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ORIGINAL ARTICLE

Circ_0006948 drives the malignant development of bladder cancer *via* activating the epithelial-mesenchymal transition

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Summary

Purpose: *To detect the expression characteristic of circ_0006948 in bladder cancer (BC), and to analyze its relationship with pathological parameters and prognosis in BC patients. In addition, molecular mechanisms of circ_0006948 on driving the malignant progression of BC by activating epithelial-mesenchymal transition (EMT) was explored.*

Methods: *Circ_0006948 levels in 72 BC and paracancerous tissues were detected, and their relationship with pathological parameters and prognosis in BC patients was analyzed by chi-square test. After establishing circ_0006948 knockdown model in 253j and T24 cells, phenotype changes were assessed by cell counting kit-8 (CCK-8), transwell and wound healing assay. Regulatory effects of circ_0006948 on EMT-associated gene expressions in BC cells were determined by Western blot. Finally, the interaction between circ_0006948 and N-cadherin was evaluated by rescue experiments.*

Results: *Circ_0006948 was upregulated in BC tissues and cell lines. High level of circ_0006948 indicated advanced tumor stage, high rates of lymph node metastasis and distant metastasis, and poor prognosis in BC. Knockdown of circ_0006948 reduced proliferative and metastatic abilities in BC cells. The key protein in the EMT signaling E-cadherin was upregulated by knockdown of circ_0006948 in BC cells, while N-cadherin, Vimentin, β-catenin and MMP-9 were downregulated. The interaction between circ_0006948 and N-cadherin was identified, and they were co-responsible for the malignant development of BC.*

Conclusions: *Circ_0006948 is upregulated in BC samples, and it is closely linked to tumor stage, metastasis and prognosis in BC patients. It drives proliferative and metastatic abilities in BC cells by activating EMT.*

Key words: *Circ_0006948, EMT, bladder cancer, malignant development*

Introduction

Bladder cancer (BC) is a common malignant tumor of the human urinary system, and bladder urothelial carcinoma is the major subtype [1]. There are about 430,000 new cases of BC and 170,000 deaths each year in the world [1,2]. Its incidence and mortality are on the rise because of aging population, changes in lifestyles and diets and environment pollution. In China, BC has the highest incidence in the urinary system [3]. Therapeutic outcomes of electrosurgical resection, bladder irrigation and radical cystectomy for primary BC have been greatly improved. However, recurrent or metastatic BC is difficult to be treated, leading to a poor prognosis [4-6]. Diagnosis and treatment of BC pose a great burden on medical cost [3,7]. Over 65% BC patients are confirmed as non-muscle invasive bladder cancer (NMIBC) at the first diagnosis, and transurethral resection of the bladder tumor (TUR-BT) is preferred [8-10]. Nevertheless, the postoperative recurrence following TUR-BT frequently occurs and results in treatment failure. Diagnosis and intervention of BC as early as possible contribute to improve the survival [10,11]. Current researches mainly focus on the regulation of relevant proteins and pathways in the development of BC, while the post-transcriptional translation of

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genes and epigenetic studies are few [11,12]. The role of non-coding RNA, especially circRNA, in BC is rarely reported [12,13].

CircRNAs, which are different from linear RNAs, are featured by the circular structure connected by the 3' and 5' ends [14,15]. They are resistant to exonuclease-mediated degradation and more stable than most linear RNAs in cells [15,16]. A recent study analyzed 27,000 circRNAs in tumor profiling using the microarrays and they found that many circRNAs are differentially expressed between tumor and normal tissues. Furthermore, they pointed out that circ_0006948 is abnormally expressed in many types of tumor tissues. Notably, the abundance of circ_0006948 is dozens of times higher than that of its parent mRNA. Circ_0006948 is considered to be a promising tumor hallmark [17].

