Original Article
Silencing of the long non-coding RNA NEAT1 suppresses ovarian cancer cell proliferation, migration and invasion

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Abstract: Increasing evidence reveals that long noncoding RNAs (lncRNAs) are involved in the development of various human cancers including ovarian cancer. LncRNA nuclear enriched abundant transcript 1 (NEAT1) is an essential structural determinant of nuclear body paraspeckles. Although aberrant expression and prognostic value of NEAT1 has been recently reported in various human cancers, NEAT1 was not fully investigated in ovarian cancer. Here, we reported that NEAT1 was upregulated in ovarian cancer tissues than noncancerous cyst tissues by real-time PCR. Furthermore, higher expression of NEAT1 was associated with more advanced tumor stage and bigger tumor size. Down-regulation of NEAT1 by siRNA transfection in two ovarian cancer cell lines, SKOV3 and OVCAR3 resulted in a significant decrease of cell proliferation via inducing cell cycle arrest and cell apoptosis. Additionally, we found that suppressing of NEAT1 expression notably repressed the migration and invasive ability of both ovarian cancer cell lines. More importantly, we investigated the possible mechanism how NEAT1 exerted its functions on ovarian cancer. NEAT1 siRNA treatment significantly affected the expression of cell apoptosis-associated proteins (Bcl-2, Bax, Caspase 3 and Caspase 9) and cell invasion-related proteins (MMP-2, MMP-9, Snail1 and TGF-β1). In summary, our study suggests that NEAT1 may serve as an oncogene in ovarian cancer.

Keywords: lncRNA, NEAT1, ovarian cancer, proliferation, migration, invasion

Introduction

Ovarian cancer is one of the most lethal types of reproductive system tumors and the fifth most common cancer for women in the world [1]. Despite advances in diagnosis and therapies, the five-year overall survival rate of patients is approximately 30% [2]. Over 75% of ovarian cancer patients were diagnosed at an advanced stage with metastasis in the pelvic and abdominal cavity and have an extremely poor prognosis [3]. In order to develop better diagnostic approaches and more effective treatment modalities, a more comprehensive understanding of the molecular mechanisms implicated in oncogenesis and progress of ovarian cancer is urgently needed.

Long non-coding RNAs (lncRNAs), which do not encode any proteins, have recently gained widespread attention in cancer research. LncRNAs may serve as oncogene or tumor sup-

pressor in the development of various human cancer [4-6], including ovarian cancer [7-10]. LncRNA nuclear enriched abundant transcript 1 (NEAT1) was firstly identified from human fibroblasts and lymphoblasts [11] and functions as an essential structural determinant of nuclear body paraspeckles [12]. Increasing evidence has revealed the aberrant expression and involvement of NEAT1 in various human cancers. Large-scale bioinformatic analysis of SAGE libraries [13] has shown that NEAT1 was downregulated in lung, liver, esophageal and gall bladder cancers, while upregulated in lymph node and prostate cancers. NEAT1 has been found as a novel prognostic marker for prostate cancer [14] and breast cancer [15]. NEAT1 may play a pivotal role in tumorigenesis and metastasis of hepatocellular carcinoma [16]. Knockdown of NEAT1 significantly decreased proliferation and the invasive properties of prostate cancer cells [14] and glioma cells [17]. In stage III serous ovarian carcinoma
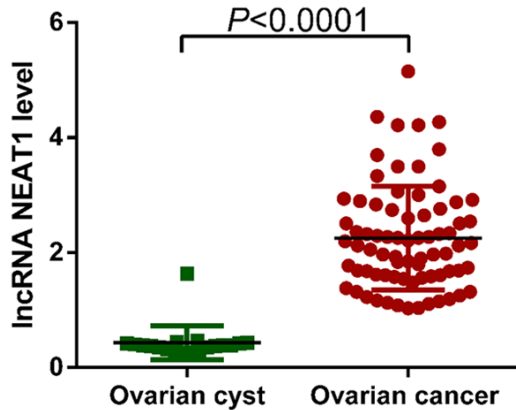
Figure 1. Expression of NEAT1 in ovarian cancer and ovarian cyst. Expression levels for NEAT1 obtained by real-time PCR analysis were normalized to GAPDH expression.

tissues, NEAT1 was found upregulated compared with normal ovary tissues [18]. However, little is known about the biological functions of NEAT1 on ovarian cancer.

