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Original Article

Long non-coding RNA SNHG1 promotes cell proliferation and invasion by competitively binding to miR-145 in breast cancer

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Abstract: Long non-coding RNAs (lncRNA) exert critical functions in the development and progression of breast cancer (BCa). In this study, we investigated the role of lncRNA SNHG1 in BCa. We found that SNHG1 was overexpressed in BCa tissues and cell lines, and increased SNHG1 expression was closely associated with poor prognosis of BCa patients. Further experiments revealed that knockdown of SNHG1 inhibited BCa cell proliferation, migration, and invasion *in vitro*, and suppressed BCa xenograft growth *in vivo*. Through bioinformatic analysis and luciferase reporter assay, we further identified that SNHG1 could bind to miR-145 at predicted binding sites. miR-145 was poorly expressed and negatively correlated with SNHG1 levels in BCa tissues. In summary, these data demonstrate that SNHG1 could act as an "oncogene" for BCa through negative regulation of miR-145.

Keywords: Long non-coding RNA, SNHG1, breast cancer, miR-145, prognosis

Introduction

Breast cancer (BCa) is the leading cause of cancer-related death among the female population worldwide [1]. In 2015, approximately 40,290 BCa-related deaths occurred in the USA [2]. The incidence of BCa is rising at an alarming rate, imposing an enormous economic burden on health care system [3]. Therefore, exploration of novel useful targets for BCa diagnosis and treatment is very important.

Long noncoding RNAs (lncRNAs) are a class of transcribed RNA molecules over 200 nucleotides with no protein-coding capacity [4]. LncRNAs have recently gained widespread attention because aberrant expression of lncRNAs may potentially alter basic cellular biological processes and contribute to tumorigenesis [5]. An increasing number of dysregulated lncRNAs in BCa have been identified in recent years. For example, overexpression of CCAT2, CRNDE, and HOXA-AS2 is capable of enhancing the abilities of proliferation and invasion of BCa cells [6-8].

Several studies have reported that small nucleolar RNA host gene 1 (SNHG1), one kind of lncRNA located on human chromosome 11q-12.3, displays increased expression in wide spectrum of human cancers, including hepatocellular carcinoma [9, 10] and glioma [11]. However, up to now, little has been known about the alteration and functional significance of SNHG1 in BCa.

In the present study, we investigated the expression pattern, biological function, and underlying mechanism of SNHG1 in BCa, and suggest that SNHG1 might be a potential novel diagnostic biomarker and therapeutic target for BCa.

Materials and methods

Tissue specimens and patient characterization

Seventy-four pairs of BCa tissues and their adjacent noncancerous tissues were collected from patients who underwent surgical resections at the First People's Hospital of Lianyungang (Lianyungang, China) after obtaining informed consent. None of the patients received any preoperative radiotherapy or chemotherapy. The clinicopathological characteristics of the BCa patients are recorded in **Table 1**. The

Table 1. Correlation between SNHG1 expression and clinicopathological features of BCa patients

Characteristics	Total number (n=74)	SNHG1 expression		*P*-value
		Low (n=36)	High (n=38)	
Age (years)				0.255
< 50	30	17 (23.0%)	13 (17.6%)	
≥ 50	44	19 (25.7%)	25 (33.8%)	
Tumor size (cm)				0.046
< 2.5	47	27 (36.5%)	20 (27.0%)	
≥ 2.5	27	9 (12.2%)	18 (24.3%)	
Histology grade				0.396
Well	16	10 (13.5%)	6 (8.1%)	
Moderate	38	18 (24.3%)	20 (27.0%)	
Poor	20	8 (10.8%)	12 (16.2%)	
TNM stage				0.035
I-II	51	29 (39.2%)	22 (29.7%)	
III-IV	23	7 (9.5%)	16 (21.6%)	
Lymph nodes metastasis				0.054
Positive	31	11 (14.9%)	20 (27.0%)	
Negative	43	25 (33.8%)	18 (24.3%)	
ER status				0.501
Positive	42	19 (25.7%)	23 (31.1%)	
Negative	32	17 (23.0%)	15 (20.3%)	
PR status				0.358
Positive	35	19 (25.7%)	16 (21.6%)	
Negative	39	17 (23.0%)	22 (29.7%)	
HER2 status				0.632
Positive	39	20 (27.0%)	19 (25.7%)	
Negative	35	16 (21.6%)	19 (25.7%)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal receptor 2.

collected samples were verified by two experienced pathologists. The samples were rapidly frozen in liquid nitrogen and then stored at -80°C. The investigation project was approved by the Ethics Committee of the First People's Hospital of Lianyungang.

