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Increased EWSAT1 expression promotes cell proliferation, invasion and epithelial-mesenchymal transition in colorectal cancer

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) have recently been identified as crucial regulators in colorectal cancer (CRC) progression. The aim of the study is to investigate the clinical role and biological effects of long non-coding RNA EWSAT1 in CRC.

PATIENTS AND METHODS: The expression of lncRNA EWSAT1 was detected in 106 cases of fresh CRC tissues and matched adjacent normal tissues by qRT-PCR analyses. The Kaplan-Meier analysis and log-rank test were used to assess the association between lncRNA EWSAT1 expression and overall survival (OS) rate of CRC patients. Cell proliferation and invasion capacity were evaluated by CCK8 assay, colony formation, and transwell invasion assays. The protein expression was detected using western blot analysis.

RESULTS: LncRNA EWSAT1 expression was abnormally higher in CRC tissues compared to matched adjacent normal tissues. Higher lncRNA EWSAT1 expression significantly associated with depth of invasion, lymph node metastasis, and advanced tumor-node-metastasis (TNM) stage in CRC patients. Patients with higher EWSAT1 expression exhibited shorter OS compared with patients with lower EWSAT1 expression. Furthermore, lncRNA EWSAT1 knockdown significantly suppressed cell proliferation and invasion in CRC. In addition, lncRNA EWSAT1 knockdown suppressed cell epithelial-mesenchymal transition (EMT) through reducing Snail1, Snail2, and N-cadherin expression, but increasing E-cadherin expression in CRC cells.

CONCLUSIONS: Downregulation of lncRNA EWSAT1 suppressed cell proliferation and invasion of CRC, which indicated that EWSAT1 may be a potential target of CRC treatment.

Key Words:
Colorectal cancer, Long non-coding RNA, EWSAT1, Prognosis, Cell invasion.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-associated mortality worldwide[1]. More than 1.2 million cases are diagnosed with colorectal cancer every year, and about 600,000 cases die from the disease[2]. Death often occurs due to tumor relapse and metastasis[3]. CRC carcinogenesis significantly associated with various alterations of oncogenes or tumor suppressor genes[4]. Thus, to explore a novel understanding of the genomic landscape involved in CRC and therapeutic targets of CRC is needed.

Long non-coding RNAs (lncRNAs) have been found to play comprehensive roles in a series of diseases including cancer[5]. Some lncRNAs function as oncogenes or tumor suppressor genes in CRC development, and participate in CRC carcinogenesis[6]. Such as, lncRNA ANCR down-regulation suppresses invasion and migration of colorectal cancer cells by regulating EZH2 expression[7]. Up-regulated NNT-AS1 contributes to proliferation and migration of colorectal cancer cells *in vitro* and *in vivo*[8]. Silencing of long non-coding RNA SBDSP1 suppresses tumor growth and invasion of colorectal cancer[9]. HOTAIRM1 is identified as a potential biomarker for diagnosis of colorectal cancer[10]. LncRNA-UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204-5p[11]. These previous study indicated that lncRNAs play crucial roles in CRC. However, the clinical significance and biological functions of long non-coding RNA EWSAT1 in colorectal cancer is little known.

We demonstrated that lncRNA EWSAT1 is significantly higher expressed in CRC tissues. CRC

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patients with higher lncRNA EWSAT1 expression levels exhibited a poor prognosis. LncRNA EWSAT1 knockdown inhibited cell proliferation, invasion, and epithelial-mesenchymal transition process of CRC. Thus, our results suggested that lncRNA EWSAT1 may be a potential target of CRC treatment.

Patients and Methods

Tissue Sample Collection

A total of 106 cases of fresh CRC tissues and pair-matched adjacent normal tissues were obtained from the Department of Colorectal Surgery, Cancer Hospital of China Medical University between March 2008 and April 2011. All of the tumor tissues derived from surgery were immediately stored in liquid nitrogen for further RNA analysis. All of the patients do not receive preoperative treatment including radiation or chemotherapy. A five-year follow-up was performed after surgery in CRC patients. The written informed consents were signed from all subjects. This research was approved by the Review Board and Ethics Committee of Cancer Hospital of China Medical University.