In the present study, we evaluated the expression of NEAT1 in ovarian cancer and ovarian cyst tissues and investigated the relationship between NEAT1 expression and clinicopathological characteristics in ovarian cancer. We also investigate the effects of silencing of NEAT1 expression on the proliferation, cell cycle distribution, cell apoptosis, migration and invasion of ovarian cancer cell lines. Our results showed that lncRNA NEAT1 was overexpressed in ovarian cancer and targeting NEAT1 might be an important therapeutic strategy for this disease.

Materials and methods

Collection of patients' samples

This study consisted of a total of 75 cases of ovarian cancer patients and 20 cases of benign ovarian cyst patients who hospitalized at Department of Gynecology, the Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine (Fujian, China) between January 2012 and December 2013. None of the participants had received hormonal therapy preoperative radiotherapy or chemotherapy. The collected tissue samples were immediately frozen in liquid nitrogen and stored at -80°C. The study was approved by Institutional Review Board of Fujian University of Traditional Chinese Medicine. All participants had given written informed consent.

Cell culture

Human SKOV3, C200, COC1, HP-8910 and OVCAR3 cells were purchased from Shanghai Institute of Cell Bank. Cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2.

Small interfering RNA (siRNA) transfection

SKOV3 and OVCAR3 cells were transfected with siRNA by using Lipofectamine™ 2000 according to the manufacturer's protocol (Invitrogen). NEAT1 siRNA (5'-GCCUUGUAAAUGCCUAUA-3') and control siRNA (NC) were synthesized by GenePharma Co. Ltd. (Shanghai, China).

Quantitative real-time PCR

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, USA) and 2 µg of RNA was used to reverse transcribed into cDNA (Fermentas, Hanover, MD, USA) following the manufacturer's instructions. The mRNA levels were quantified by real-time PCR using SYBR Green qPCR mix (Thermo, Rockford, IL, USA). GAPDH was served as an internal control. Primers used in PCR amplifications were: NEAT1, 5'-GGGATGATGCAAACAATTAC-3' and 5'-TACCA-TACAGAGCAACATAC-3'; GAPDH, 5'-CACCCACTC-CTCCACCTTTG-3' and 5'-CCACCACCCTGTTGC-TGTAG-3'. Real-time PCR and data collection were performed with an ABI PRISM 7300 detection system under the following reaction conditions: 95°C denaturation for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds.

Western blotting

Total proteins were extracted from cells with protein lysis buffer and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The protein concentration was quantified by BCA Kit (Thermo). Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to PVDF membranes. The membranes were then blocked with 5% non-fat dry milk at room temperature for 1 h, incubated with primary antibodies overnight at 4°C, and then incubated with corresponding secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Immunoreactivity was detected using enhanced chemiluminescence (Millipore Biotech., Bredford, MA, USA). The densitometry of the bands was quantified

Table 1. Correlations of NEAT1 expression with clinico-pathological features in patients with ovarian cancer

| Variables | Total | NEAT1 expression | | P value |
		Low (n=38)	High (n=37)	
Age (years)				
<60	49	26	23	0.6321
≥60	26	12	14	
Histologic type				
Serous	42	20	22	0.4723
Endometrioid	12	9	3	
Mucinous	9	4	5	
Clear cell	7	3	4	
Mixed	5	2	3	
Tumor grade				
I-II	42	27	15	0.0107*
III	33	11	22	
Tumor size				
<5 cm	36	24	12	0.0110*
≥5 cm	39	14	25	

P values are from chi-square test. *P<0.05.

using the Image J software (USA) with GAPDH as the loading control. The sources of primary antibodies were as follows: anti-Caspase 3, anti-Caspase 9, anti-MMP-2 and anti-TGF-β1 were from Abcam (Cambridge, MA, USA); anti-Bcl-2, anti-Bax and anti-MMP-9 were from Santa Cruz (Santa Cruz, CA, USA); anti-Snail1 and anti-GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell proliferation measured by cell counting kit (CCK-8)

The SKOV3 and OVCAR3 cells were seeded at a density of 5×10^3/well onto 96-well plates. At 0, 24 and 48 hours post transfection with NEAT1 siRNA or control siRNA, cell proliferation was measured by adding CCK-8 reagent (Beyotime, Shanghai, China) to each well one hour prior to detection. The spectrophotometric absorbance was determined at 450 nm by using Model 550 microplate reader (BioRad, Richmond, CA, USA).

