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Up-regulation of Long Noncoding RNA BSN-AS2 is Correlated with Tumor Progression and Poor Prognosis in Patients with Bladder Cancer

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Abstract

OBJECTIVE: The purpose of this study was to explore the associations between long noncoding RNA BSN-AS2 (BSN-AS2) expression and clinical significance of bladder cancer (BC) patients.

PATIENTS AND METHODS: The expressions of BSN-AS2 were determined in 168 BC tissues and matched non-tumor samples using qRT-PCR. The receiver operating characteristic (ROC) curve was performed to estimate the diagnostic significance of BSN-AS2 in BC patients. The association between BSN-AS2 expression and clinicopathological parameters was analyzed. Kaplan-Meier curves and multivariate assays were applied to examine the impact of clinical factors on clinical survivals.

RESULTS: The expression of BSN-AS2 in BC tissues was distinctly higher than that of their matched non-tumor specimens ($p < 0.001$). High BSN-AS2 levels were observed to be robust in differentiating BC specimens from non-tumor bladder tissues (AUC= 0.7698, $p < 0.001$). BSN-AS2 expression was significantly associated with T stage ($p = 0.016$) and grade ($p = 0.007$). Survival data indicated that high expression of BSN-AS2 had a decreased overall survival ($p = 0.0017$) and disease-free survival ($p < 0.0001$). Multivariate analysis revealed that increased BSN-AS2 expression was an independent risk factor for BC patients.

CONCLUSIONS: Our findings, for the first time, demonstrated that BSN-AS2 expression in BC could be a useful prognostic and diagnostic marker.

Key words: LncRNA BSN-AS2, Bladder cancer, Diagnosis, Prognosis.

1. Introduction

Bladder cancer (BC) is a type of malignancy ranking ninth around the world. As estimated, 386,000 cases are diagnosed with BC worldwide on a yearly basis[1, 2]. About 70% of BC patients will be preliminarily diagnosed with nonmuscle-invasive BC (NMIBC), while 50-70 % of BC patients will relapse and nearly 10-20% of BC patients will develop to muscle-invasive BC (MIBC)[3, 4]. In recent decades, there has been a rapid development about the therapeutic strategies of BC. Standard therapy includes intra-vesical instillation, extensive tumor resection, as well as adjuvant chemotherapy. Nevertheless, BC patients still suffer a very low curative rate5, 6. The exploration of proper therapies for improving the survival rates of BC made a limited progression due to the unclear development and progression mechanism of BC7. On that account, the research on the biological features as well as pathogenesis of BC

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is of vital significance for effectively detecting and treating BC in an early stage.

With great advances of high-throughput RNA sequencing technology, many reports have shown that most of human transcriptome could be classified as long noncoding RNAs (lncRNAs)[8]. Long noncoding RNAs (lncRNAs) refer to RNAs with length over 200 nucleotides and have limited or no capacity to code protein[9]. Although lncRNAs can not be characterized as obviously as microRNAs, more and more evidences have found that lncRNAs can greatly affect different cellular processes like the pluripotency and differentiation of stem cell, the development of heart and body wall, cell apoptosis, as well as cancer metastasis[10, 11]. Metastasis, a key process in tumor progression, is a complex process that involves aberrant proliferation and changes the migratory properties of tumor cells[12]. In recent years, relevant studies have characterized lncRNAs associated with cancer as well as confirmed their biological activities and potential molecular mechanisms that affect the tumorigenesis[13, 14]. However, a large number of lncRNAs remained to be functionally identified in BC.

Long noncoding RNA BSN-AS2 (BSN-AS2) was firstly identified to be involved in the progression of osteosarcoma by Zhou and his group[15]. However, to our best knowledge, the expression and function in other remained unclear to a larger extent. The study for the first time identified BSN-AS2 as a novel BC-related lncRNA which may used as a novel biomarker for the diagnosis and prognosis of BC patients.

2. Patients and Methods

Patients and Tissue Samples

We obtained 168 paired of BC tissues together with matched adjacent normal bladder tissues from hospital, and these cases were histologically confirmed. There were 91 males and 77 females, and the medium age was 44.1 (range 25.6-69.2) years. None of these patients had previously been diagnosed with any type of malignancy. After being removed from patients, all tissue samples were quickly frozen in liquid nitrogen followed by being stored at -80°C. Detailed demographic and clinicopathological data such as gender, age, size of tumor, T stage, N stage and grade were gathered and summarized. The study has obtained the approval of the Ethics Committee of Hospital, as well as obtained patients' written informed consent.

