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LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway

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Abstract. – OBJECTIVE: The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA (lncRNA) that plays a key role in the malignant phenotype of tumors. Although abnormal regulation of lncRNA MALAT1 impacts clinical prognostic and tumor metastasis, its function remains unclear in ovarian cancer.

PATIENTS AND METHODS: We collected 64 samples of surgical EOC tissues and 30 samples of normal ovarian tissues at the Department of Gynecology of Harbin Medical University (Harbin, China). The 30 control samples of ovarian surface epithelial tissues were obtained from patients diagnosed with uterine fibroids and scheduled hysterectomy with oophorectomy.

RESULTS: The present study discovered that MALAT1 was upregulated in tumor tissues and ovarian cancer cell lines. Further, the 5-year overall survival was higher in the lower expression of the MALAT1 group. MALAT1 inhibition impeded cell proliferation, invasion and metastasis, and promoted cell apoptosis in both *in vivo* and *in vitro*. Furthermore, silencing of MALAT1 hindered the expression of epithelial-to-mesenchymal transition (EMT)-related genes and MMPS. The evidence showed that MALAT1 induce EMT via PI3K/AKT pathway.

CONCLUSIONS: Our research suggests that MALAT1 transforms metastasis in EOC and may be a prospective therapeutic target.

Key Words:
LncRNA MALAT1, Ovarian cancer, EMT, PI3K/AKT.

Introduction

Ovarian cancer is currently the fifth leading cause of death among females and the most lethal gynecologic malignancy[1]. The standard treatment for advanced ovarian cancer is cytoreduction surgery and platinum-based chemotherapy; however, the overall survival rate remains unsatisfactory with a five-year survival of only 30%[2]. The most common origin of ovarian cancer is epithelial ovarian cancer (EOC), which represent over 85% of all cases[3]. The precise molecular alterations underlying EOC metastasis are still unknown. Therefore, a better understanding of the pathogenesis and the molecular alterations in EOC will help improve the treatment of ovarian cancer. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides with no protein-coding capacity[4]. LncRNAs have been recently investigated for their involvement in carcinogenesis and cancer progression, processed dominated by genetic expression regulation including transcription, posttranscriptional processing, genomic imprinting, chromatin modification, and the regulation of protein function[5-7]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), an evolutionarily conserved lncRNA, has been shown to regulate tumor cell proliferation, migration, invasion, and metastasis in hepatocellular carcinoma, cervical cancer, breast cancer, ovarian cancer, and colorectal cancer[8-11]. Although diverse functions have been discovered for MALAT1 in different cancers, the potential role for MALATI in the invasion and metastasis of ovarian cancer is not understood yet. The epithelial to mesenchymal transition (EMT) provides epithelial cells with mesenchymal properties such as reduced cell-cell adhesion and increased motility[12]. Moreover, increasing evidence showed that EMT is involved in many vital processes, including tumor invasion, metastasis, inhibition of cell apoptosis, and acquisition of stem-like properties[13,14]. Several studies suggest that MALAT1 plays a pivotal role in malignancy phenotypes of cancer, making it a new gene associated with cancer growth and metastasis[15]. Therefore, we conjecture that MALAT1 could promote tumor initiation in EOC by inducing EMT and conferring cancer cells stem cell properties. In this study, we found the clini-

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cal significance of the expression of MALAT1 in EOC, which correlated with prognoses and recurrence rate. Inhibition of MALAT1 using RNA interference altered tumorous cell proliferation, apoptosis, invasion, and metastasis in EOC. We further discovered that MALAT1 is involved in EMT by the underlying signaling pathway in the progression of EOC. Therefore, MALAT1 represents a potential therapy for inhibiting ovarian cancer progression.

Patients and Methods

Patient Data and Tissue Specimens

We collected 64 samples of surgical EOC tissues and 30 samples of normal ovarian tissues at the Department of Gynecology of Harbin Medical University (Harbin, China) between January 2011 and December 2012. Patients read and signed an informed consent before the use of the samples. The 64 EOC cases were pathologically confirmed and histologically graded in accordance with the World Health Organization classification. Exclusion criteria: borderline ovarian cancer, two or more different malignancies, and patients received hormonal therapy, preoperative radiotherapy, or chemotherapy. The 30 control samples of ovarian surface epithelial tissues were obtained from patients diagnosed with uterine fibroids and scheduled hysterectomy with oophorectomy. Exclusion criteria: previous ovarian surgery or ovarian cysts. All fresh surgical samples were immediately frozen in liquid nitrogen and stored at -80°C until processed. This study was approved by the Ethic Committee of The First Affiliated Hospital of Harbin Medical University..