Evaluation of cell cycle and apoptosis by flow cytometry

The cells were transfected with NEAT1 siRNA or control siRNA. After 48 h, cell cycle distribution and apoptosis was determined by flow cytometry analysis. For cell cycle evaluation, cells were detached and fixed with 70% cold ethanol at -20°C overnight. Subsequently, the cells were rinsed with PBS and then incubated with 25 µg/mL RNase A and 50 µg/mL propidium iodide (PI, Sigma, St. Louis, MO, USA) for 30 minutes in the dark. For apoptosis analysis, cells were collected, resuspended in Annexin V binding buffer and then incubated with Annexin V-FITC and PI (eBioscience, San Diego, CA, USA) following the manufacture's instruction. The samples were then analyzed using a BD Accuri C6 Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell migration and invasion assay

Cell migration and invasion was evaluated by using Transwell chambers without and with Matrigel (BD Bioscience), respectively. Briefly, 48 h after transfected with siRNA, SKOV3 and OVCAR3 cells were trypsinized and seeded onto the upper chambers of a Transwell plate with serum-free medium (5×10^4 per well). Culture medium supplemented with 10% FBS was then added into the lower chamber. After 24 h of incubation, cells on the upper side were completely removed. Migrated cells on the lower side were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in randomly selected five fields on each chamber were counted under an inverted microscope.

Statistical analysis

All statistical calculations were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Each *in vitro* experiment was performed at least in triplicate. All data are presented as means ± standard deviation (SD). Student's *t* test was carried out to compare the levels of the parameters between two groups. *P* value less than 0.05 was regarded statistically significant.

Results

Overexpression of NEAT1 in ovarian cancer

We assessed the expression of NEAT1 for 75 cases of ovarian cancer and 20 cases of ovarian cyst by real-time PCR. The relative expression of NEAT1 compared with GAPDH were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described [17]. As shown in **Figure 1**, expression of NEAT1 was significantly higher in ovarian cancer than in ovarian cyst (*P*<0.0001).
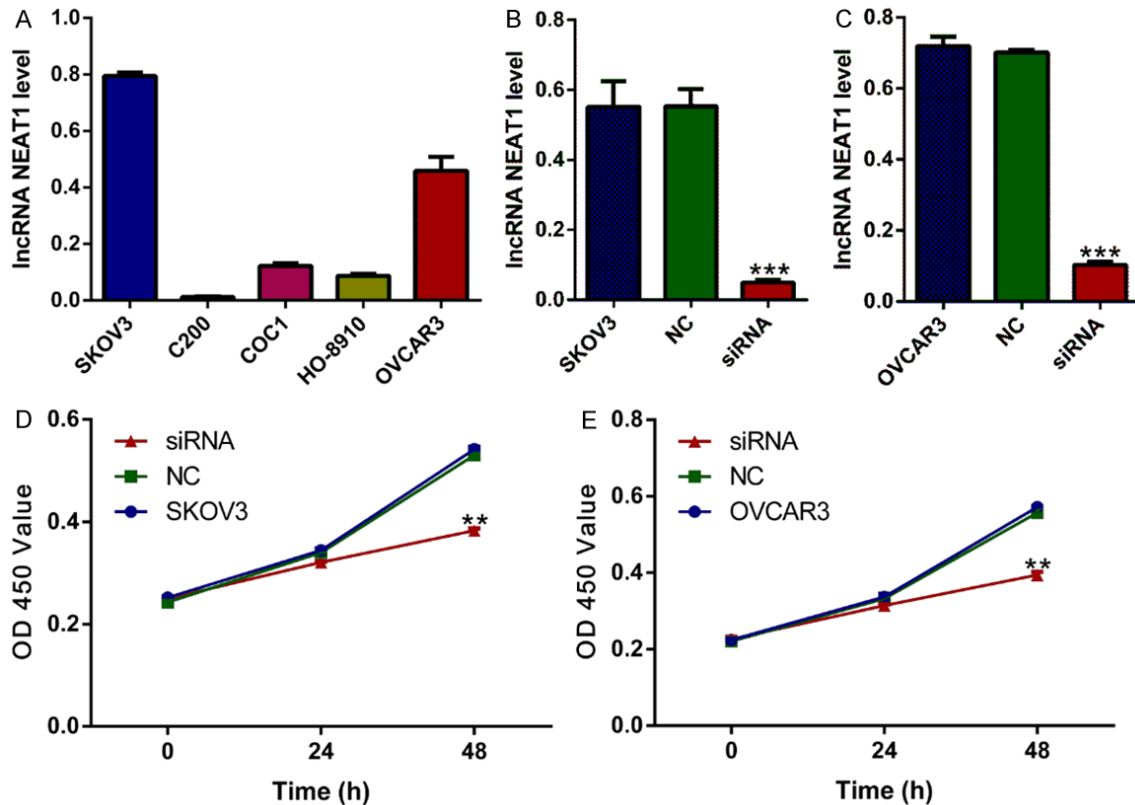
Figure 2. Silencing of NEAT1 inhibited cell growth of ovarian cancer cells. A. NEAT1 expression level in five ovarian cancer cell lines was analyzed by real-time PCR. B, C. Expression of NEAT1 in SKOV3 and OVCA3 cells was analyzed by real-time PCR. D, E. Cell proliferation was detected in SKOV3 and OVCA3 cells. WT: wild-type cells; NC: scrambled shRNA transfected cells; siRNA: NEAT1 siRNA transfected cells. **$P<0.01, ***$P<0.001$ VS NC.