Cell Culture

Three human CRC cell lines (SW480, HT29, and SW620) and an intestinal epithelial cell line (NCM460) used in this study were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). CRC cell lines were incubated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Quantitative Real Time-PCR (QRT-PCR) Analysis

Total RNA was extracted from CRC tissues or cells using TRIzol Reagent (Takara Biotechnology Co., Ltd, Dalian, China). The 1 μg of total RNA was reversed to complementary DNA (cDNA) by using Prime Script RT reagent kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd, Dalian, China). QRT-PCR was performed on an ABI 7500 Real Time-PCR system (Applied Biosystems, Foster City, CA, USA) by using a SYBR Premix EX Taq™ Kit (TaKaRa Biotechnology Co., Ltd, Dalian, China). The reaction condition was as follow: pre-denaturation at 95°C for 32 sec, followed by 40 cycles of 95°C for 5 sec, 55°C for 30 sec, and 72°C for 31 sec. The primers used in the study are as follows: lncRNA EWSAT1 forward, 5'-GTGTCTGGCAAGGAACACTA-3', and lncRNA EWSAT1 reverse, 5'-GGTGGAGAAGAGGGACAATAAG-3'. GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3', and GAPDH reverse, 5'-GCCCAATACGACCAAATCC-3'. The mRNA expression was determined using $2^{-\Delta\Delta Ct}$ methods.

Cell Transfection

Lentivirus expressing shRNAs against lncRNA EWSAT1 and negative control shRNA were synthesized and purchased from Shanghai GenePharma Co., Ltd (Shanghai, China). Cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of the two designed lncRNA EWSAT1 shRNAs were as follows: shRNA-lncRNA EWSAT1-1 (sh-1), 5'-TTGGGCTCTCAATGGTATCAT-3' and shRNA-lncRNA EWSAT1-2 (sh-2), 5'-AAGGGAGGGTTACTAACTTTA-3'.

Cell Proliferation Assay

Cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. Briefly, transfected cells (2000 cells/per well) were seeded in 96-well plates. After cells were cultured at 1, 2, 3, and 4 days, the CCK-8 solution was added to each well and were maintained for 2 h at 37°C in a humidified atmosphere with 5% CO_2. The absorbance was measured at 450 nm by a microplate reader victor (BioTek Instruments, Inc., Winooski, VT, USA).

Colony Formation Assay

The transfected cells (500 cells/per well) were seeded in 12-well plates. Then, cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 for 14 days. The cells were then fixed with methanol, stained using crystal violet, and the colonies number was counted. The experiments were performed at least three times.

Transwell Invasion Assay

Cell invasion ability was evaluated using a 24-well transwell chamber (Costar, MA, USA) with Matrigel (1 mg/ml) (BD Biosciences, Franklin Lakes, NJ, USA). 1×10^5 transfected cells in serum-free medium were seeded on the upper

chambers. Medium with 10% fetal bovine serum (FBS) was added on the lower chamber contained. Then, cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 for 24h. The cells on the lower chamber were fixed and stained using crystal violet and counted using a microscope (Olympus Corp., Tokyo, Japan).

Western Blot Analysis

Transfected cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China). The equal quantity (40 μg) protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretic transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk and then were incubated with specific primary antibodies with Snail1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Snail2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (1:1000, Cell Signaling Technology, MA, USA, 1:1000), N-cadherin (1:1000, Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:2000, Cell Signaling Technology, Danvers, MA, USA). Following by incubation with horseradish peroxidase (HRP)-linked secondary antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA), the proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA). The density of the blots was quantified using Image J software with the ChemiDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of β-actin was used as a control for normalization.

Statistical Analysis

Statistical analysis was carried out using SPSS software (version 20.0; IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). The results are presented as the mean ± standard deviation. Differences between two groups were analyzed using the Student's *t*-test. Differences among more than two groups were analyzed using one-way analysis of variance (ANOVA) and post-hoc test (Student-Newman-Keuls method) were used following the performance of ANOVA. Kaplan-Meier analysis and log-rank test were used for survival analysis. A $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of lncRNA EWSAT1 is Higher in Human CRC Tissues and Cell Lines

The relative expression of lncRNA EWSAT1 was examined by qRT-PCR assay in 106 CRC tissue and pair-matched adjacent normal tissues samples. The lncRNA EWSAT1 mRNA expression normalized to GAPDH. As shown in Figure 1A, the results showed that the expression of lncRNA EWSAT1 was significantly higher in CRC tissues compared with matched adjacent normal tissues samples ($p < 0.01$). Furthermore, we detected the expression of lncRNA EWSAT1 in three human CRC cell lines (SW480, HT29, and SW620) and an intestinal epithelial cell line (NCM460). Results analysis showed that lncRNA EWSAT1 expression was notably higher in human CRC cell lines than that in NCM460 cell line ($p < 0.05$, Figure 1B). Additionally, we analyzed the association between expression of lncRNA EWSAT1 and clinicopathological data. As listed in Table I, the results showed that higher lncRNA EWSAT1 expression associated with depth of invasion ($p = 0.011$), lymph node metastasis ($p = 0.014$), and tumor-node-metastasis (TNM) stage ($p = 0.008$) in CRC patients. Patients with higher EWSAT1 expression levels exhibited shorter overall survival rates compared with patients with lower EWSAT1 expression levels (log-rank= 8.931, $p < 0.05$, Figure 1C).