Cell Culture and Transfection

We obtained the human ovarian cancer cell lines SKOV3, OVCAR3, HO8910, A2780, and HO8910PM from the Pathology Laboratory of Basic Medical of Harbin Medical University (Harbin, China). Cells were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) complemented with 10% fetal bovine serum (FBS) and 100 U/mL streptomycin/penicillin and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. We transfected human ovarian cancer cells with 20 nM siRNA targeting MALAT1 or scrambled negative controls (GenePharma, Shanghai, China) using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations.

Quantitative Real-time PCR

We extracted total RNA from ovarian cancer tissues, normal tissues, or cells with TRizol (Invitrogen, Carlsbad, CA, USA), and performed reverse transcription reactions using PrimeScript one step RT-PCR kit (TaKaRa, Dalian, China). Real-time PCR was carried out using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the Bio-Rad System (Bio-Rad, Hercules, CA, USA). The primer sequences were: MALAT1 Fw-AGCGGAAGAACGAATGTAAC and Rv-GAACAGAAGGAAGAGCCAAG; GAPDH Fw-TGTTGCCATCAATGACCCCTT and Rv-CTCCACGACGTACTCAGCG. The results were expressed as log 10 ($2^{-\Delta\Delta Ct}$).

Cell Proliferation Assay

Cells were transfected with 20 nM siRNA targeting HOTAIR (HOTAIR-siRNA) or a scrambled negative control (siRNA-NC). 24 h later cells were seeded into 96-well plates (10^3 cells per well) and transfected with MALAT1 siRNA1-3, control siRNA, or no siRNA. After 24 h incubation, cells were transfected for 0, 1, 2, 3, 4, and 5 days as described above, followed by the addition of 10 μL CCK-8 kit at each time point. The cells were then cultured for 1 h at 37°C. Then, optical density was calculated at 450 nm (Bio-Rad Laboratories, Hercules, CA, USA). The survival rate of cells (%) = experimental group OD value-blank group OD value/control group OD value-blank group OD value. The experiments were performed in triplicate.

Cell Apoptosis Assay

Cells were seeded into six-well plates (10^6 cells per well) were transfected with 20 nM HOTAIR-siRNAs or siRNA-NC for 48 h. The cells were harvested by trypsinization and washed twice with ice-cold PBS after centrifugation at 2000 rpm for 5 min. 10^5 cells were suspended with 100 μl binding buffer, followed by 5 μl AnnexinV-FITC and 5 μl PI staining solution, and the resulting mixture was held at 4°C in the dark for 10 min. Cell apoptosis was measured by flow cytometry by a BD FACS Caliber instrument (BD Biosciences, San Jose, CA, USA). The experiments were performed in triplicate.

Transwell Assay

Cancer cell migration assay was performed using transwell membranes coated without Matrigel (Millipore, Billerica, MA, USA), and the transwell membranes coated with Matrigel BD Biosciences (Franklin Lakes, NJ, USA) were used

in cell invasion assay. The transfected cells were seeded at 10^4-10^5 per well in the top chamber of transwell assay inserts in 200 μL of serum-free RPMI 1640 medium. The inserts were then placed in the lower chamber filled with RPMI 1640 with 20% FBS. 48 h later, cells remaining in the upper chamber were scrubbed with a sterile cotton swab, while invading cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, examined, counted, and imaged using digital microscopy. The experiments were performed in triplicate.

Wound-healing Assay

The transfected cells were seeded in six-well plates at 10^6 cells per well in 10% FBS medium until confluence reached 90%. The wound was scratched with a 10 μl micropipette tip, and PBS was used to wash and remove the cellular debris. The cells were continued to culture at 37°C. The size of the wound was measured daily. The experiments were performed in triplicate.