During the process of malignant tumors that are derived from epithelial cells, the epithelial-mesenchymal transition (EMT) is a vital event [18,19]. Characteristics of adhesion, junction and cell polarity in epithelial cells are lost, and then characteristics of mesenchymal cells (i.e. deformation and migratory potentials) are acquired. EMT has been highlighted in the development of human tumors [20,21]. EMT leads to the enhancement of metastasis potential and drug resistance in tumor cells [21,22]. In this article, we mainly explored the role of circ_0006948 in the malignant development of BC and the involvement of EMT.

Methods

BC patients and tumor samples

BC and paracancerous tissues were surgically resected from 72 BC patients. Tumor staging of BC was diagnosed according to the TNM criteria. None of them had preoperative anti-cancer treatment. This study got approval by Ethics Committee of Hanyang Hospital Affiliated to Wuhan University of Science and Technology (approval number HB-03-WH-HY-13560) and it was conducted after getting signed informed consent from each patient.

Cell lines and reagents

BC cell lines (EJ, T24, 253j, J82, 5637) and the urothelial cell line (SV-HUC-1) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added in the culture medium.

Transfection

Cells were inoculated in 6-well plates and cultured to 30-50% confluence. They were transfected with plasmids constructed by GenePharma (Shanghai, China) using Lipofectamine 3000 (Invitrogen, USA). Transfection efficacy was tested by quantitative real-time polymerase chain reaction (qRT-PCR) at 48 h.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in 96-well plates with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

Transwell migration assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μL of suspension (2.0×10^5 cells/mL) was added in the upper layer of a chamber with 500 μL of medium containing 20% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 5 random selected fields per sample.

Wound healing assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 h later, wound closure was captured for calculating the percentage of wound healing.

QRT-PCR

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary DNAs (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). Primers used in qRT-PCR were synthesized using Primer 5.0 software. The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of β-actin. Circ_0006948: Forward: 5'-AGCCGGTCCAGTACACCTTT-3', Reverse: 5'-GGAAAGCACCGTCTGTTGTT-3'; β-actin: Forward: 5'-CCTGGCACCCAGCACAAT-3', Reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Primary and secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were applied for indicated time. Band exposure and analyses were finally conducted.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the t-test. The relationship between circ_0006948 level and pathological parameters in BC patients was evaluated by Chi-square test. $P<0.05$ was considered as statistically significant.

Results

Highly expressed circ_0006948 in BC tissues and cell lines

Compared with paracancerous tissues, circ_0006948 was upregulated in BC tissues (Figure 1A). Meanwhile, it was also highly expressed in BC cell lines than the urothelial cell line, especially 253j and T24 cell lines (Figure 1B).

Circ_0006948 expression was correlated with tumor staging and metastasis in BC patients

Recruited BC patients were divided into two groups according to the median level of circ_0006948 in BC tissues, and the case number was respectively calculated. Chi-square analysis showed that circ_0006948 level was positively correlated to tumor staging and the rates of lymph node metastasis and distant metastasis, while it was unrelated to other pathological parameters in BC patients (Table 1). It is suggested that circ_0006948 could be an oncogene during the development of BC.

Knockdown of circ_0006948 weakened proliferative and metastatic potentials in BC

We successfully constructed circ_0006948 knockdown model in 253j and T24 cells by transfection of sh-circ_0006948 (Figure 2A). Compared with those transfected with sh-NC, BC cells transfected with sh-circ_0006948 had a lower viability, indicating the suppressed proliferative ability (Figure 2B). In addition, both transwell assay and wound healing assay showed that knockdown of circ_0006948 decreased the migratory potential in BC cells (Figure 2C, 2D).