Decreased lncRNA EWSAT1 Suppresses CRC Cell Growth and Colony Formation

Furthermore, we assessed the effects of lncRNA EWSAT1 expression on cell proliferation. The SW480 and SW620 cell lines were transfected with sh-EWSAT1-1 and sh-EWSAT1-2 in order to induce lncRNA EWSAT1 silencing. The sh-EWSAT1-1 could markedly reduce lncRNA EWSAT1 expression (89%) and used in the following experiments (Figure 2A-2B). Growth curves determined by CCK8 analysis showed that lncRNA EWSAT1 knockdown significantly repressed cell proliferation rate, compared with control group in SW480 and SW620 cells ($p < 0.05$, Figure 2C-2D). Moreover, the cell colonies assay revealed that colony formation rates were significantly reduced, when compared with control group in SW480 and SW620 cells ($p < 0.05$, Figure 2E-2F). Thus, these results indicated that decreased lncRNA EWSAT1 exerted the potential to inhibit cell proliferation of CRC.

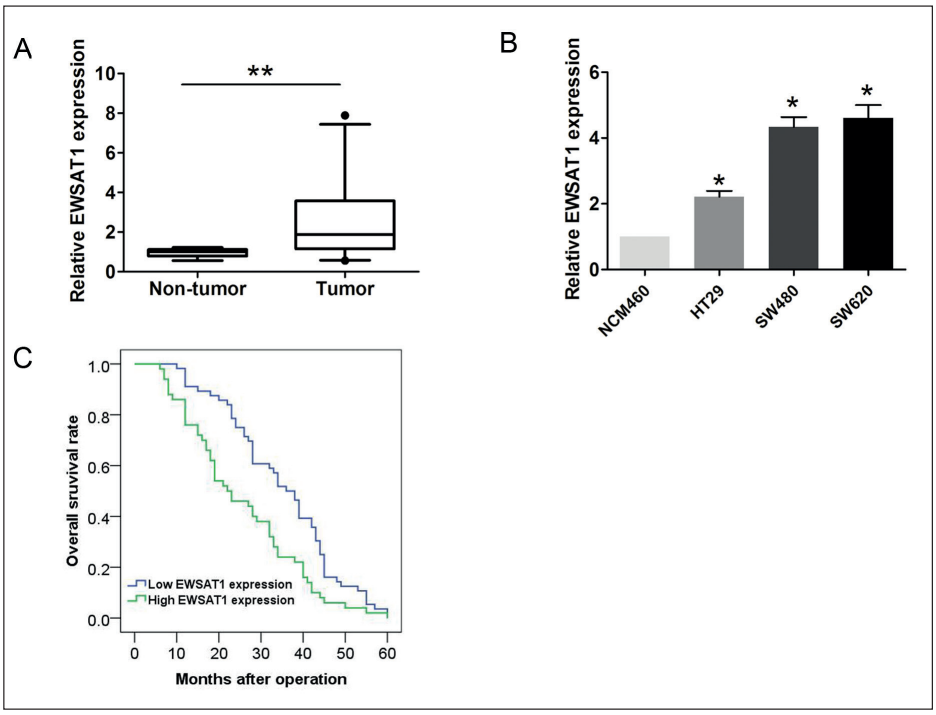

Figure 1. Expression of lncRNA EWSAT1 is higher in human CRC tissues and cell lines. ***A***, The relative expression of lncRNA EWSAT1 was examined by qRT-PCR assay in 106 CRC tissue and pair-matched adjacent normal tissues samples, with mRNA expression normalized to GAPDH, $^{**}p < 0.01$. ***B***, The overall survival time of the higher lncRNA EWSAT1 expression group (n = 50) of CRC patients was significantly shorter compared with the lower expression group (n = 56). ***C***, The relative expression of lncRNA EWSAT1 was examined by qRT-PCR assay in three human CRC cell lines (SW480, HT29, and SW620) and an intestinal epithelial cell line (NCM460) used in this study, $^{*}p < 0.05$.