Western Blot Assay

We performed Western blot using procedures described in previous work[16]. For protein extraction, we homogenized the cells in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). The proteins (40 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Scientific, Waltham, MA, USA), blocked for 1 h in 5% skim milk, and incubated with a primary antibody at 4°C overnight. Then, the membrane was washed 3 times with PBS before incubating with a secondary antibody for 2 h, and the signal was developed and measured using the Quantity One software (Bio-Rad, Hercules, CA, USA). Primary antibodies: rabbit anti-E-cadherin (Cell Signaling; 1:500); anti-N-cadherin (Cell Signaling; 1:500); anti-vimentin (Cell signaling; 1:500); anti-snail (Cell Signaling; 1:500); anti- total Akt (Abcam, Cambridge, MA, USA; 1:500); anti- pAkt Ser473 (Abcam, Cambridge, MA, USA; 1:500); anti- PI3Kp85a (Abcam, Cambridge, MA, USA; 1:500); anti-human MMP2 (Abcam, Cambridge, MA, USA; 1:500); anti-MMP9 (Cell Signaling; 1:500), and mouse anti-GAPDH (Millipore, Billerica, MA, USA; 1:10.000).

Xenograft Tumors in Nude Mice

The animal protocol was approved by the Institutional Animal Care and Use Committee at Harbin Medical University. Female balb/c nude mice aged six to eight weeks and weighting 20-22 g (Slac Laboratory Animal, Shanghai, China) were randomly assigned into two groups: negative control (sh-NC) group (n=8) and the MALAT1 knockdown (sh-MALAT1) group (n=6). Mice were intraperitoneally injected with lentivirus, and we measured tumor volume using calipers every week. All mice were executed after 33 days, and tumors were collected, paraffin-embedded and immunostained.

Statistical Analysis

Values were expressed as means ± SE. Statistical analysis was performed by ANOVA, χ^2-test, or Student's *t*-test using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). *p*-values less than 0.05 were considered statistically significant ($p<0.05$).

Results

Expression of MALAT1 is Upregulated in EOC

MALATI is upregulated in several tumors. To determine the role of MALAT1in EOC, we compared the expression levels of MALAT1 in 68 EOC and 30 normal ovarian surface epithelial tissues by qRT-PCR, and normalized to GAPDH. We first found that MALAT1 is highly expressed in EOC than in normal tissue (Figure 1A, $p<0.001$). Further, according to the median relative MALAT1 expression value in EOC, the 68 EOC patients were classified into two groups: high (n = 34) and low (n = 34) groups (Figure 1B). As shown by the Kaplan-Meier survival analysis (Figure 2D), patients with higher MALAT1 expression (n = 34) had significantly reduced overall survival compared with patients with lower MALAT1 expression (n = 34) ($p=0.052$). Further, we compared the MALAT1 expression levels between cancer and non-cancerous tissues and confirmed that the expression of MALAT1 in EOC tumor tissue increased by 95.59% (65/68) compared to normal tissue (Figure 1C, $p<0.001$). These results suggested that MALAT1 was upregulated in EOC tumors, supporting a potential role in EOC progression.

MALAT1 Inhibition Slows EOC cell Proliferation in vitro

To explore the functional effect of MALAT1 in EOC, we analyzed the expression of MALAT1 in diverse EOC cell lines by qRT-PCR. The five