Knockdown of circ_0006948 reduced the activity in the EMT signaling

To analyze the potential mechanisms of circ_0006948 on regulating the proliferative and

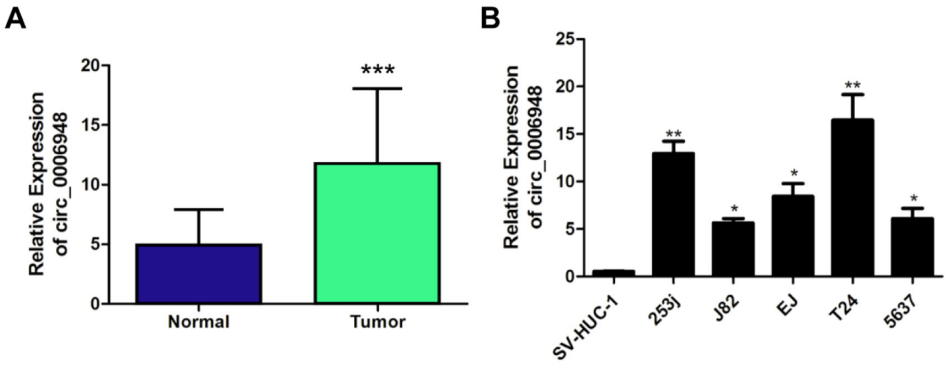

Figure 1. Highly expressed circ_0006948 in BC tissues and cell lines. **A:** Circ_0006948 levels in BC tissues and paracancerous ones. **B:** Circ_0006948 levels in BC cell lines. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

Table 1. Clinicopathologic characteristics of the individuals with bladder cancer in Low- and High-expression groups of circ_0006948

Items	*Number of cases*	*circ_0006948 expression*		*p value*
		Low (n=42)	*High (n=30)*	
Age (years)				0.467
<60	30	19	11	
≥60	42	23	19	
T stage				0.014
T1-T2	41	29	12	
T3-T4	31	13	18	
Lymph node metastasis				0.017
No	43	30	13	
Yes	29	12	17	
Distant metastasis				0.043
No	48	32	16	
Yes	24	10	14	