Further, 75 cases of ovarian cancer patients were classified into NEAT1 low expression group (n=38) and NEAT1 high expression group (n=37). **Table 1** showed the relationship between NEAT1 expression and clinicopathological characteristics. Patients in NEAT1 high expression group were more likely to have more advanced disease (P=0.0107) and bigger tumor size than those in NEAT low expression group. These data suggest that NEAT1 may be associated with the progression of ovarian cancer.

Silencing efficiency of NEAT1 by siRNA transfection in SKOV3 and OVCAR3 cells

We evaluated the expression level of NEAT1 in five ovarian cancer cell lines, SKOV3, C200, COC1, HP-8910 and OVCAR3, by real-time PCR (**Figure 2A**). Two cell lines, SKOV3 and OVCAR3, showed higher expression of NEAT1 and chosen for the following assays.

Specific siRNA targeting NEAT1 was used to knock down its expression in SKOV3 and

OVCAR3 cells. The knock-down efficiency was then evaluated by real-time PCR analysis (**Figure 2B** and **2C**). A notably reduced expression of NEAT1 was observed in SKOV3 and OVCAR3 cells transfected with NEAT1 siRNA.

NEAT1 siRNA inhibits cell proliferation in SKOV3 and OVCAR3 cells

The effect of NEAT knockdown on the proliferation of SKOV3 and OVCAR3 cells was determined by CCK-8 assay. As shown in **Figure 2D** and **2E**, compared with cells transfected with control siRNA (NC), the viability of SKOV3 and OVCAR3 transfected with NEAT1 siRNA was reduced to 70.6% and 68.9% at 48 h (P<0.01), respectively. In other words, NEAT1 silencing effectively reduces the proliferation of ovarian cancer cells.

