Original Article
The long non-coding RNA H19 promotes invasion and metastasis of ovarian cancer cell through EMT-related protein regulation

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Abstract: The long non-coding RNA (lncRNA) H19 is reported to be involved in invasion and metastasis of ovarian cancer (OC). In addition, the epithelial-mesenchymal transition (EMT) plays a role in the progression and metastasis of OC. However, whether lncRNA H19 involvement in OC invasion and metastasis is associated with EMT remains unclear. Eight cases of OC tissues and the corresponding adjacent normal (AN) tissues were included. The expressions of lncRNA H19 and EMT-related proteins (E-cadherin, TWIST1, Slug, and Snail) were detected by RT-qPCR and immunohistochemistry. Human OC cell lines (SKOv3, OVCAR3, CoC1, A2780, and HO-8910PM) and human normal ovarian surface epithelial HOSEpiC cells were obtained from ATCC. SKOv3 cells that silence the lncRNA H19 expression (silncRNA H19) were established via a transfection of recombinant lentivirus. The expression of lncRNA H19 and EMT-related proteins were significantly increased in human OC tissues compared with relative AN tissues (except for a decline in the level of E-cadherin) assessed through RT-qPCR and western blot. The lncRNA H19 and both EMT-related protein and mRNA levels (TWIST1, Slug, and Snail) in OC cell lines including SKOv3, OVCAR3, CoC1, A2780, and HO-8910PM were dramatically elevated compared with HOSEpiC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05); however, the expression of E-cadherin protein and mRNA were decreased, which is consistent with the results in vivo. Inhibition of lncRNA H19 expression in SKOv3 cells abolished the effects of lncRNA H19 on EMT-associated protein regulation. Importantly, Transwell assay suggested that invasion and metastasis ability were significantly reduced in SKOv3+silncRNA H19 group compared with SKOv3 group (P < 0.01). This study demonstrates a novel mechanism by which lncRNA H19 modulates OC cell invasion and metastasis, and this mechanism is involved in the regulation of EMT-related proteins.

Keywords: LncRNA H19, ovarian cancer, invasion, migration, EMT

Introduction
Ovarian cancer (OC) is the seventh most common type of cancer in gynecologic malignancies, ranking eighth highest in mortality rate of cancers that threaten women’s health [1]. The high mortality rate of OC is due to its high metastasis and recurrence rates [1]. OC cells can invade or spread to other parts of the body. There may be little or no symptoms in the early stages, and the symptoms gradually appear as the cancer progresses [2]. Epithelial-mesenchymal transition (EMT) participates in the progression and metastasis of ovarian cancer, in which the epithelial cells loses their cell polarity and cell-cell adhesion, and acquire migration and invasion properties in the process of becoming mesenchymal stem cells [3]. Therefore, EMT plays an important role in OC invasion and metastasis and is considered as a critical event in cancer progression and metastasis [4]. The induction of EMT is accomplished by several transcription factors including TWIST1, Slug, and Snail that inhibit the expression of E-cadherin in various forms of human cancers and are responsible for aggressive tumor behavior and poor prognosis [5-9].

Long non-coding RNA (lncRNA) is a non-coding RNA with a length of more than 200 nucleotides, which regulate various normal life activities [10, 11]. However, the abnormal expression
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and distribution in various human cancer tissues are related to the occurrence and progress of cancer [12]. Previous studies have revealed that the expression of IncRNA H19 in OC tissues and cell lines is abnormally elevated, which promotes the migration and invasion of cancer cells [13]. In addition, IncRNA H19 regulates cisplatin resistance through glutathione metabolism regulation in high-grade serous OC [14]. These results suggest that IncRNA H19 plays an oncogene role in ovarian cancer, but the underlying mechanism in terms of the relationship between IncRNA H19 effects on OC invasion and metastasis and EMT-related protein regulation remains poorly understood.

Thus, the aims of this study were: (1) to testify to the expression of IncRNA H19 and EMT-associated protein and mRNA in OC tissues and cells; (2) to investigate the roles of IncRNA H19 on EMT-related protein regulation in OC cells; (3) to determine the effects of IncRNA H19 inhibition on OC cell invasion, migration and viability.