Cell culture and transfection

BCa cell lines MDA-MB-231, MDA-MB-468, and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The normal human breast cell line MCF-10A was incubated in DMEM/F12 (1:1) (Hyclone). All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Three small interfering RNAs (siRNAs) targeting SNHG1 (si-SNHG1-1, 5'-CCTTAAAGTGTTAGCAGACACAGAT-3', si-SNHG1-2, 5'-GATTAAGACACTGGGAGCCAATGAA-3' and si-SNHG1-3, 5'-GGGAGCCAATGAAACAGCAGTTGAG-3') and scrambled negative control siRNA were synthesized by Shanghai Genepharma Co., Ltd. (Shanghai, China). BCa cells were seeded into six-well plates, and transfection was performed using Lipofectamine 2000 (Invitrogen). 48 hours post-transfection, transfection efficacy was analyzed by real-time quantitative RT-PCR (qRT-PCR).

RNA extraction and qRT-PCR

Total RNA was extracted from clinical tissue samples and cultured cells using Trizol Reagent (Invitrogen). For miR-145 detection, RNA was reverse transcribed into cDNA using One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA, USA). For SNHG1 detection, cDNA was synthesized using the Primer Script RT reagent Kit (Takara, Dalian, China). SYBR Premix Ex Taq (TaKaRa, Dalian, China) was then used to conduct qRT-PCR on an ABI 7900 system (Applied Biosystems, Foster City, CA, USA). The sequences of the primers are summarized in **Table 2**. The qRT-PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ method [12] and normalized to U6 snRNA or GAPDH mRNA expression.

CCK-8 assay

Cell proliferation was documented every 24 h for 4 days using the Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, MD, USA). Cells were seeded in 96-well plates at 2×10^3 cells per well. 10 μl of CCK-8 solution was added into each well and incubated for another 2 hours at 37°C. Absorbance values were detected at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Table 2. The sequences of the primers

Gene name	Primer sequences
SNHG1 Forward primer	TAACCTGCTTGGCTCAAAGGG
SNHG1 Reverse primer	CAGCCTGGAGTGAACACAGA
GAPDH Forward primer	CGAGATCCCTCCAAAATCAA
GAPDH Reverse primer	TTCACACCCATGACGAACAT
miR-145 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAT
U6 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA
miR-145 Forward primer	GTCCAGTTTTCCCAGGA
miR-145 Reverse primer	GTGCAGGGTCCGAGGT
U6 Forward primer	CTCGCTTCGGCAGCACATATACT
U6 Reverse primer	ACGCTTCACGAATTTGCGTGTC

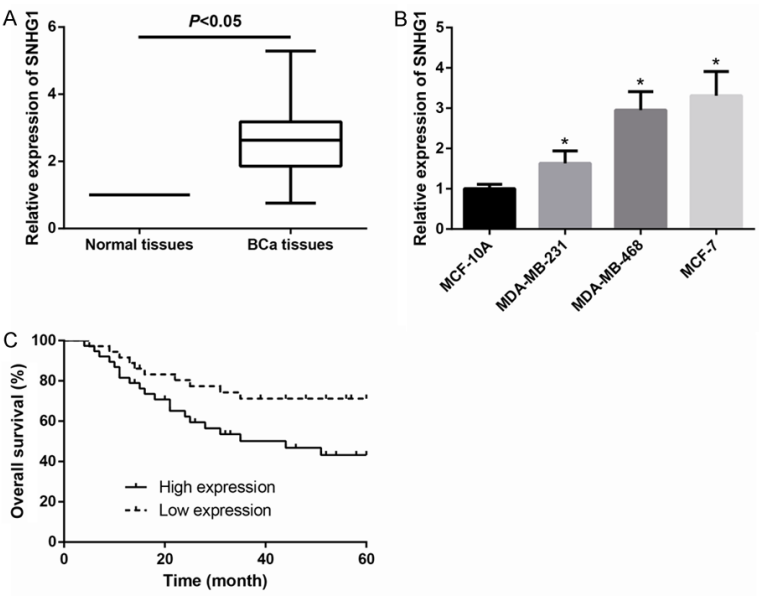

Figure 1. SNHG1 is upregulated in BCa and predicts poor prognosis. A. The expression of SNHG1 in BCa tissues was increased than that in adjacent non-tumor tissues. B. Expression of SNHG1 in BCa cell lines was significantly increased than that in normal MCF-10A cells. Data are presented as the mean ± SD from at least three independent experiments. **P* < 0.05. C. BCa patients with high-expressing SNHG1 showed a significantly reduced overall survival (*P*=0.028).