NEAT1 silencing affects the cell cycle distribution of ovarian cancer cells

We next examined the effect of NEAT1 knockdown on cell cycle distribution in SKOV3 and
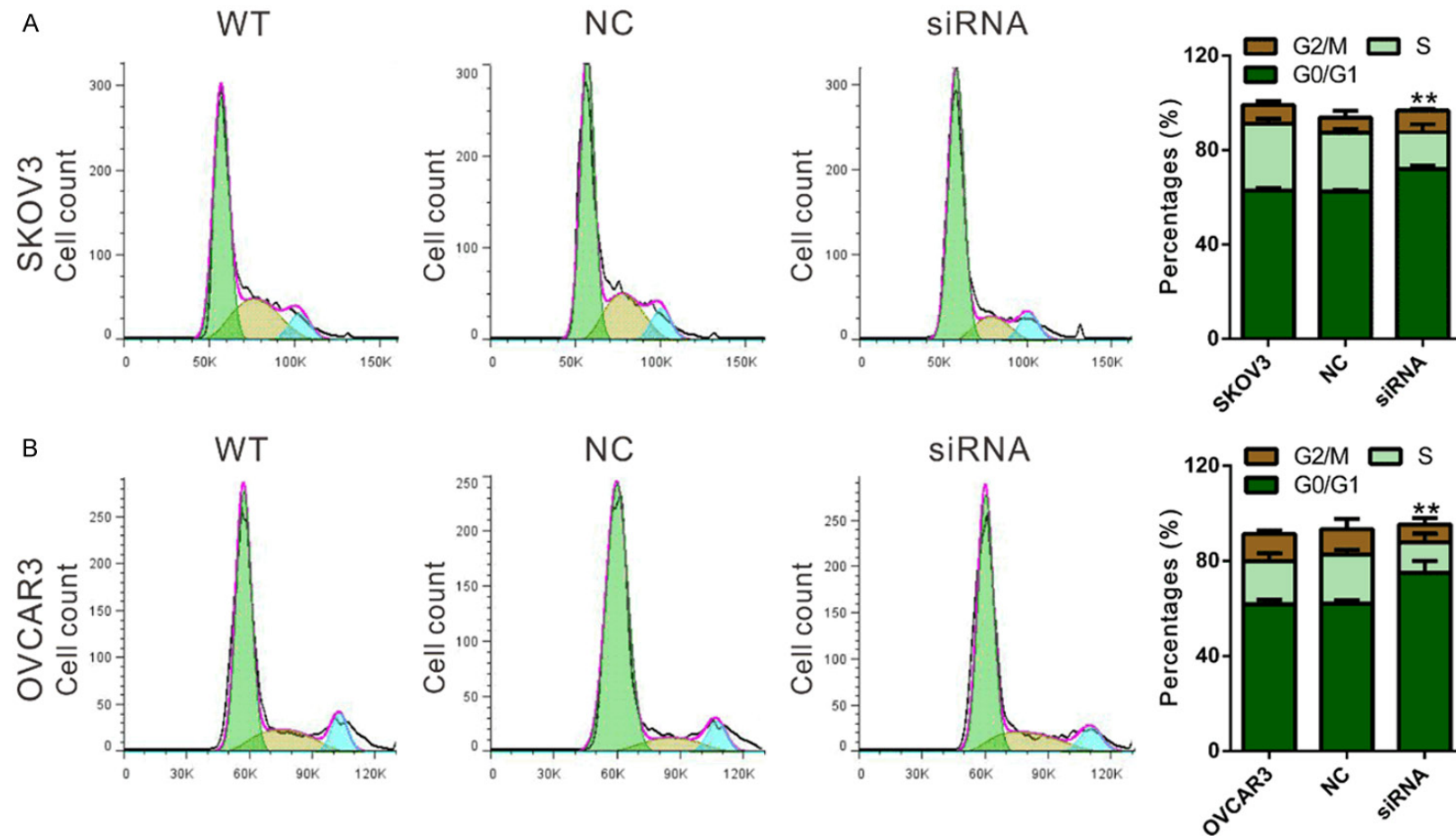
Figure 3. Silencing of NEAT1 inhibited G1/S phase transition in ovarian cancer cells. Cell cycle profile was analyzed using flow cytometry in SKOV3 (A) and OVCA3 cells (B). Representative images (left panel) and quantitative results (right panel) were shown. WT: wild-type cells; NC: scrambled shRNA transfected cells; siRNA: NEAT1 siRNA transfected cells. **P<0.01 VS NC.
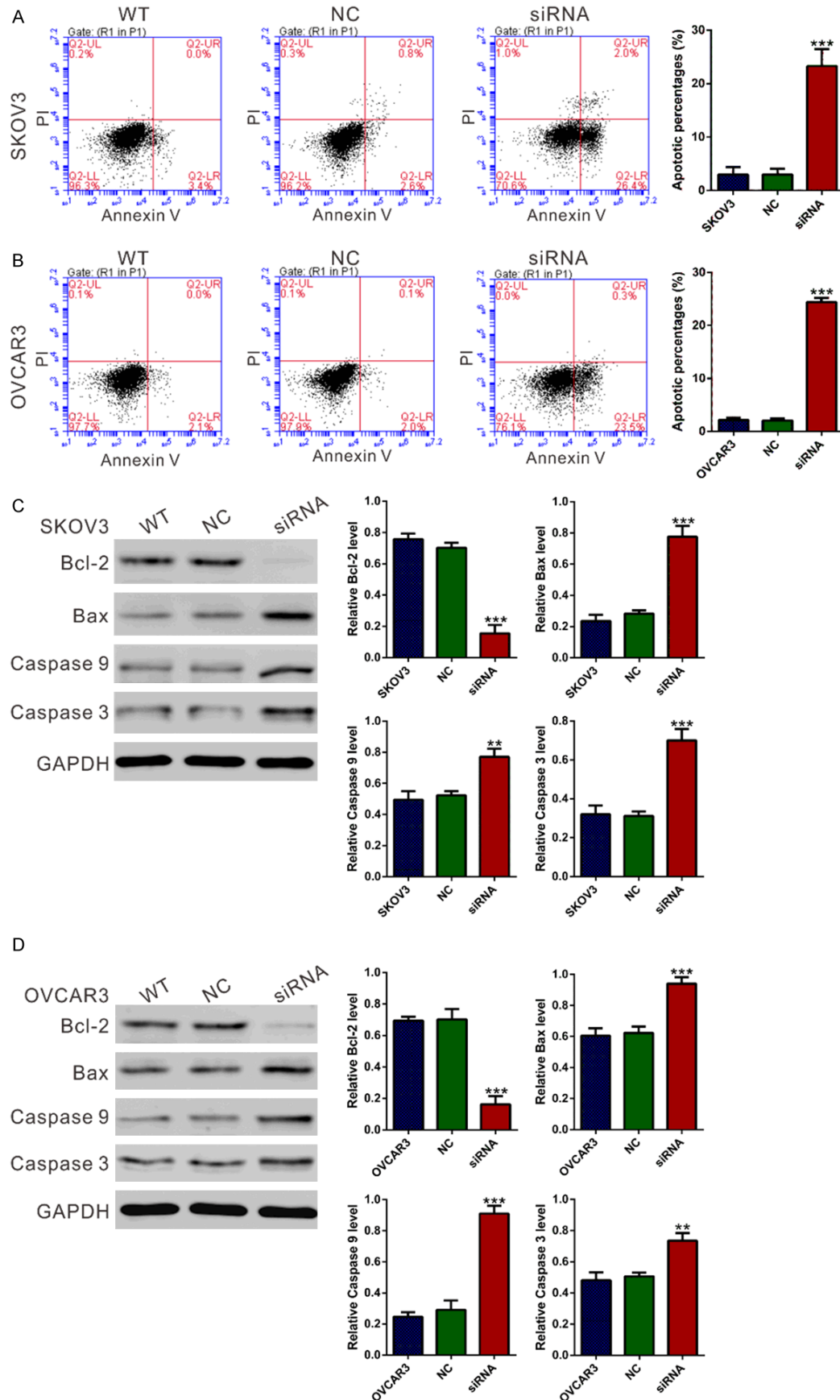
Figure 4. Suppressing NEAT1 expression induced cell apoptosis in ovarian cancer cells. A, B. SKOV3 and OVCA3 cells were stained with Annexin V-FITC/PI and apoptosis rates was analyzed using flow cytometry. C, D. Expression of apoptotic factors Bcl-2 and Bax was evaluated by western blot. Representative images (left panel) and quantitative results (right panel) were shown. WT: wild-type cells; NC: scrambled shRNA transfected cells; siRNA: NEAT1 siRNA transfected cells. **P<0.01, ***P<0.001 VS NC.