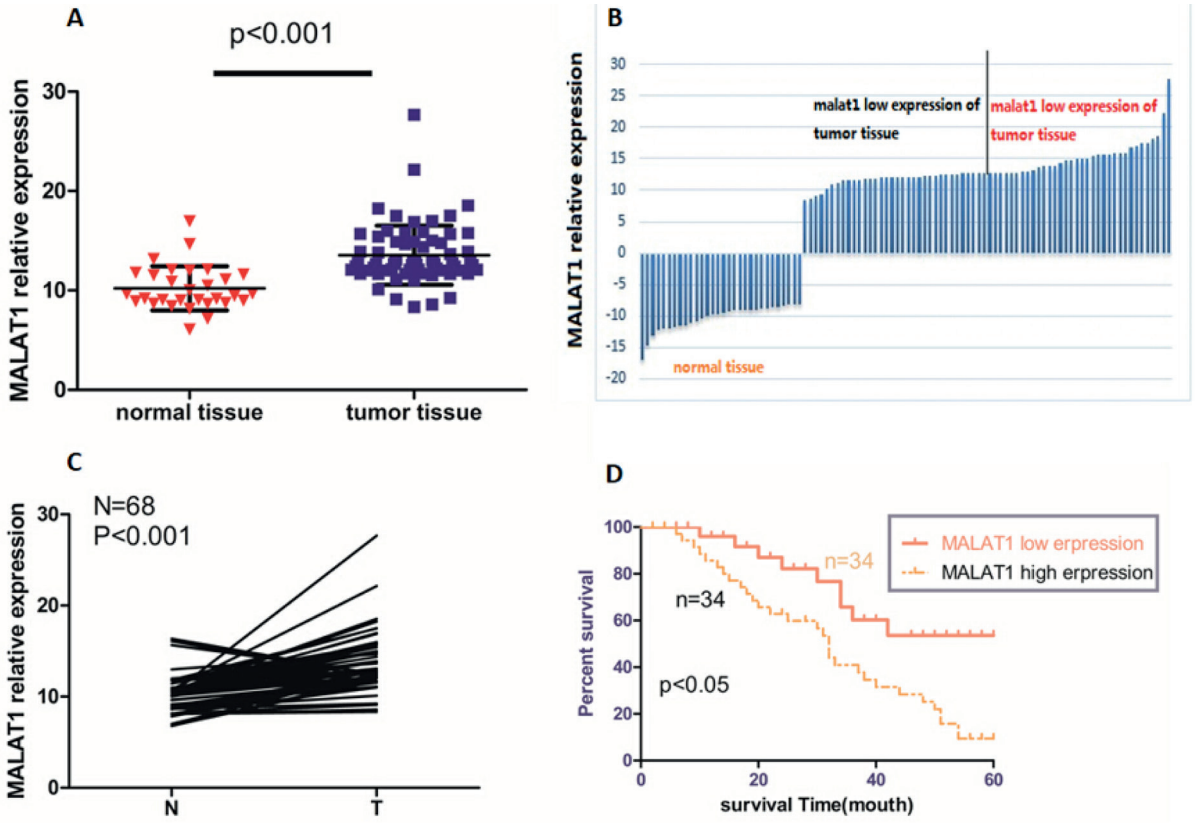

Figure 1. Upregulation of MALAT1 in epithelial ovarian cancer tissues. ***(A)*** relative expression of MALAT1 in EOC and normal ovarian surface epithelial tissues analyzed by qPCR ($p<0.01$); ***(B)*** expression of MALAT1 in EOC and normal tissues were divided into two groups on the basis of expression level in EOC tissues; ***(C)*** expression of MALAT1 in EOC tissues and corresponding non-cancerous tissues ($p<0.01$); ***(D)*** Kaplan-Meier survival analysis for patients with different levels of MALAT1 in EOC ($p<0.05$). MALAT1 expression was normalized against GAPDH expression.

lines we tested had different levels of MALAT1, with SKOV3 showing the lowest and HO8910PM showing the highest (Figure 2A). Next, we transiently transfected siRNA MALAT1 and found A2780 and HO8910 cell lines had the highest transfection efficiency based on the reduction of MALAT1 expression (Figure 2B). A2780 and HO8910 cells transfected with siRNA MALAT1 exhibited significantly reduced cell proliferation (Figure 2C). Moreover, flow cytometric analysis revealed that silencing of MALAT1 markedly promoted cell apoptosis both in A2780 and HO8910 cell lines (Figure 2D).

MALAT1 Silencing Inhibits EOC Cell Metastasis in vitro

We next examined the ability of MALATI to regulate tumor cell metastasis *in vitro*. The transwell assay revealed that the number of cells with migrating and invading activities decreased greatly in MALAT1 knockdown cells (Figure 2E-F). The wound-healing activity of A2780 and HO8910 was inhibited in cells transfected with MALAT1 siRNA (Figure 3A). Collectively, these results indicated that elevated MALAT1 expression levels prevent cell apoptosis and promote cell proliferation, migration, and invasion of EOC cells.