Transwell assay

Cell migration and invasion assays were performed in a 24-well transwell plate with 8-μm polyethylene terephthalate membrane filters (Costar, Corning, MA, USA). Cells in serum-free medium were added to the upper chambers with either uncoated or Matrigel-coated membranes. Lower chamber was filled with medium containing 10% FBS. After 24 h of incubation, the non-migrated cells on the upper sides of the filters were scraped off, and the cells located in the lower filters were fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. Migrated or invaded cells were counted in five randomly chosen fields per well under a microscope.

In vivo tumorigenesis assay

Five-week-old Balb/c-nu nude male mice, maintained in the specific pathogen-free (SPF) conditions, were randomized to the control or experimental group (6 mice/group). MCF-7 cells (2×10^6) were stably transfected with sh-SNHG1 and sh-NC and then implanted subcutaneously into the back of each nude mice. Tumors were measured every three days and the volume was calculated following the formula: $V=0.5 \times \text{length} \times \text{width}^2$. All mice were sacrificed after 4 weeks, and the tumors were excised and weighed. All experimental procedures were approved by the Ethics Committee of the First People's Hospital of Lianyungang.

Bioinformatics prediction and dual-luciferase reporter assay

The potential microRNA binding sites of SNHG1 predicted by computer-aided algorithms were obtained from starBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). The putative miR-145 target binding sequence in SNHG1 and its mutant of the binding sites were ampli-