OVCAR3 cells by using flow cytometry (**Figure 3**). NEAT1 siRNA showed similar effects on the cell cycle distribution in SKOV3 and OVCAR3 cells. Compared with NC cells, cell population in G0/G1 phase was increased 15.4% and 20.8% in SKOV3 and OVCAR3 transfected with NEAT1 siRNA, respectively (P<0.01).

NEAT1 siRNA induces cell apoptosis in SKOV3 and OVCAR3 cells

To explore whether NEAT1 siRNA suppressed cell viability was due to apoptosis induction, Annexin V-FITC/PI staining assay was performed (**Figure 4A** and **4B**). In SKOV3 cells, apoptosis rate was 2.97 ± 1.40%, 2.97 ± 1.10% and 23.27 ± 3.20% in wild-type (WT) cells, NC cells and NEAT1 siRNA-transfected cells, respectively. In OVCAR3 cells, apoptosis rate was 2.17 ± 0.40%, 2.07 ± 0.40% and 24.40 ± 0.82% in wild-type (WT) cells, NC cells and NEAT1 siRNA-transfected cells, respectively. The apoptotic rate was significantly increased by NEAT1 siRNA transfection in both cells (P<0.001).

We further investigated the expression of apoptosis associated proteins, Bcl-2, Bax, Caspase 9 and Caspase 3 (**Figure 4C** and **4D**). It demonstrated that silencing of NEAT1 expression significantly reduced Bcl-2 expression, but remarkably increased expression of Bax, Caspase 9 and Caspase 3 (P<0.01, P<0.001).