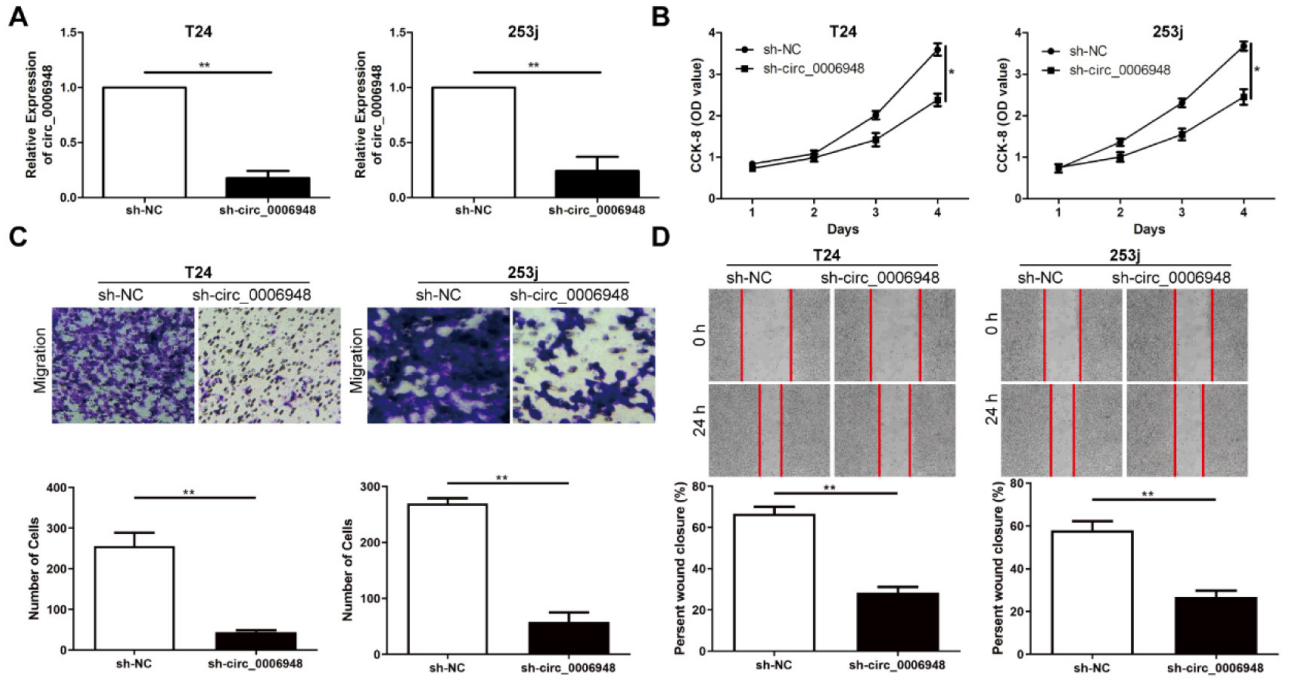

Figure 2. Knockdown of circ_0006948 weakened proliferative and metastatic potentials in BC. **A:** Transfection efficacy of sh-circ_0006948 in 253j and T24 cells. **B:** Viability in 253j and T24 cells transfected with sh-NC or sh-circ_0006948. **C:** Migratory cell number in 253j and T24 cells transfected with sh-NC or sh-circ_0006948. **D:** Wound healing percentage in 253j and T24 cells transfected with sh-NC or sh-circ_0006948. Data were expressed as mean±SD. *p<0.05, **p<0.01.

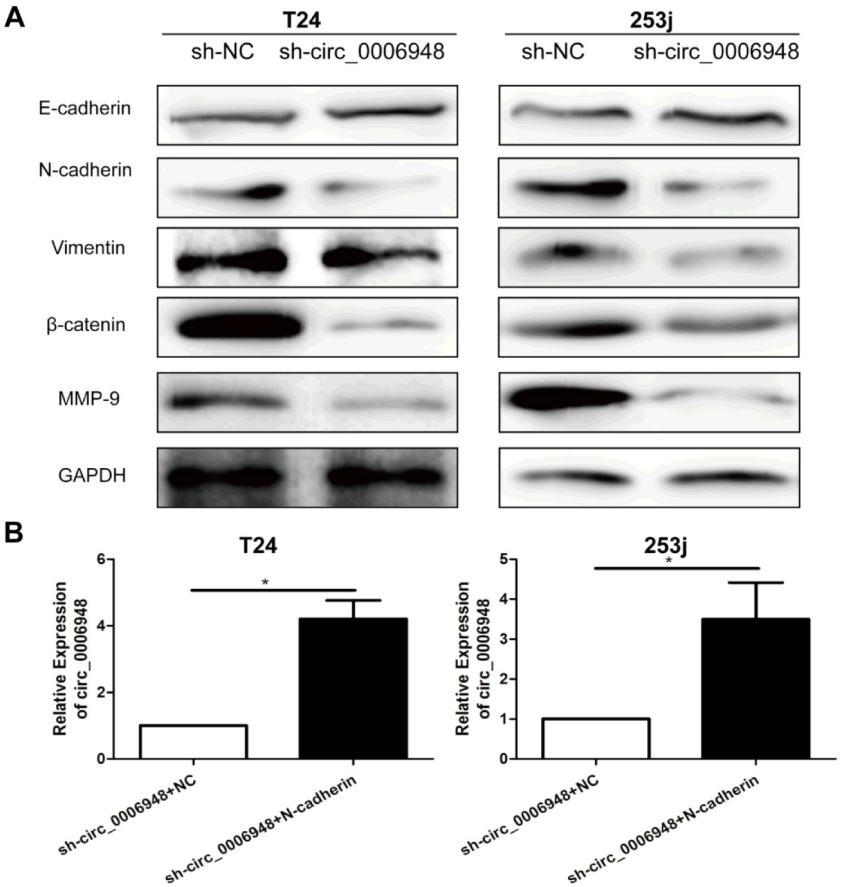

Figure 3. Knockdown of circ_0006948 reduced the activity in the EMT signaling. **A:** Protein levels of E-cadherin, N-cadherin, Vimentin, β-catenin and MMP-9 in 253j and T24 cells transfected with sh-NC or sh-circ_0006948. **B:** N-cadherin level in 253j and T24 cells co-regulated by circ_0006948 and N-cadherin. Data were expressed as mean±SD. *p<0.05.