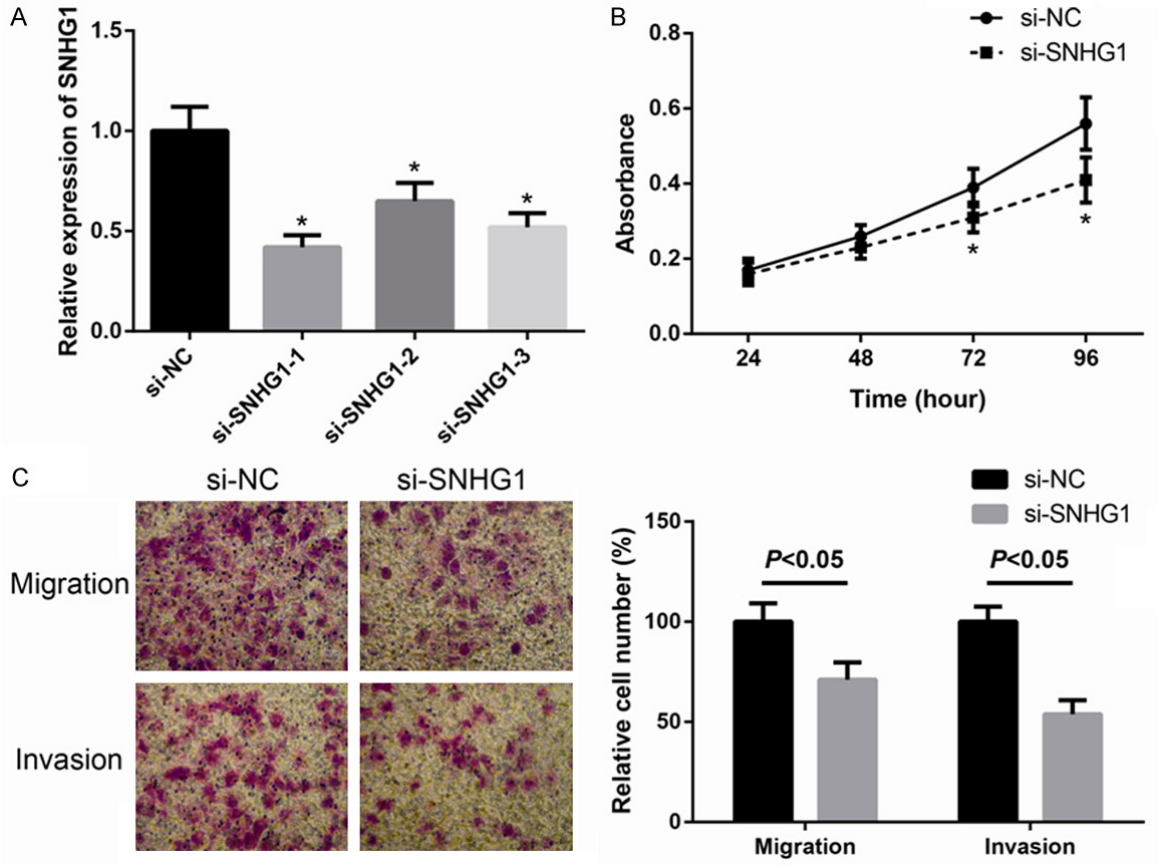

Figure 2. SNHG1 promotes the proliferation, migration and invasion of BCa cells. A. SNHG1-siRNAs could significantly reduce SNHG1 expression in MCF-7 cells. B. CCK-8 assay was performed to evaluate the effect of SNHG1 on MCF-7 cell proliferation. C. Transwell assay was performed to evaluate the effect of SNHG1 on MCF-7 cell migration and invasion. Data are presented as the mean ± SD. *$P < 0.05$.

fied and inserted into downstream of the firefly luciferase gene in a pGL3promoter vector (Promega, Madison, WI, USA), named as: SNHG1-WT or SNHG1-MUT, respectively. For dual-luciferase assay, the luciferase reporter gene vector with SNHG1-WT or SNHG1-MUT, together with miR-145 mimics or mimics control, were co-transfected into BCa cells. At 48 h after transfection, luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase.

Statistical analysis

Each experiment was performed in triplicate and repeated at least three times. The data are expressed as the mean ± SD. Kaplan-Meier plots and log-rank tests were used for survival analysis. All statistical analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism (version 6.01) software (GraphPad Software, Inc., La Jolla, CA, USA). *P* value < 0.05 is considered significant.

Results

SNHG1 is upregulated in BCa and predicts poor prognosis

In order to fully understand the functions of SNHG1 in BCa, we first examined expression of SNHG1 in 74 pairs of BCa tissues and their adjacent non-cancerous tissues by qRT-PCR. We observed that, as demonstrated in **Figure 1A**, SNHG1 levels were markedly higher in 74 BCa tissues in their counterparts. We then examined SNHG1 expression in BCa cell lines, and found that SNHG1 was more highly expressed in BCa cell lines (MDA-MB-231, MDA-MB-468 and MCF-7) than that of in the normal human breast cell line MCF-10A (**Figure 1B**).

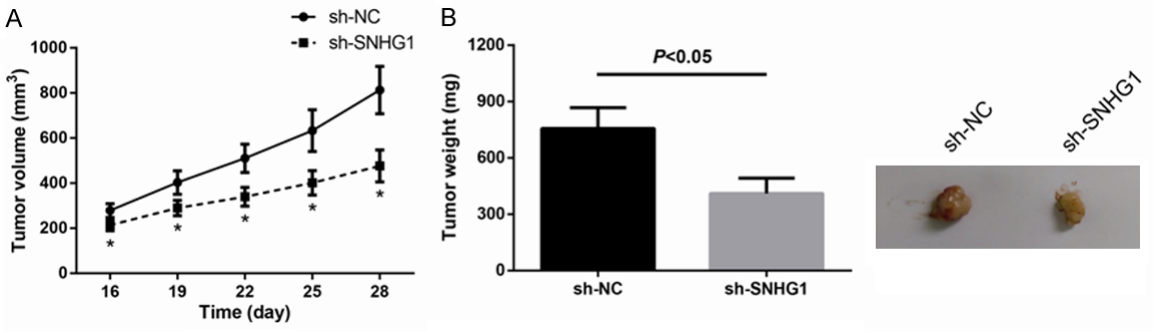

Figure 3. SNHG1 promotes the growth of BCa xenografts *in vivo*. A. Tumor volume was calculated every 3 days. B. Four days after implantation, tumors were excised and weighted. Data are presented as the mean ± SD (n=6 per group). **P* < 0.05.