NEAT1 silencing reduces the migration and invasive capacity of SKOV3 and OVCAR3 cells

To examine whether the down-regulation of NEAT1 in ovarian cancer cells affected their migration and invasive capacity, we conducted Transwell analysis (**Figure 5A** and **5B**). The results showed that the number of migrated and invaded cells was reduced in NEAT1 siRNA-transfected SKOV cells by 57.4% (P<0.001) and 56.3% (P<0.01), respectively, compared with NC cells. Similar results were observed in OVCAR3 cells.

We further investigated the expression of important factors to regulate migration and

invasion (**Figure 5C** and **5D**). NEAT1 knockdown significantly downregulted the expression of MMP-2, MMP-9, Snail1 and TGF-β1. These data indicated that the NEAT1 played an important role in the migration and invasion of ovarian cancer cells.

Discussion

Emerging evidence has revealed the involvement of lncRNA in malignancy [4-6]. LncRNA NEAT1 was reported overexpressed in prostate cancer [14], breast cancer [15], hepatocellular carcinoma [16], glioma [17] and stage III serous ovarian carcinoma [18]. In the current study, we demonstrated that NEAT1 was overexpressed in ovarian cancer tissues (I-III) by real-time PCR analysis on 75 cases of ovarian cancer and 20 cases of ovarian cyst tissues (**Figure 1**). Moreover, NEAT1 expression level was associated with tumor stage and tumor size (**Table 1**). We also investigated the effects of NEAT1 siRNA on the biological behavior of ovarian cancer cell lines, SKOV3 and OVCAR3 (**Figures 2**-**5**). Our results suggested that NEAT1 may act as an oncogene on ovarian cancer.