Real-time PCR

TRIzol reagent (Invitrogen, Suzhou, Jiangsu, China) were used to extract total RNA from BC and normal samples. The Prime-Script™ one step RT-PCR kit (TaKaRa, Dalian, China) helped to reverse transcribe RNA into cRNA. The levels of BSN-AS2 in 168 pairs of BC specimens and normal bladder specimens were detected by qRT-PCR. An ABI7500 system (Applied Biosystems, Shenzhen, Guangdong, China) together with SYBR Green PCR Master Mix (Takara, Changsha, Hunan, China) assisted in carrying out the RT-PCR reactions. The study took GAPDH as the endogenous control. The $2^{-\Delta\Delta Ct}$ methods were employed to measure the relative quantitative value. Experimenters performed every experiment in triplicates and repeated them for three times. The primer sequences were shown in Table I.

Table I The used sequences of primer for RT-PCR

Primer name	Sequences
BSN-AS2: Forward	GCTATATCTCAGCTACGCTA
BSN-AS2: Reverse	ACTTGCTACCTTGTGACCA
GAPDH: Forward	ACTTTGGTATCGTGGAAGG
GAPDH: Reverse	GCCATCACGCCACAGTTTC

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) coupled with GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. χ^2-tests helped to evaluate the interplay between the clinicopathological parameters and the expression of BSN-AS2. Receiver-operating characteristic (ROC) assisted in assessing the potential value exhibited by BSN-AS2 for BC diagnosis. The overall survival (OS), the disease-free survival (DFS), and their difference in BC patients were determined by Kaplan-Meier methods and log-rank tests. Cox's proportional hazard model was adopted for the multivariate assays of independent prognostic indicators. A *p*-value of 0.05 denotes the degree of statistical significance.

3. Results

BSN-AS2 expression was increased in BC patients

We firstly determine the expression levels of BSN-AS2 in 168 BC patients by RT-CPR. As shown in Figure 1, our results indicated that BSN-AS2 presented a distinct up-regulation in BC tissues compared with the adjacent normal bladder specimnes ($p < 0.01$), suggesting BSN-AS2 as a positive regulator in the progression of BC.

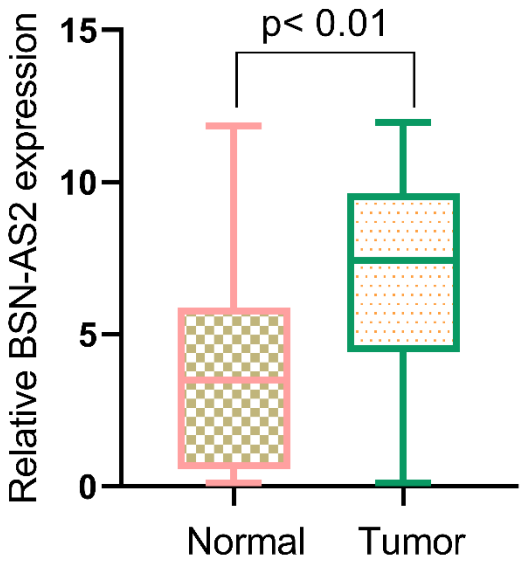

Figure 1 Relative expression levels of BSN-AS2 in 168 paired human BC and normal bladder specimens were measured by qRT-PCR. BSN-AS2 expression was increased in BC tissues compared with bladder specimens ($p < 0.001$).

The diagnostic significance of overexpression of BSN-AS2 in BC

Then, we explored whether BSN-AS2 levels had a diagnostic potential. According to the ROC assays, high BSN-AS2 expression had an AUC value of 0.7698 (95% CI: 0.7189 to 0.8208) for BC (Figure 2). The sensitivity and specificity of BSN-AS2 expressions for distinguishing BC samples from normal samples was 65.31%/82.17%, indicating BSN-AS2 as an indicator for the diagnosis of BC patients.

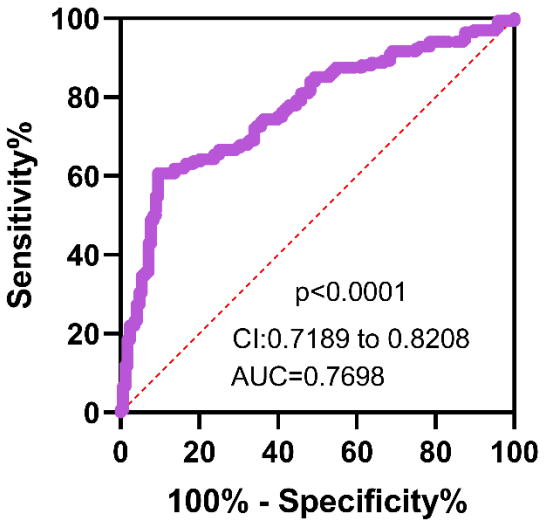

Figure 2 ROC assays for BSN-AS2 as a diagnostic marker for BC patients.