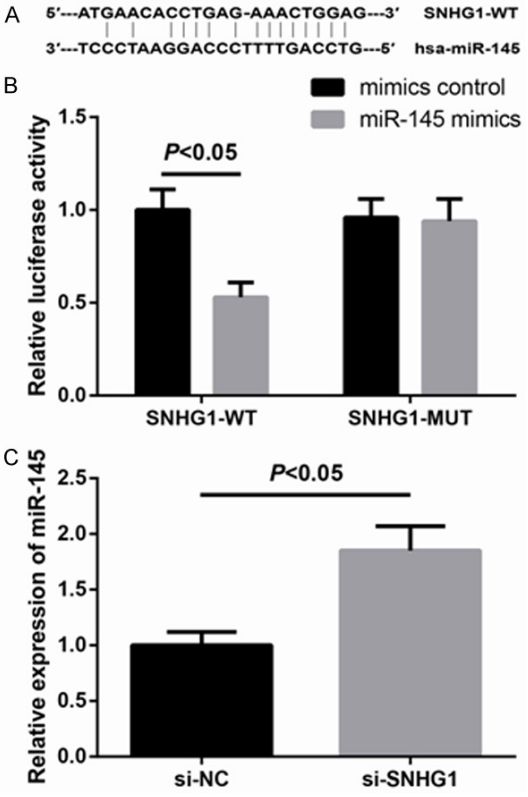

Figure 4. SNHG1 functions as a ceRNA for miR-145 in BCa cells. A. The predicted miR-145 binding sites in SNHG1 are shown. B. Luciferase assay shows that miR-145 mimics reduced the luciferase activity of SNHG1-WT in MCF-7 cells. C. The expression of miR-145 was increased in MCF-7 cells with SNHG1 inhibition. Data are presented as the mean ± SD from at least three independent experiments.

To further explore the clinical significance of SNHG1 in BCa, the association between SNHG1 expression and the clinicopathological status of 74 BCa patients was analyzed. BCa patients were divided into a high expression group (≥ mean, n=38) and a low expression group (< mean, n=36) on the basis of the cutoff value of SNHG1 expression. As indicated in **Table 2**, increased SNHG1 expression level in BCa tissues was significantly associated with tumor size (*P*=0.046) and TNM stage (*P*=0.035). Kaplan-Meier analysis revealed that high SNHG1 expression was closely related to a poorer overall survival in BCa patients (log-rank test, *P*=0.028; **Figure 1C**).

SNHG1 promotes the proliferation, migration and invasion of BCa cells

Since our clinical data showed that SNHG1 was high expressed in BCa, we further investigated its oncogenic features and effects on BCa cell lines. MCF-7 cells were transiently transfected with three siRNAs targeting SNHG1, which efficiently silenced endogenous expression of SNHG1 (**Figure 2A**). si-SNHG1-1 caused the lowest level of SNHG1 and was accordingly selected for further study. CCK-8 assay were performed to assess the role of SNHG1 in BCa cell proliferation and the results showed that the BCa cells transfected with si-SNHG1 grew slower compared to control cells (**Figure 2B**). Furthermore, as shown in **Figure 2C**, MCF-7 cells with reduced SNHG1 expression exhibited decreased migration and invasion compared to control cells. These findings indicate that down-regulation of SNHG1 inhibits BCa cell proliferation, migration, and invasion.

SNHG1 promotes growth of BCa xenografts in vivo

To confirm the above data *in vivo*, MCF-7 cells stably transfected with sh-SNHG1 or sh-NC were injected subcutaneously into nude mice, respectively. Xenografts tumor volume was measured every three days after a palpable

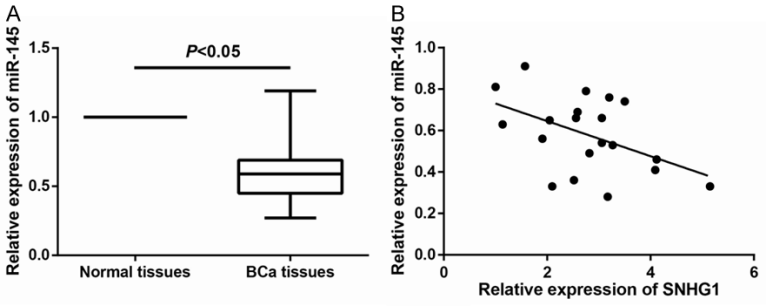

Figure 5. miR-145 expression is negatively associated with SNHG1 expression in BCa tissues. A. Expression of miR-145 in BCa tissues was reduced compared to that in adjacent non-tumor tissues. B. Pearson correlation analysis shows a negative association between SNHG1 and miR-145 expression in BCa tissues.

tumor formed, and mice were killed four weeks after cell implantation. As shown in **Figure 3A**, tumors derived from the sh-SNHG1 group grew at a slower rate than sh-NC group, and the tumor weight in the sh-SNHG1 group was significantly less than sh-NC group (**Figure 3B**). Thus, silencing of SNHG1 could markedly inhibit tumorigenesis of BCa cells *in vivo*.