MALAT1 Silencing Suppressed Tumorigenicity of EOC Cells in Nude Mice

After analyzing the function of MALAT1 in cultured cells, we next moved to perform experiments *in vivo*. To research the effect of MALAT1 in tumorigenicity of vivo, we constructed a xenograft tumor model. We stably transfected A2780-NC and A2780-M KD cells and injected them intraperitoneally into nude mice to establish abdominal metastatic ovarian tumors. After 12 days, we measured the volumes of the tumors every three days (Figure 3B). All mice were executed after 33 days, and volume and weight of

A2780-M KD group were compared against the negative control group. The growth of tumors in the MALAT1siRNA group was significantly smaller than those in the control group (Figure 3B). Western blot analysis indicated that EMT-related proteins, N-cadherin, vimentin, and snail were significantly inhibited in tumor tissue from the MALAT1 siRNA group (Figure 3C). In contrast, E-cadherin expression was elevated when MALAT1was silenced.

MALAT1 Silencing Hinders cell Migration and Invasion via the PI3K/AKT Pathway

EMT implicates invasion, metastasis, and stem cell behavior that contribute to cancer progression[17-19]. To further understand the function of MALAT1, we used Western blot analysis to examine EMT-related proteins in two cell lines. We discovered that expression of E-cadherin increased whereas the expression of N-cadherin, snail and vimentin decreased in MALAT1 siRNA in A2780 and HO8910 cell lines (Figure 4A and B). Moreover, matrix metalloproteases (MMPs) such as MMP2 and MMP9, which are involved in migration/invasion[20], were also reduced (Figure 4A-B). Previous results suggested that EMT regulates migration/invasion via PI3K/AKT pathway[21,22]. Western blot analysis indicated that the expression of p-AKT was dramatically reduced whereas the expression of total AKT had no change. These data suggests that inhibition of MALAT1 impeded EMT by downregulating the PI3K/AKT pathway in EOC.