Uncontrolled proliferation is a critical feature of cancer cells. Here, we found that knockdown of NEAT1 in ovarian cancer cells significantly impaired cell growth (**Figure 2**), which was consistent with previous findings in prostate cancer cells [14] and glioma cells [17]. Abnormal cell proliferation is controlled by cell cycle progression. Knockdown of NEAT1 in both ovarian cancer cell lines was able to impede the G1 cells from entering the S phase (**Figure 3**), thus inhibiting cell proliferation. The anti-apoptosis role of NEAT1 has been reported in human glioma cells [17]. In line with this finding, silencing of NEAT1 significantly induced the apoptosis of ovarian cancer cells (**Figure 4**), which was further confirmed by the expression analysis of apoptosis associated proteins, Bcl-2, Bax, Caspase 3 and Caspase 9 (**Figure 4C** and **4D**). The proteins of the Bcl-2 family either promote (e.g., Bax) or inhibit cell apoptosis (e.g., Bcl-2) [19]. Increased expression of Caspase 3 and Caspase 9 indicated higher apoptotic rates
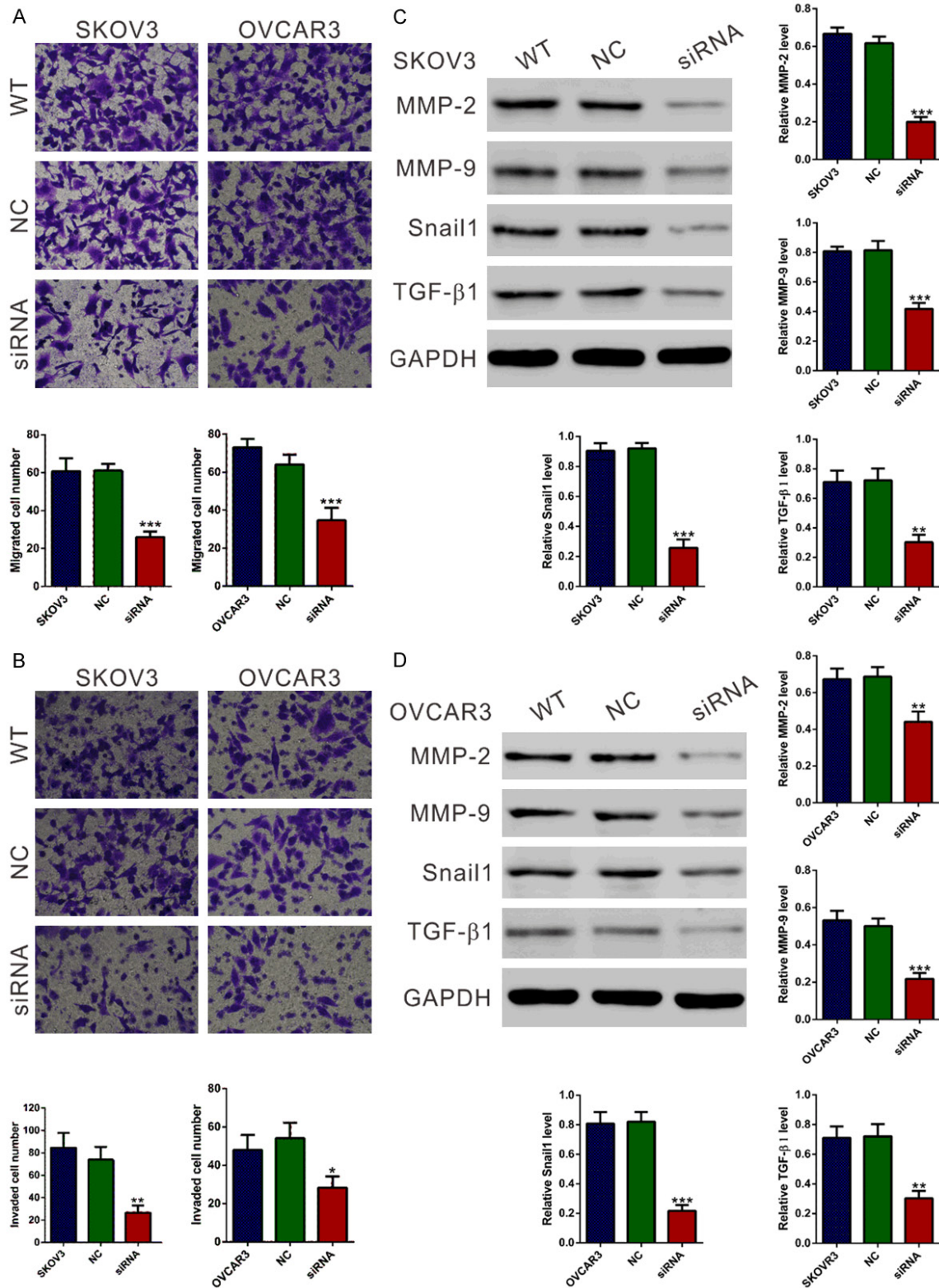
Figure 5. Silencing of NEAT1 inhibited cell migration and invasion in ovarian cancer cells. A, B. The migration and invasive capacity was determined by Transwell assay. C, D. Expression of migration and invasion related factors, MMP-2, MMP-9, Snail and TGF-β1 was evaluated by western blot. Representative images (left panel) and quantitative results (right panel) were shown. WT: wild-type cells; NC: scrambled shRNA transfected cells; siRNA: NEAT1 siRNA transfected cells. *P<0.05, **P<0.01, ***P<0.001 VS NC.

[20]. NEAT1 knockdown significantly enhanced the expression of Caspase 3, Caspase 9 and Bax, but notably reduced Bcl-2 expression (**Figure 4C** and **4D**).

Migration and invasion are the essential processes during the metastasis of ovarian cancer. The promoted effect of NEAT1 on the migration and invasion has been revealed in glioma cells [17]. Here, our data also demonstrated that NEAT1 siRNA notably repressed the migration and invasive ability of ovarian cancer cells (**Figure 5A** and **5B**). Matrix metalloproteinases, such as MMP-2 and MMP-9, are important regulators during tumor invasion and metastasis [21].

Higher expression of Snail is associated with lower overall survival of ovarian cancer patients [22]. Ectopic expression of Snail leads to epithelial-mesenchymal transition (EMT), and induced motility and invasiveness of ovarian cancer cells [23]. TGF-β1 is involved in EMT and cancer cell invasion [24]. In the present study, NEAT1 siRNA remarkably reduced the expression of MMP-2, MMP-9, Snail 1 and TGF-β1 (**Figure 5C** and **5D**). Taken together, we speculated that NEAT1 may exert the invasion-promoting function by regulating TGF-β1, Snail1, MMP-2 and MMP-9.

In summary, this may be the first study to explore the role and function of lncRNA NEAT1 in ovarian cancer. We found that the overexpression of NEAT1 in ovarian cancer tissues was associated with tumor stage and tumor size. In addition, our data suggested that NEAT1 played a key role in the proliferation, migration and invasion of ovarian cancer cells.

Disclosure of conflict of interest

None.

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