migratory potentials in BC cells, protein levels of EMT-associated genes were detected by Western blot. As the results showed, the key protein in the EMT signaling, E-cadherin was upregulated by knockdown of circ_0006948 in BC cells, while N-cadherin, Vimentin, β-catenin and MMP-9 were downregulated (Figure 3A). To further identify the interaction between N-cadherin and circ_0006948, we co-transfected sh-circ_0006948 and pcDNA-N-cadherin in BC cells. Overexpression of N-cadherin markedly upregulated circ_0006948 in BC cells with circ_0006948 knockdown (Figure 3B).

N-cadherin was involved in BC cell phenotypes regulated by circ_0006948

We thereafter speculated that N-cadherin was closely involved in the malignant development of BC regulated by circ_0006948. Transfection efficacy of pcDNA-N-cadherin was examined in BC cells (Figure 4A). Compared with BC cells with circ_0006948 knockdown, those co-transfected with sh-circ_0006948 and pcDNA-N-cadherin had a higher viability (Figure 4B). As expected, the weakened migratory ability induced by knockdown of circ_0006948 was partially abolished by overexpression of N-cadherin (Figure 4C, 4D).

Discussion

Metastasis is the major reason for BC death. About 70% of patients develop recurrence after TUR-BT. The 5-year survival of NMIBC following TUR-BT remains 60-70% [3-6]. It must be recognized that life quality in BC patients is severely affected after surgery or chemotherapy/radiotherapy [6,7]. Nowadays, molecular biotherapy is becoming a viable salvage treatment for BC patients who are poorly responders to conventional treatment [7,8]. However, low efficacy, poor targeting and unstable effect of molecular biotherapy greatly limit its clinical application [8-10]. Searching for effective and sensitive hallmarks of BC is beneficial to improve the diagnostic and therapeutic efficacy [11-13].

CircRNAs is a class of non-coding RNAs without 3' and 5' ends. They are in a circular structure by covalent bonding [14]. Unlike traditional linear RNAs, circRNAs are more stable and less prone to degradation [15-17]. The functional roles of circRNAs remain largely unclear [16,17]. It is reported that circRNAs may be related to atherosclerotic vascular diseases, neurological diseases and cancers. They are potential biomarkers for cancer diagnosis and treatment [23,24]. A recent report has