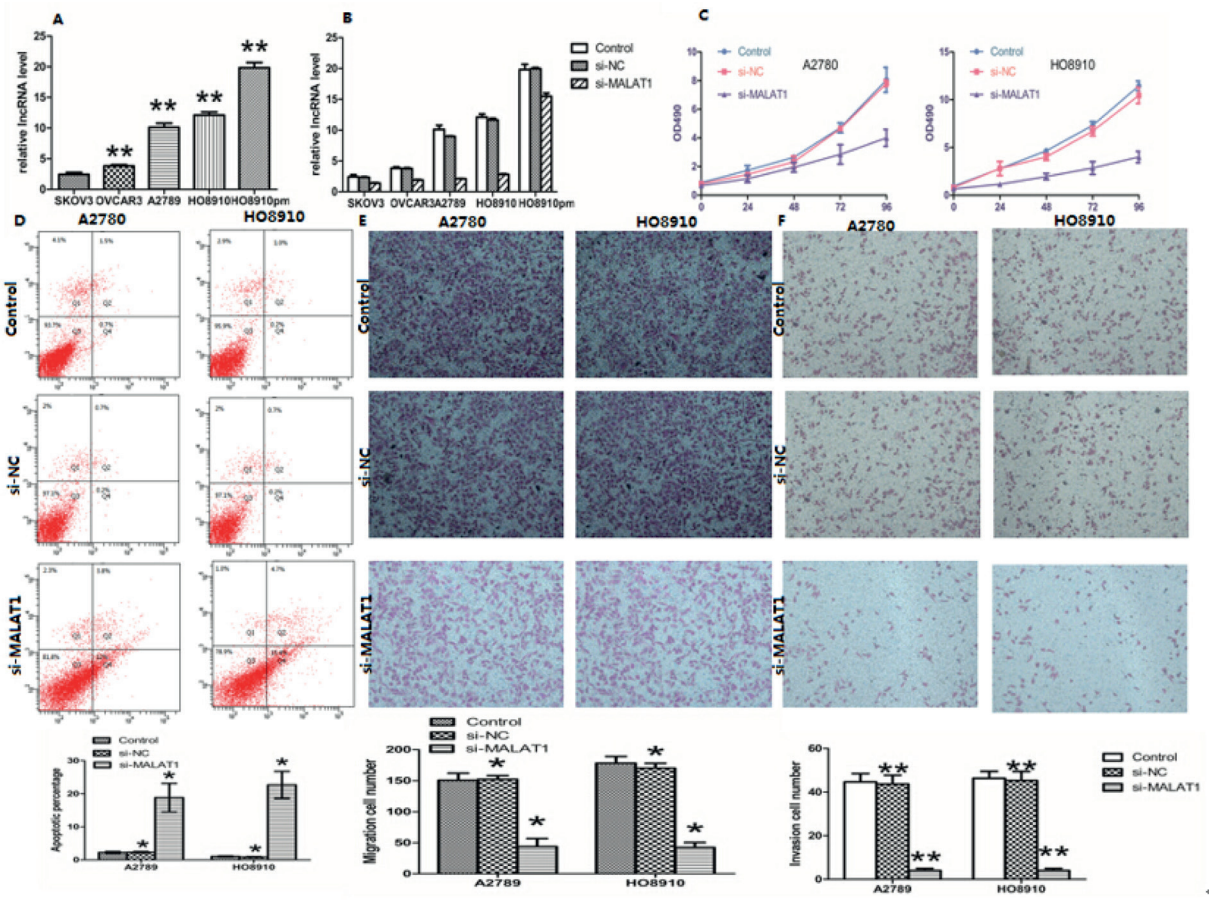

Figure 2. Silencing of MALAT1 enhances EOC cells malignant phenotype. ***(A)*** MALAT1 expression in five ovarian cancer cell lines was analyzed by qPCR; ***(B)*** knockdown efficiency ascertained by qPCR in five ovarian cancer cell lines transfected with si-NC or MALAT1-si RNAs. The efficiency of MALAT1 silencing was higher in A2780 and HO8910; ***(C)*** Knockdown MALAT1 inhibited cell proliferation in A2780 and HO8910 cell lines by CCK-8 assay; ***(D)*** knockdown MALAT1 promoted tumor cells apoptosis in A2780 and HO8910 cell lines by flow cytometry; ***(E-F)*** transwell assay testified silencing of MALAT1 restrained cell invasion and metastasis. The data represent the means ± standard deviations (SDs) from three independent experiments. The error bars denote the SDs. *$p<0.05$, **$p<0.01$.