Materials and methods

Clinic tissue preparation

Eight cases of clinical specimens form OC patients were collected. All patients were hospitalized in the Department of Obstetrics and Gynecology, in the Changzheng Hospital Affiliated with the Naval Military Medical University from October 2017 to November 2018. Tumor samples were obtained during the first operation without chemotherapy, and the tissues were confirmed as serous epithelial OC pathologically after the operation. The corresponding adjacent normal (AN) tissues were also acquired from the same patient. These patients ranged in age from 45 to 58 years, with an average age of 49.6 years. This study was approved by the Medical Ethics Committee of Changzheng Hospital, and all patients signed the informed consent.

Lentivirus (LVs) and plasmids preparation

Recombinant lentivirus (LVs) was prepared from GeneChem (Shanghai, China). Short interference RNA (siRNA) vector-LVs against IncRNA H19 (GenePharma, Shanghai, China) were successfully constructed and were then packaged in 293T cells. The siRNA sequence for IncRNA H19 was: 5'-UAAGUCAUUUGCUGGUU-3'. Meanwhile, a scrambled locus siRNA was constructed with LVs for the vehicle group.

Cell culture and transfection

The human ovarian cancer cell lines (SKOv3, OVCAR3, CoC1, A2780, and HO-8910PM) and human normal ovarian surface epithelial HOSEpiC cells were acquired from ATCC (Manassas, USA). Cells were plated on 60 mm dishes (Corning, New York, USA) in RPMI-1640 medium (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin-Streptomycin solution (Invitrogen, Carlsbad, USA), cultured at 37°C in a humidified atmosphere containing 5% CO₂. When the confluence reached approximately 80%, silncRNA H19-LVs was transfected following the manufacturer’s protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TRizol reagent (Invitrogen, Carlsbad, USA) was used to extract the total RNA from OC tissues and cells and HOSEpiC cells. The RNA was then subjected to reverse transcription to cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Afterwards, 20-uL reactions with IncRNA H19, E-cadherin, TWIST1, Slug, or Snail primers (GENEWIZ, South Plainfield, USA) were detected using a PikoReal 96 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) with SYBR Green PCR Master Mix (Applied Biosystems, Waltham, USA). Relative quantitative analysis in mRNA expression was obtained using the $\Delta\Delta$CT method and normalized to GAPDH. The sequences of the primers are demonstrated in the Table 1.

Immunohistochemical staining

OC sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% H₂O₂ at room temperature for 30 min, and the sections were then washed with phosphate-buffered saline (PBS; pH 7.4). Primary antibody against E-cadherin, TWIST1, Slug, and Snail (Abcam, Cambridge, UK; 1:100) were used and incubated at 4°C overnight. The slides were then washed with PBS and incubated with the secondary antibody from the streptavidin-peroxidase secondary antibody kit (ZSGB-BIO, Beijing, China) according to the
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manufacturer’s instructions. After chromogenic development with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-BIO, Beijing, China), the slides were then counterstained with haematoxylin, dehydrated through a graded alcohol, and mounted with neutral gum.

Western blot

OC and HOSEpiC whole cell proteins were extracted using a Cell Total Protein Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer’s protocol. Protein concentrations were measured using a bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Equivalent amount of protein was prepared and separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electro-transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany). Then anti-E-cadherin (1:1000), TWIST1 (1:50), Slug (1:1000), Snail (1:500) or GAPDH (1:10,000) (Abcam, Cambridge, UK) antibodies were incubated at 4°C overnight, and probed with relative secondary antibodies at room temperature for 2 h. Finally, signals were detected by Odyssey Infrared Imaging System (Li-Cor, Lincoln, USA). Protein expression levels were calculated from the ratio of protein/GAPDH ×100%.

Boyden chamber Transwell migration assay

The migration ability of SK0v3 and HOSEpiC cells were testified by a Transwell chamber culture system (8 μm pore; Corning). Briefly, SK0v3 and HOSEpiC cells were seeded in a Boyden chamber Transwell without matrigel-coating at a concentration of 2×10^5 cells/well in serum-free Opti-MEM medium (Gibco). Then, the lower chamber were added with complete growth medium supplemented with 10% FBS. After incubation at 37°C for 1 d, the cells attached to the lower insert filter were stained with 0.1% crystal violet (Solarbio, Beijing, China) at room temperature for 10 mins. Finally, the migrated cells were measured and counted by Image-Pro Plus 6 software (Media Cybernetics, Rockville, USA).