Table I. Correlation with EWSAT1 expression and clinical characteristics in 106 CRC patients.

Characteristics	Case number (n = 106)	lncRNA EWSAT1 expression		*p*-value
		Lower (n = 56)	Higher (n = 50)	
Gender				0.958
Female	40	21	19	
Male	66	35	31	
Age				0.092
≤ 60	72	34	38	
> 60	34	22	12	
Tumor size				0.102
< 5 cm	47	29	18	
> 5 cm	59	27	32	
CEA (ng/ml)				0.090
> 5	43	27	16	
≤ 5	63	29	34	
Tumor location				
Rectum	52	26	26	0.567
Colon	54	30	24	
Depth of invasion				
T1-T2	41	28	13	0.011*
T3-T4	65	28	37	
Lymph node metastasis				0.014*
Negative	64	40	24	
Positive	42	16	26	
Differentiation				0.092
Well, moderate	72	34	38	
Poor	34	22	12	
TNM stage				0.008*
I-II	67	42	25	
III	39	14	25	

TNM, tumor-node-metastasis, *represents the *p*-values < 0.05.

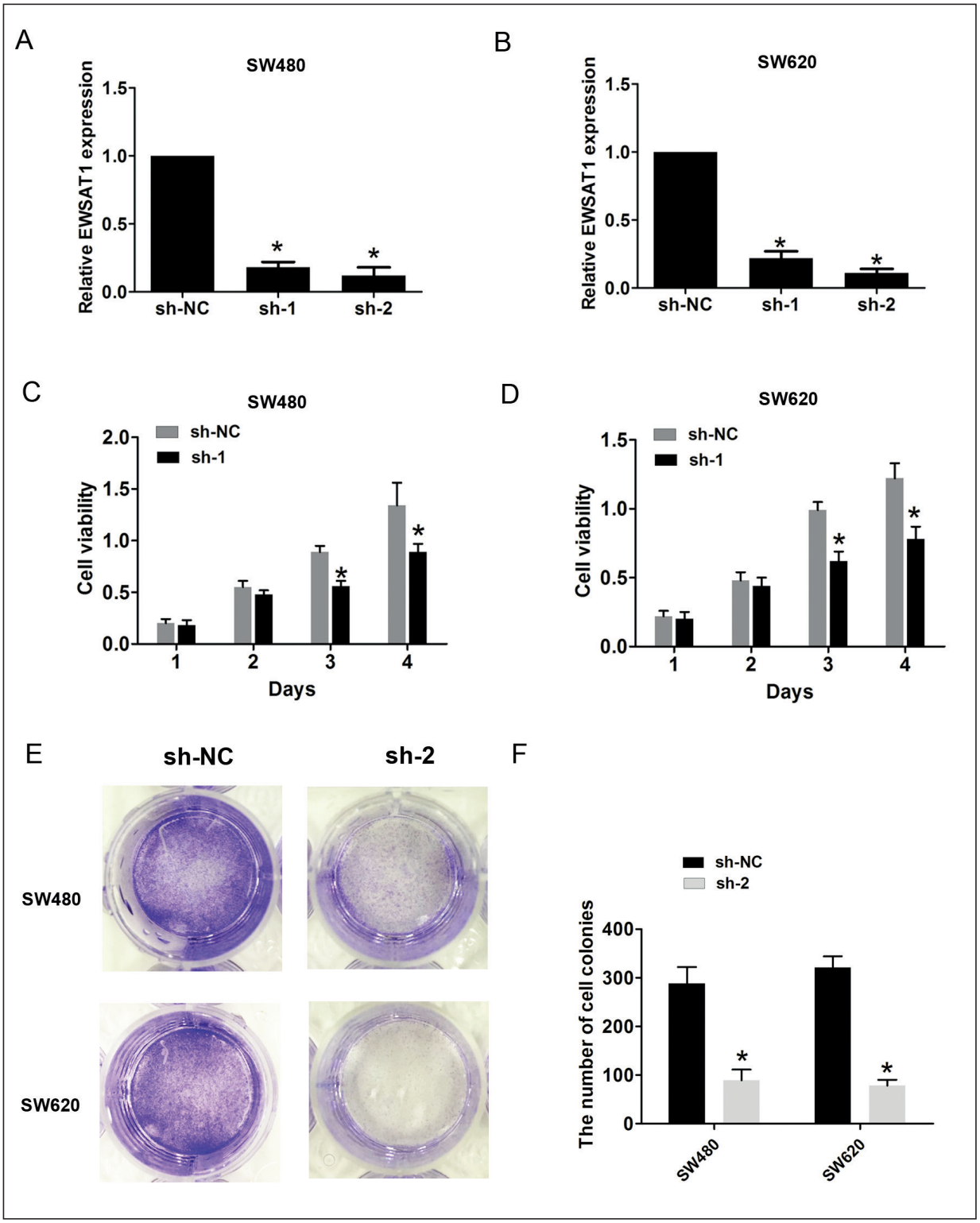

Figure 2. Decreased lncRNA EWSAT1 suppresses CRC cell growth and colony formation, *p <0.05. ***A-B,*** The relative expression of lncRNA EWSAT1 was examined by qRT-PCR assay following treatment with sh-NC and sh-EWSAT1-2 (sh-2) in SW480 and SW620. ***C-D,*** A CCK8 assay was performed to determine the proliferation rate of SW480 and SW620 following treatment with sh-NC and sh-EWSAT1-2 (sh-2). ***E-F,*** A colony formation assay was performed following treatment with sh-NC and sh-EWSAT1-2 (sh-2) in SW480 and SW620. The data represent the means ± SD from three independent experiments, *p < 0.05.

LncRNA EWSAT1 Regulates CRC Cell Invasion and Epithelial-Mesenchymal Transition (EMT)

Cell invasion and EMT process correlated with CRC metastasis[12]. Next, we detected whether lncRNA EWSAT1 expression affected cell invasion and EMT pathway. Transwell invasion assay showed that the number of invading cells was significantly reduced when lncRNA EWSAT1 was knocked down compared with control group in SW480 and SW620 cells ($p < 0.05$, Figure 3A-3B). Western blot was performed to analyze the expression of EMT-related markers in SW480 and SW620 cells after lncRNA EWSAT1 knockdown. As showed in Figure 3C-3D, lncRNA EWSAT1 knockdown markedly reduced the expression of Snail1, Snail2, and N-cadherin, meanwhile greatly increased the expression of E-cadherin in SW480 and SW620 cells. Thus, these results indicated that the decreased lncRNA EWSAT1 suppressed cell invasion and EMT pathway.

Discussion

LncRNAs, an important ncRNA, have been reported to aberrantly express in human CRC and are involved in CRC carcinogenesis[13]. The