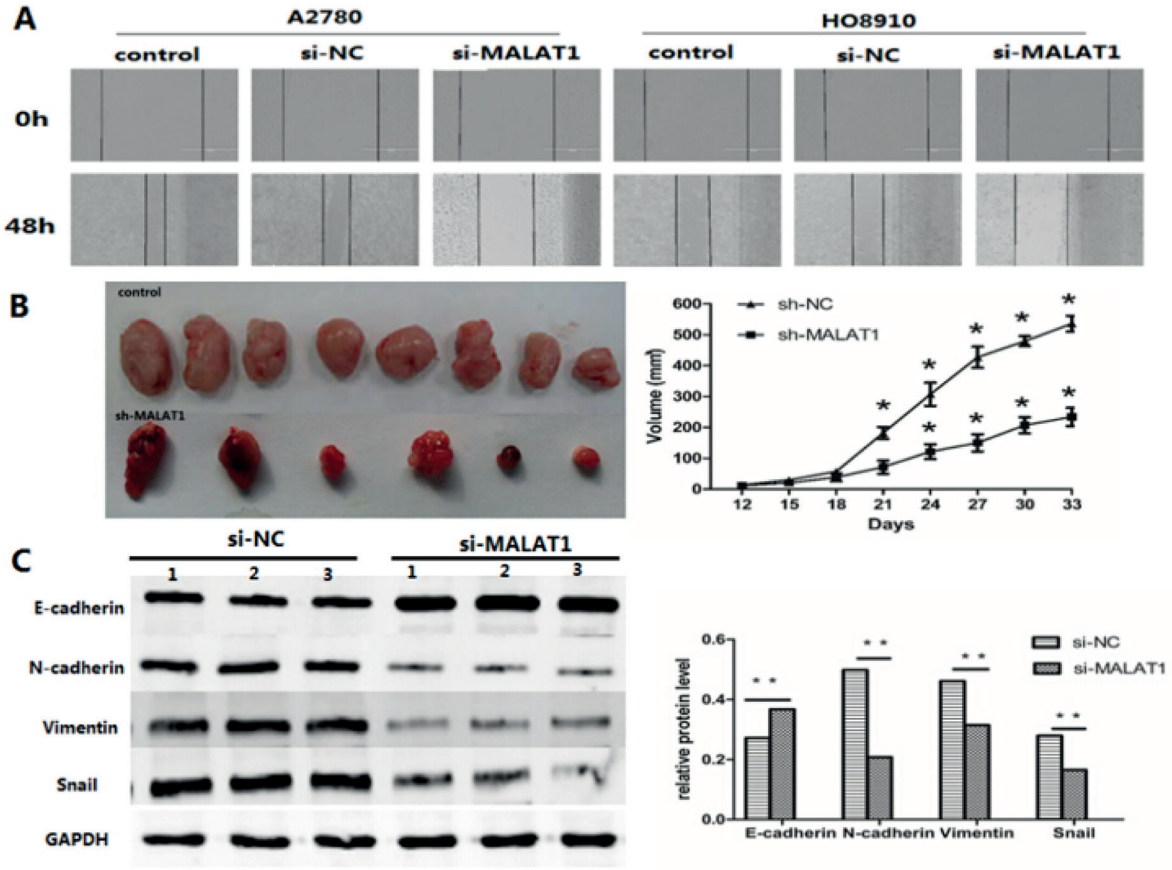

Figure 3. Inhibition of MALAT1 hinders proliferation and invasion. ***(A)*** wound-healing assay suggested MALAT1 knockdown affected migration of EOC cells in A2780 and HO8910 cell lines; ***(B)*** tumors induced by A2780-shMALAT1 cells and A2780-NC cells were excised from nude mice after 33 days and volumes were measured every three days. The tumors were smaller in A2780-shMALAT1 mice; ***(C)*** EMT related proteins, including E-cadherin, N-cadherin, Vimentin, and snail, were verified by western blot analysis. All the experiments were performed in triplicate. *$p<0.05$, **$p<0.01$.

Discussion

Ovarian cancer is the most lethal gynecological malignancy in worldwide, and more than 200,000 women are diagnosed with ovarian cancer every year[23]. The epithelial origin of these tumors comprises over 85%, which are typically diagnosed at an advanced stage[24,25]. Accordingly, the field is in dire need of the new molecular mechanisms that can help with the design of new and effective therapies. Recent studies indicated that lncRNAs play a vital role in transcription and translation[26,27]. Recent evidence suggested that lncRNAs might be significant contributors to abnormalities of cell and tumorigenesis[28,29]. MALAT1 was initially reported to promote tumor cell migration/invasion in non-small cell lung cancer and is overexpressed in many human tumors, including lung cancer[10,30], breast cancer[31,32], hepatocellular carcinoma[33], pancreatic cancer cells[5], prostate cancer[34] cervical cancer[35], and osteosarcoma cells[36,37]. MALAT1 promotes cell proliferation, regeneration, invasion, and metastasis, influences revascularization and inhibits apoptosis[38,39]. Despite these results, the contribution of MALAT1 to EOC is still mostly unknown, but we hypothesize that MALAT1 may be a significant therapeutic target for ovarian cancer. Here, we confirmed that MALAT1 levels were highly expressed in EOC tissue compared with normal tissue. Our research showed that the 5-year- survival rate of the patients with high MALAT1 was dramatically reduced compared with the low MALAT1 group. These results suggest that MALAT1 can be used as a new maker for early diagnosis. Reduced expression of MALAT1 in A2780 and HO8910 cells hindered cell proliferation, invasion, and metastasis, and were promoted apoptosis. Xenographs in nude mice validated the role of MA-