BSN-AS2 associations with clinic and pathological characteristics

To assess the clinical relevance of BSN-AS2 expression in BC, the median expression level of SN-AS2 was used as a cutoff point to divide all 168 patients into two groups (high groups and low groups). Then, we examined the association

between the expression of BSN-AS2 and clinicopathological parameters. Based on Table II, high expression of BSN-AS2 was observed to be distinctly associated with T stage (p = 0.016) and grade (p = 0.007). Nevertheless, other characteristics did not show any significant difference.

Table II Relationship within BSN-AS2 expression and clinicopathological parameters of BC patients.

Characteristic		number	BSN-AS2 expression		*p* value
			Low	High	
Age, years	<60	91	41	50	0.118
	≥60	77	44	33	
Sex	Female	77	39	38	0.990
	Male	91	46	45	
Tumor size (cm)	<3	99	55	44	0.123
	≥3	69	30	39	
T stage	T1	112	64	48	0.016
	T2- 4	56	21	35	
N stage	N0	101	55	46	0.219
	N1	67	30	37	
Grade	Low	110	64	46	0.007
	High	58	21	37	

BSN-AS2 expression is a prognostic biomarker in patients with BC

Kaplan-Meier survival analysis assisted in confirming the association between the expression of BSN-AS2 and 168 BC patients' prognosis. As presented in Figure 3, we observed that patients with high expression of BSN-AS2 had poorer OS (p = 0.0017, Figure 3) and DFS (p < 0.001, Figure 4) compared with those with low BSN-AS2 group. Moreover,

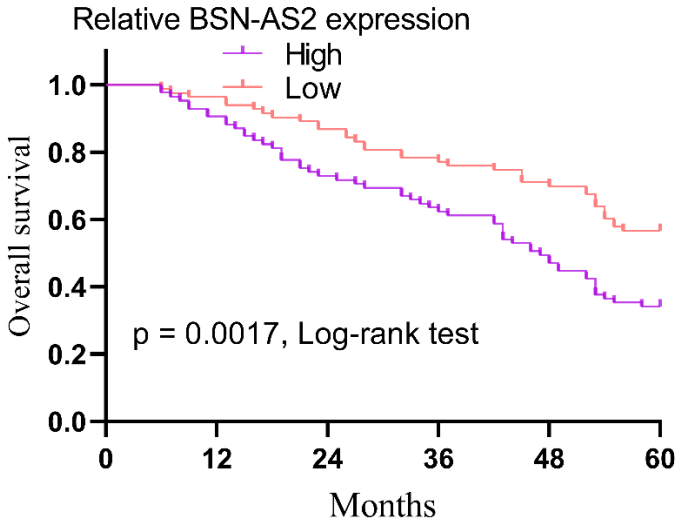

Figure 3 The association between BSN-AS2 expression and OS. The survival analysis revealed that the BC patients with higher expression level of BSN-AS2 had worse 5-year OS (p = 0.0017).

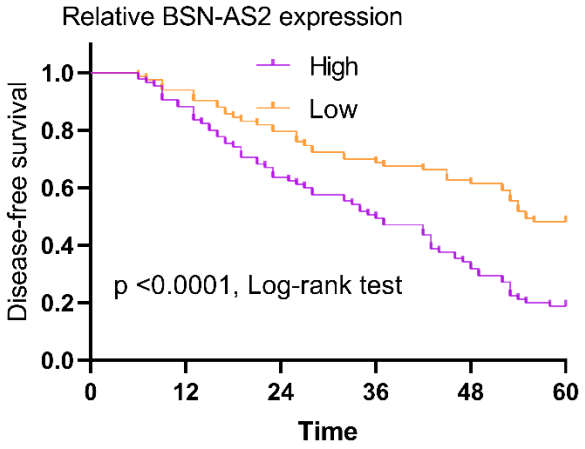

Figure 4 The association between BSN-AS2 expression and DFS. The survival analysis revealed that the BC patients with higher expression level of BSN-AS2 had worse 5-year DFS ($p < 0.0001$).

multivariate assays were performed to determine whether BSN-AS2 was independent factors for prognostic prediction in BC patients. Importantly, the results indicated that expression of BSN-AS2 could be used to independently predict the prognosis of patients in terms of OS (HR=2.782, 95% CI: 1.372-4.652, $p = 0.007$) and DFS (HR=3.018, 95% CI: 1.488-5.121, $p = 0.002$) (Table III).

