Figure 2. The knockdown of SPRY4-IT1 inhibits proliferation, migration and invasion in J82 cells. A. qRT-PCR revealed that SPRY4-IT1 was efficiently knocked down by treatment with si-SPRY4-IT1 in J82 cells. B. J82 cells trasfected with si-SPRY4-IT1 displayed significantly lower proliferation ability compared with those transfected with si-NC. C. J82 cells trasfected with si-SPRY4-IT1 displayed significantly lower migration ability compared with those transfected with si-NC. D. J82 cells trasfected with si-SPRY4-IT1 displayed significantly lower invasion ability compared with those transfected with si-NC. Results are expressed as means ± SD for three replicate determinations. *$P < 0.05$.

IT1 in patients with UCB. Our results revealed that SPRY4-IT1 expression levels were higher in UCB tissues than in adjacent non-tumor tissues, and elevated SPRY4-IT1 expression levels were associated with an advanced tumor stage, high histological grade, and positive lymph node metastasis, indicating that SPRY4-IT1 over-expression may promote an aggressive phenotype and UCB growth and metastasis. Elevated SPRY4-IT1 expression levels were also correlated with poor overall survival of UCB patients. The multivariate survival analysis indicated that the over-expression of SPRY4-IT1 was independently associated with overall survival in patients with UCB. These findings highlight the clinical significance of SPRY4-IT1 in patients with UCB and imply a potentially important role for SPRY4-IT1 in predicting the progression of UCB. However, the mechanism of SPRY4-IT1 in bladder cancer is still unclear.

So, to further understand the mechanism of SPRY4-IT1 in bladder cancer, in vitro experiments were conducted. siRNA-mediated knockdown of SPRY4-IT1 significantly decreased proliferation, migration and invasion capability of bladder cancer cells compared with si-NC group, suggested that knockdown of SPRY4-IT1 can suppress the development of bladder cancer. These results indicated that high expression level of SPRY4-IT1 can promote the malignant phenotypes of bladder cancer cells.

In conclusion, the expression level of the oncogenic lncRNA SPRY4-IT1 is increased in UCB tissues adjacent to non-tumor tissues. Elevated SPRY4-IT1 expression has been associated with poor prognosis, likely due to the ability of SPRY4-IT1 to induce cell growth and metastasis in bladder cancer cells. Cumulatively, these findings indicate that SPRY4-IT1 plays a vital role in the development and progression of bladder cancer. The development of SPRY4-IT1 based therapeutic strategies for the down-regulation of such oncogenic lncRNAs may provide a novel and promising alternative therapeutic approach for future cancer treatment.

Disclosure of conflict of interest

None.

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References

[1] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[2] Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, Kiemeney L, Kriegmair M, Montironi R, Murphy WM, Sesterhenn IA, Tachibana M and Weider J. Bladder cancer: epidemiology, staging and grading, and diagnosis. Urology 2005; 66: 4-34.

[3] Stenzl A, Cowan NC, De Santis M, Kuczyk MA, Merseburger AS, Ribal MJ, Sherif A and Witjes JA. Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines. Eur Urol 2011; 59: 1009-1018.

[4] Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, Skinner E, Bochner B, Thangathurai D, Mikhail M, Raghavan D and Skinner DG. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. J Clin Oncol 2001; 19: 666-675.

[5] Prensner JR and Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov 2011; 1: 391-407.

[6] Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12: 861-874.

[7] Costa FF. Non-coding RNAs: Meet thy masters. Bioessays 2010; 32: 599-608.

[8] Wang KC and Chang HY. Molecular mechanisms of long noncoding RNAs. Mol Cell 2011; 43: 904-914.

[9] Geng YJ, Xie SL, Li Q, Ma J and Wang GY. Large intervening non-coding RNA HOTAIR is associated with hepatocellular carcinoma progression. J Int Med Res 2011; 39: 2119-2128.

[10] Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G, Revenko A, Arun G, Stentrup M, Gross M, Zornig M, MacLeod AR, Spector DL and Diederichs S. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res 2013; 73: 1180-1189.

[11] Han Y, Liu Y, Gui Y and Cai Z. Long intergenic non-coding RNA TUG1 is overexpressed in urothelial carcinoma of the bladder. J Surg Oncol 2013; 107: 555-559.

[12] Khaitan D, Dinger ME, Mazar J, Crawford J, Smith MA, Mattick JS and Perera RJ. The melanomaupregulated long noncoding RNA SPRY4-IT1- modulates apoptosis and invasion. Cancer Res 2011; 71: 3852-3862.

[13] Zhang HM, Yang FQ, Yan Y, Che JP and Zheng JH. High expression of long non-coding RNA SPRY4-IT1 predicts poor prognosis of clear cell renal cell carcinoma. Int J Clin Exp Pathol 2014; 7: 5801-5809.

[14] Xie HW, Wu QQ, Zhu B, Chen FJ, Ji L, Li SQ, Wang CM, Tong YS, Tuo L, Wu M, Liu ZH, Lv J, Shi WH, Cao XF. Long noncoding RNA SPRY4-IT1 is upregulated in esophageal squamous cell carcinoma and associated with poor prognosis. Tumor Biol 2014; 35: 7743-54.

[15] Zou Y, Jiang Z, Yu X, Sun M, Zhang Y, Zuo Q, Zhou J, Yang N, Han P, Ge Z, De W and Sun L. Upregulation of long noncoding RNA SPRY4-IT1 modulates proliferation, migration, apoptosis, and network formation in trophoblast cells HTR-8SV/neo. PLoS One 2013; 8: e79598.

[16] Yang FQ, Huang JH, Liu M, Yang FP, Li W, Wang GC, Che JP and Zheng JH. Argonaute 2 is upregulated in tissues of urothelial carcinoma of bladder. Int J Clin Exp Pathol 2014; 7: 340.

[17] Bolenz C and Lotan Y. Molecular biomarkers for urothelial carcinoma of the bladder: challenges in clinical use. Nat Clin Pract Urol 2008; 5: 676-685.

[18] Ponting CP, Oliver PL and Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.

[19] Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F and Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene 2009; 28: 195-208.

[20] Sun M, Xia R, Jin F, Xu T, Liu Z, De W and Liu X. Downregulated long noncoding RNA MEG3 is associated with poor prognosis and promotes cell proliferation in gastric cancer. Tumour Biol 2014; 35: 1065-1073.

[21] Ma MZ, Kong X, Weng MZ, Zhang MD, Qin YY, Gong W, Zhang WJ and Quan ZW. Long noncoding RNALET is a positive prognostic factor and exhibits-- tumorsuppressive activity in gallbladder cancer. Mol Carcinog 2014; [Epub ahead of print].