SNHG1 functions as a ceRNA for miR-145 in BCa cells

Recent studies have reported lncRNAs could act as molecular sponges or ceRNAs to regulate the biological functions of miRNAs. To further clarify the molecular mechanisms of SNHG1 on the biological phenotypes of BCa cells, we searched for the target miRNAs using starBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). miR-145 was thus selected as one of the candidate targets of SNHG1, because of the putative target sequences, as shown in **Figure 4A**. To further confirm whether SNHG1 is a functional target for miR-145, luciferase activity assay was performed. Through the luciferase activity assay, we found that miR-145 mimics markedly inhibited SNHG1-WT reporter activity, while it had nearly no inhibitory effect on the SNHG1-MUT reporter activity (**Figure 4B**). Additionally, we found that inhibition of SNHG1 led to remarkably increased expression of miR-145in MCF-7 cells (**Figure 4C**).

miR-145 expression is negatively associated with SNHG1 expression in BCa tissues

To further determine the association between SNHG1 and miR-145 in BCa, we examined expression of miR-145 in BCa tissues and normal tissues by qRT-PCR. As shown in **Figure 5A**, miR-145 expression was significantly reduced in BCa tissues compared with that in normal tissues. Furthermore, as shown in **Figure 5B**, miR-145 expression was negatively correlated with the relative SNHG1 expression in BCa tissues (r=-0.478, *P*=0.033).

Discussion

Despite many efforts in recent years, the survival rate of BCa still remains dismal. The etiology of BCa involves a complex interplay of various factors. Recently, accumulating studies have indicated that lncRNAs play important roles in regulating various cellular processes [13] and act as drivers of tumor suppressive and oncogenic functions in the development and progression of BCa [14]. Therefore a better understanding of correlation between lncRNAs and tumor etiology will provide novel therapeutic targets for the early diagnosis and treatment of BCa. SNHG1 is a recently discovered lncRNA, which has not yet been extensively explored in human BCa.

In the present study, we studied the role of SNHG1 in BCa progression. Based on the results of qRT-PCR, we discovered that the expression of SNHG1 was significantly increased in BCa tissues and cell lines, which is consistent with earlier studies in colorectal carcinoma [15] and prostate cancer [16]. Further, we explored the association between SNHG1 expression and clinical characteristics of BCa patients, and observed that patients with highly expressed SNHG1 had more aggressive characteristics and unfavorable prognosis.

In order to highlight the function of SNHG1 in BCa, we further explored the critical roles of SNHG1 in the progression of BCa by loss-of-function analysis. Our data showed that knockdown of SNHG1 contributed to significant inhibition of BCa cell proliferation, migration, and invasion *in vitro*. Additionally, downregulation of SNHG1 could inhibit the BCa tumorigenesis in murine model. Taken together, these findings suggest that SNHG1 might function as a potential oncogene to promote BCa development and progression.

The ceRNA hypothesis is gaining attention. This hypothesis suggests that lncRNA could function as a competing endogenous RNA (ceRNA) or a molecular sponge in regulating the expression patterns and biological functions of miRNA [17]. It has been reported that miR-101-3p is a direct target of SNHG1 in non-small cell lung cancer [18], indicating that SNHG1 might be a natural sponge for miRNAs. In the present study, miRNA complementary base pairing with SNHG1 was predicted by bioinformatics method, and we discovered miR-145 might form complementary base pairing with SNHG1. MiR-145 was previously shown to be a tumor repressor, including BCa [19, 20], and we therefore speculated that SNHG1 might promote BCa progression in a miR-145-dependent manner.

In summary, the present study offers the first direct investigation of a relationship between SNHG1 and BCa progression. We also observed that SNHG1 might function as a ceRNA to attenuate the endogenous function of miR-145 in BCa. However, other possible mechanisms by which SNHG1 participates in BCa remain to be further explored.

Disclosure of conflict of interest

None.

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