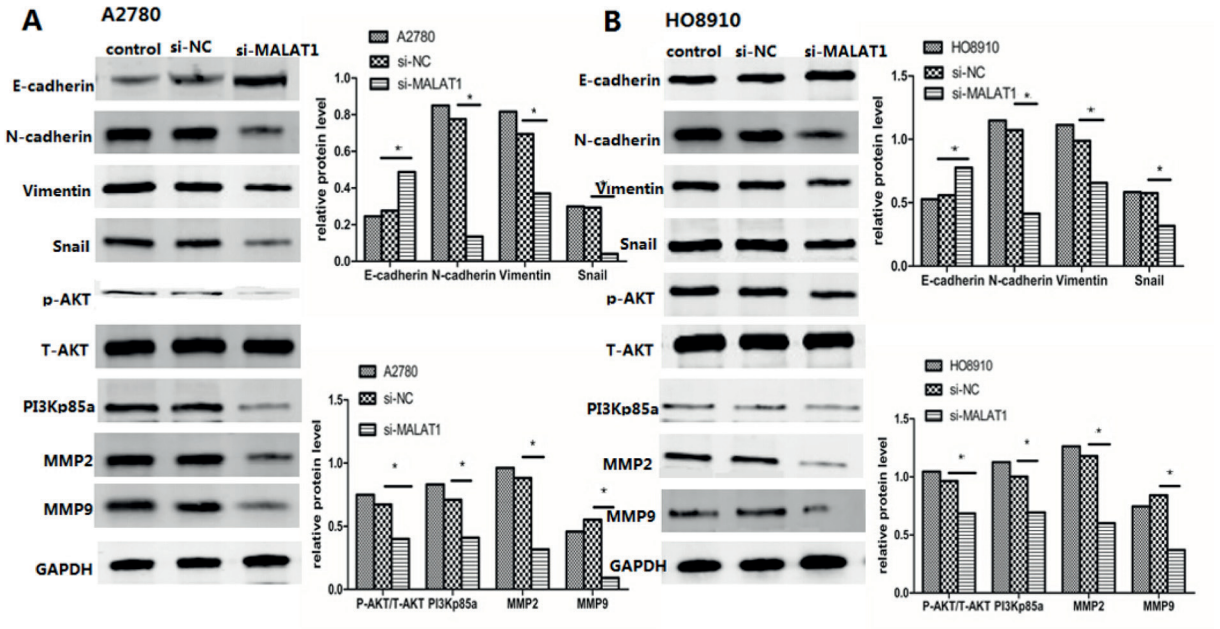

Figure 4. MALAT1 participates in EMT via PI3K/AKT pathway. ***(A-B)*** Western blot analysis shows EMT-related and MMPs proteins altered by MALAT1 knockdown. PI3Kp85a and phospho-AKT were decreased in both knockdown of MALAT1 of A2780 cell line and HO8910 cell line. All the experiments were performed in triplicate. *$p<0.05$.

LAT1 in tumor growth. Because of obvious effects in invasion and metastasis, we hypothesized that MALAT1 was involved in EMT. EMT maintains the mesenchymal cell phenotype and promotes migration and invasion[40,41] through E-cadherin, N-cadherin, vimentin, and snail[42]. The expression of these proteins was significantly perturbed in knockdown MALAT1 cell lines. Furthermore, downregulation of p-AKT in knockdown MALAT1 cells revealed that MALAT1 may influence the EMT in EOC by activating the PI3K-AKT pathway. Consistently, our studies confirmed that the expression of proteins of nude mice was adjusted as same as knockdown MALAT1 cell. Notably, the protein levels of MMP2 and MMP9 also decreased in knockdown MALAT1 cell. MMPs are zinc-dependent endopeptidases that dominate invasion and metastasis in ovarian cancer[43]. Although additional experiments are required to prove the relationship between MALAT1 and EMT, this preliminary evidence indicates that MALAT1 regulates a series of EMT-associated genes by the inactivation of the PI3K/Akt pathway.

Conclusions

We showed that MALAT1 effects on proliferation and metastasis in EOC induce EMT by activation of the PI3K-AKT pathway. High MALAT1 expression was associated with poor prognosis, whereas MALAT1 knockdown inhibited invasion, metastasis, and EMT-related genes in EOC cells in *in vitro* and *in vivo*. These results strongly suggest that MALAT1 can become an effective target for the diagnosis and treatment of ovarian cancer.

Conflict of interest

The authors declare no conflicts of interest.

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