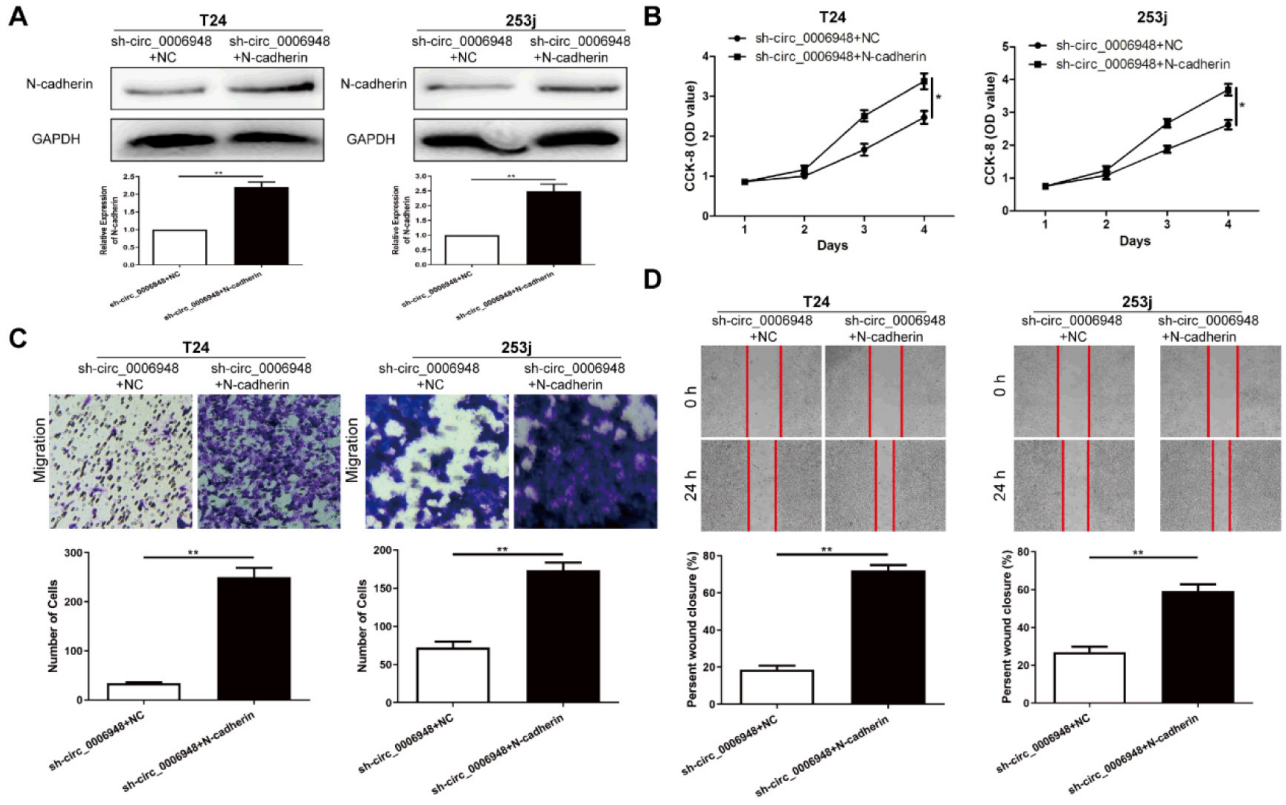

Figure 4. N-cadherin was involved in BC cell phenotypes regulated by circ_0006948. **A:** Transfection efficacy of pcDNA-N-cadherin in 253j and T24 cells. **B:** Viability in 253j and T24 cells co-regulated by circ_0006948 and N-cadherin. **C:** Migratory cell number in 253j and T24 cells co-regulated by circ_0006948 and N-cadherin. **D:** Wound healing percentage in 253j and T24 cells co-regulated by circ_0006948 and N-cadherin. Data were expressed as mean±SD. *p<0.05, **p<0.01.

found the vital role of circ_0006948 in a variety of diseases, including tumors [17]. In this paper, we explored the biological functions of circ_0006948 in BC cell behaviors and its potential influence on the prognosis in BC. By detecting circ_0006948 level in 72 BC tissues we collected, it was found that circ_0006948 was markedly upregulated. In addition, its level was related to tumor staging, lymph node metastasis, distant metastasis and poor prognosis in BC patients. Subsequently, we constructed circ_0006948 knockdown model to uncover its *in vitro* effects on BC cells. Functional experiments results revealed the promotive effects of circ_0006948 on the proliferative and metastatic potentials in BC.

EMT helps tumor cells gain invasiveness through a series of morphological and physiological changes [18-20]. The initiation process of EMT includes the loss of E-cadherin and expression changes of EMT regulators, such as Snail, Slug, Twist, etc. [20-22]. Here, knockdown of circ_0006948 upregulated E-cadherin, and downregulated N-cadherin, Vimentin, β-catenin and MMP-9, suggesting the regulatory effects of circ_0006948 on EMT in BC cells. Furthermore, overexpression of N-cadherin could reverse the weakened proliferative and metastatic abilities in BC cells with circ_0006948 knockdown. We believed that circ_0006948 exerted regulatory effect on EMT, thus driving the malignant development of BC. Our findings provide experimental evidence for diagnosis, treatment and prognosis evaluation in BC patients.

Conclusions

Circ_0006948 is upregulated in BC samples, and it is closely linked to tumor staging, metastasis and prognosis in BC patients and it drives proliferative and metastatic abilities in BC cells by activating EMT.

Conflict of interests

The authors declare no conflict of interests.

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