Table III multivariate analysis of survival in BC patients.

Variables	Overall survival			Disease free survival		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	0.892	0.432-1.893	0.215	1.132	0.673-2.213	0.116
Sex	1.259	0.671-2.411	0.326	0.965	0.562-2.325	0.231
Tumor size	1.482	0.778-2.328	0.218	1.281	0.982-2.218	0.147
T stage	2.893	1.332-4.364	0.016	2.982	1.442-4.563	0.008
N stage	1.123	0.776-1.983	0.213	0.998	0.561-2.135	0.134
Grade	3.026	1.336-4.832	0.006	3.214	1.442-5.012	0.003
BSN-AS2 expression	2.782	1.372-4.652	0.007	3.018	1.488-5.121	0.002

4. Discussion

In China, BC presents the highest incidence rate among all tumors related to urinary system and its mortality has exhibited an obvious increase in the past decade[16]. It has been demonstrated that the early diagnosis could contribute to the systematic optimization of therapeutic schedule in clinical practice, resulting in a favorable clinical outcome for the majority of BC patients[4, 17]. In clinical practice, only a few factors have been used for the clinical screening and the prediction of prognosis of BC patients[18]. However, the poor specificity and sensitivity of those biomarkers limited their clinical application, which needed to be further improved. In recent years, more and more literatures have found the differential expression of lncRNAs in different cancer types, and more and more cellular experiments have confirmed lncRNAs as important regulators in tumor progression, which highlighted their potential used as novel diagnostic and prognostic biomarkers for tumor patients[19, 20].

In recent years, more and more studies have suggested that alteration of the expression or structure of lncRNAs may promote the formation, progression, and metastasis of various tumors, including BC[21, 22]. For instance, as reported by Chen et al, lncRNA MST1P2 was distinctly overexpressed in BC, and its knockdown could resensitize DDP-resistant BC cells to DDP treatment by decreasing miR-133b expression, thus modulating Sirt1/p53 signaling[23]. Yang et al[24] provided evidences that lncRNA LINC00319 was highly expressed in BC, and its overexpression promoted the proliferation and metastasis via the modulation of miRNA-

4492/ROMO1 axis. LncNRA MNX1-AS1, a recent identified BC-related lncRNA, was reported to be overexpressed in BC and predict a poor clinical outcome, indicating its potential to be used as a diagnostic and prognostic biomarker for BC patients. In their lost-of-function study, knockdown of lncNRA MNX1-AS1 was shown to suppress the growth and migration of BC cells via modulating miRNA-218-5p/RAB1A axis[25]. Recently, a novel tumor-associated lncRNA, BSN-AS2, was functionally identified in osteosarcoma. This lncRNA was shown to exhibit a high level in osteosarcoma specimens compared to normal bone specimens. Functional assays confirmed that BSN-AS2 depletion reduced cell proliferation and invasion by regulating miRNA-654-3p/SYTL2[15]. However, whether BSN-AS2 expression was also dysregulated in BC, and its clinical significance have not been investigated.

In this study, we collected 168 pairs of BC specimens and matched normal tissues for the RT-PCR assays which revealed that BSN-AS2 expression was distinctly increased in BC specimens compared to normal bladder specimens. The diagnostic study revealed that high BSN-AS2 expression in the tumor specimens enabled the discrimination of BC patients from normal lung tissues with an AUC of 0.7698, suggesting it as a potential diagnostic biomarker for BC. A clinical study indicated that increased expression of BSN-AS2 was associated with T stage and grade, indicating its positive roles in regulating the clinical progress of BC. Moreover, survival assays confirmed that high BSN-AS2 expression level could lead to a shorter OS and DFS of BC patients. Finally, multivariate assays identified that high BSN-AS2 expression could independently indicate the poor prognosis for BC patients. Our findings suggested that BSN-AS2 is a novel biomarker for the diagnosis and prognosis of BC patients. However, further *in vitro* and *in vivo* assays and a large sample size were needed to confirm our assessment.

5. Conclusions

Our findings revealed that BSN-AS2 was overexpressed in BC and might serve as novel biomarkers to distinguish patients with BC, as well as help us judge the progression of BC.

Conflict of interest

The authors declare that no conflicts of interest exist in the paper.

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