Cell counting kit-8 (CCK-8) assay

SK0v3 and HOSEpiC cells were seeded into 96-well plates (Corning) at 1×10^4 cells/mL. Cell viability was assessed using a cell counting kit-8 (CCK-8; DOJINDO, Tokyo, Japan). Absorbance at 450 nm was measured with a microplate reader (BioTek Synergy 2, Vermont, USA). The means of the optical density (OD) measurements from 6 wells were used to calculate the percentage of cell viability.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using PASW Statistic 23 (SPSS Inc., Chicago, USA). Statistical comparison was carried out with two-tailed Student’s t test or one-way analysis of variance. Analyses were performed using GraphPad prism 6 (La Jolla, USA). Significance was defined as P < 0.05.

Results

Overexpression of IncRNA H19, TWIST1, Slug, Snail and low-expression of E-cadherin are observed in human ovarian cancer tissues

Ovarian cancer (OC) tissues and relative adjacent normal (AN) tissues were obtained from clinical ovarian cancer patients, and the level of IncRNA H19 was detected by RT-qPCR. Results revealed that H19 transcription level in OC tissues was significantly higher than that in AN tissues (P < 0.01; Figure 1A). Furthermore, the immunohistochemical assay demonstrated that the EMT-associated protein expression including TWIST1, Slug, and Snail were increased in the OC group compared with AN tissues.
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**Figure 1.** LncRNA H19 and EMT-related protein expression in human ovarian cancer tissues. A. The expression of lncRNA H19 was measured by RT-qPCR in ovarian cancer (OC) tissues and relative adjacent normal (AN) tissues, n = 8. **P < 0.01 vs. indicated group. B.** Immunohistochemical staining for TWIST1, Slug, Snail, and E-cadherin expression in AN and OC tissues, bar = 1 mm. Data were compared using two-tailed Student’s t test.

As expected, the level of E-cadherin in the OC group was declined compared with AN group.

**Up-regulation of lncRNA H19, TWIST1, Slug, Snail and down-regulation of E-cadherin RNA levels are observed in ovarian cancer cell lines**

The expression of lncRNA H19 in all OC cell lines (SKOv3, OVCAR3, CoC1, A2780, and HO-8910PM) was significantly elevated compared with human normal ovarian surface epithelial HOSEpiC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05; Figure 2A). In addition, the mRNA expression of TWIST1, Slug, and Snail were promoted, and E-cadherin mRNA expression was reduced in all OC cell lines compared with HOSEpiC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05; Figure 2B-E).

**EMT-associated protein expression in OC cell lines and HOSEpiC cells**

Consistent with the mRNA results, the protein levels of E-cadherin in OC cell lines were dramatically decreased compared with HOSEpiC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05; Figure 3A and 3B). Additionally, another EMT-related protein expression such as TWIST1, Slug, and Snail were notably up-regulated in OC cell lines compared with HOSEpiC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05; Figure 3A and 3C-E).

**LncRNA H19 is responsible for the EMT-related protein regulation in SKOv3 cell line**

As the lncRNA H19 and EMT-associated protein expression were most notable in SKOv3 cell
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We therefore used the SKOv3 cell line for the next study. To further determine the relationship between lncRNA H19 and EMT-related proteins, we silenced the expression of lncRNA H19 (silncRNA H19) in SKOv3 cell line and detected the corresponding changes of EMT-related protein and mRNA expression. As shown in Figure 4A there was no difference of lncRNA H19 expression between SKOv3 and SKOv3+vehicle group. Silencing the lncRNA H19 expression results in a significant increase in protein and mRNA expression of E-cadherin compared with SKOv3 (normal) group (P < 0.01; Figure 4A-C). Furthermore, both protein and mRNA levels of TWIST1, Slug, and Snail were dramatically reduced in silncRNA H19 group compared with normal group (P < 0.01).

**Down-regulation of lncRNA H19 inhibits the invasion and metastasis and cell viability in SKOv3 cell line**

Finally, the relationship between lncRNA H19 and invasion and metastasis and cell viability of OC cells were determined through Transwell migration assay and CCK-8 assay, respectively. As shown in Figure 5A and 5B, the invasion and metastasis ability of the SKOv3 cell line were significantly increased compared with HOSEpiC cells (P < 0.01). However, for the cell viability there was no significance between SKOv3 and HOSEpiC cells (Figure 5C). Interestingly, the SKOv3 cell line with lncRNA H19 expression silencing lead to a sharp decline both in invasion and metastasis ability and cell viability compared with normal (SKOv3) group (P < 0.01).