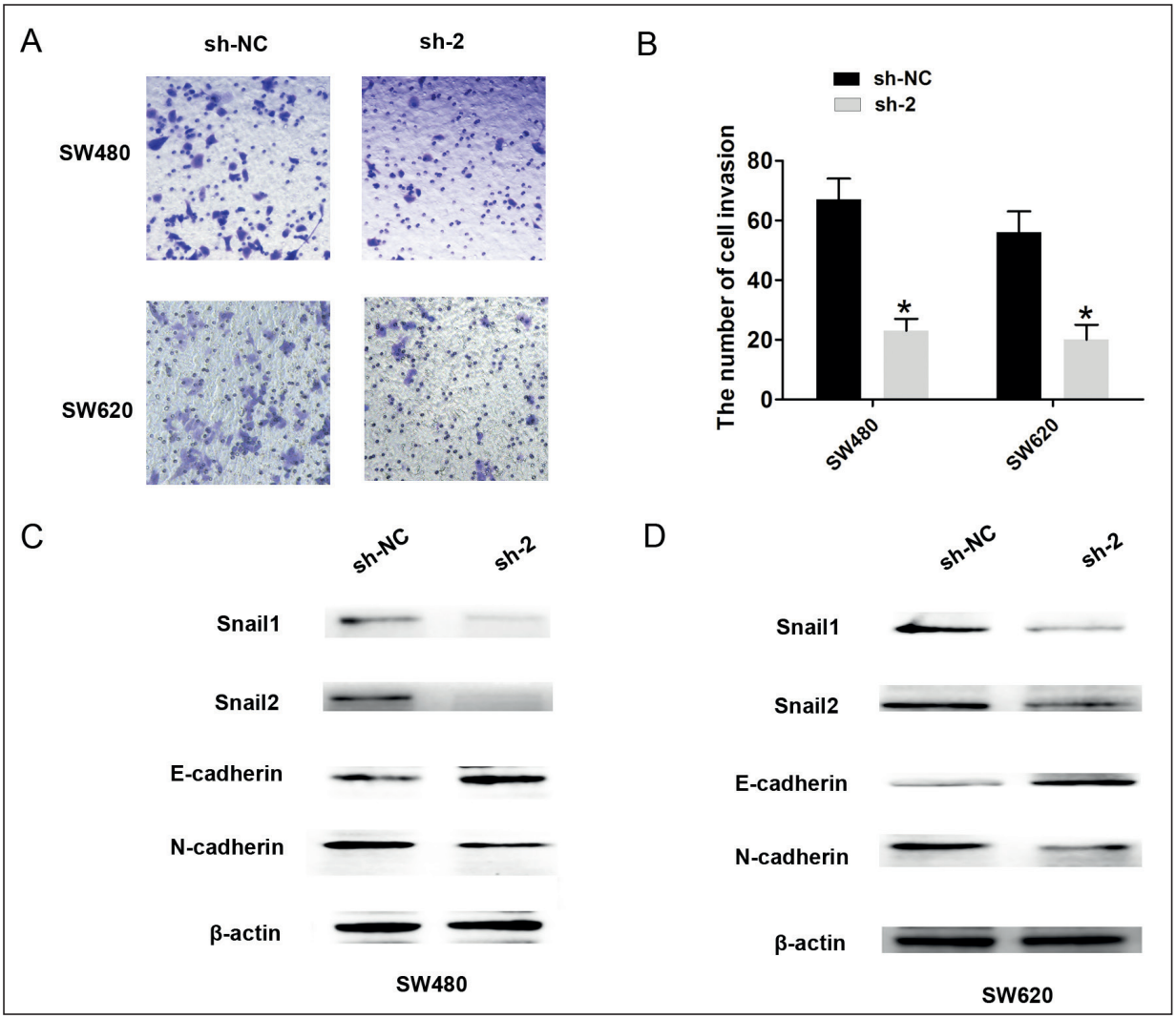

Figure 3. LncRNA EWSAT1 regulates CRC cell invasion and epithelial-mesenchymal transition (EMT). ***A-B,*** A transwell invasion assay was performed to determine the cell invasion ability of SW480 and SW620 following treatment with sh-NC and sh-EWSAT1-2 (sh-2). ***C-D,*** The relative protein expression of Snail1, Snail2, N-cadherin and E-cadherin were detected by western blot following treatment with sh-NC and sh-EWSAT1 in SW480 and SW620. The data represent the means ± SD from three independent experiments, $*p < 0.05$.

underlying functions and molecular mechanisms of some lncRNAs have been found previously[14]. However, little known molecular and biological functions were reported for lncRNA EWSAT1 in CRC. In this study, we demonstrated that lncRNA EWSAT1 expression was significantly higher in CRC tissues when compared to adjacent tissues, and associated with depth of invasion, lymph node metastasis, and tumor-node-metastasis (TNM) stage in CRC patients. Patients with higher EWSAT1 expression levels exhibited shorter overall survival rates compared with patients with lower EWSAT1 expression levels.

In the previous research, the long non-coding RNA EWSAT1 had been reported to associate with tumor progression. Analysis of RNAseq data from primary human Ewing sarcoma further supported a role for EWSAT1 in mediating gene repression to facilitate Ewing sarcoma oncogenesis[15]. The long non-coding RNA EWSAT1 promotes human nasopharyngeal carcinoma cell growth *in vitro* by targeting miR-326/-330-5p[16]. The long ong non-coding RNA EWSAT1 promotes osteosarcoma cell growth and metastasis through suppression of MEG3 expression[17]. We demonstrated that cell proliferation and invasion ability were significantly suppressed when lncRNA EWSAT1 knockdown compared with the control group in SW480 and SW620 cells. Due to little is known for the underlying molecular mechanism of lncRNA EWSAT1, here, we investigated whether lncRNA EWSAT1 expression affected the EMT pathway, which is an essential process for tumor invasion and metastasis[18]. The Western blot analysis results demonstrated that lncRNA EWSAT1 knockdown could suppress CRC cell epithelial-mesenchymal transition through inhibiting Snail1, Snail2, and N-cadherin expression and increasing the expression levels of E-cadherin.

Conclusions

We demonstrated that lncRNA EWSAT1 is significantly higher in CRC tissues and cells, and higher lncRNA EWSAT1 expression predicted a poor outcome. Moreover, inhibition of lncRNA EWSAT1 expression inhibited cell proliferation, invasion epithelial-mesenchymal transition process. Thus, EWSAT1 may be a potential target of CRC treatment.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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