**Discussion**

OC is the leading cause of mortality in women worldwide, and epithelial ovarian cancer (EOC) accounts for nearly 90% of all ovarian malignancies. The hysteretic diagnosis is responsible for the high mortality of OC after it has reached end-stage and a high rate of therapy resistance [2, 15]. Approximately 75% of patients suffer from metastatic disease, in which cancer has spread in the peritoneal cavity because of the lack of specific symptoms and effective imaging techniques [16]. In addition, several studies have demonstrated that the invasion and migration ability of OC cells were elevated after losing epithelial features and gaining a mesenchymal characteristic which is known as EMT [17]. EMT is a process in which epithelial cell exhibit mesenchymal...
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features, which are important for wound healing and stem cell differentiation, and pathologically promote fibrosis and cancer progression [18]. Therefore, EMT promotes the OC cells development as invasive, and move from the systemic circulation to be a new host tissue. EMT is achieved through down-regulating E-cadherin [19], cytokeratin, laminin-1, MUC-1 and microRNAs (miR-200 family) and up-regulating transcription factors including Snail1, Snail2, TWIST1, miR-10b and miR-21 [20]. Additionally, EMT is induced by a variety of signals such as growth factors, Wnt/β-catenin, integrin, Notch transcription factors, prostaglandin E2, cyclooxygenase-2, and hormones [21]. EMT increases the invasion and metastasis of OC cells by modulating matrix metalloproteinase production and altering cytoskeletal organization [22]. These studies suggest that EMT plays a vital role in OC progression and can be a potential target for preventing OC progression [23, 24].

LncRNA H19 is a carcinoembryonic antigen that is only slightly expressed in normal human tissues. Furthermore, lncRNA H19 plays an important role in cell proliferation and development of cancer cells [25]. The expression of lncRNA H19 is up-regulated in a variety of tumors such as adrenal cortical tumors, choriocarcinoma [26], hepatocellular carcinoma, bladder cancer, ovarian serous epithelial cancer, endometrial cancer [27], breast cancer, acute T cell leukemia [28]. In addition, lncRNA H19 is involved in the metastasis of these tumors [29]. In T24 bladder cancer cells, lncRNA H19 increases gene expression related with angiogenesis and tumor metastasis. In CRC colorectal cancer cells, lncRNA H19 promotes the expression of EMT-related
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markers such as vimentin, ZEB1 and ZEB2, and induces miR-138 and miR-200a to promote cell migration [30]. In addition, after subcutaneous injection of SGC7-901 cells in combination with lncRNA H19 into nude mice, rapid tumor growth and an increase in the number of nodules in the peritoneal cavity were observed [31]. These studies have revealed that lncRNA H19 modulates the migration and metastasis of OC, but whether its role on OC is correlated to the regulation of EMT-related proteins has not been clearly reported.

In the present study, we indicated lncRNA H19 increases OC cell invasion and metastasis via modulating the EMT-related proteins. The expression of lncRNA H19 and EMT-associated protein (TWIST1, Slug, and Snail) levels were dramatically up-regulated, except E-cadherin levels, in human OC tissues compared with rel-
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Consistently, both EMT-related protein and mRNA levels (except a decrease of E-cadherin protein and mRNA expression) and expression of lncRNA H19 in SKOv3, OVCAR3, CoC1, A2780, and HO-8910PM OC cells were all promoted compared with HOSeqPC cells (SKOv3 and OVCAR3: P < 0.01; CoC1, A2780, and HO-8910PM: P < 0.05). Furthermore, there is a significant decrease of TWIST1, Slug, and Snail expression and a sharp increase of E-cadherin expression in SKOv3 silncRNA H19 group compared with SKOv3 group (P < 0.01). It is notable that inhibition of lncRNA H19 expression attenuated the invasion and metastasis of OC cells and reduced the OC cell viability in SKOv3+silncRNA H19 group compared with SKOv3 group (P < 0.01). These results indicate lncRNA H19 induces OC cell invasion and metastasis and promotes cell viability through regulating EMT-related protein expression both in mRNA and protein levels. However, the potential signaling pathway for the regulation of EMT protein by lncRNA H19 deserves further investigation. In addition, whether lncRNA H19 also plays a similar role in chemotherapy-resistant OC cells remains to be verified.

In general, our study provided novel insight into understanding the mechanism underlying OC cell invasion and metastasis and lncRNA H19. Additionally, the effects of lncRNA H19 on OC cell viability and invasion and metastasis is associated with regulation of EMT-related proteins. Inhibition of lncRNA H19 may be a promising therapeutic target for overcoming the threat of OC.

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Disclosure of conflict of interest

None.

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