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Long non-coding RNA H19 correlates with unfavorable prognosis and promotes cell migration and invasion in ovarian cancer

Hainong Ma, Li Gao, Huimin Yu, Xu Song

Hwamei Hospital, University of Chinese Academy of Sciences, Ning Bo, China

ABSTRACT

Objectives: The purpose of this study is to investigate the expression pattern of lncRNA H19 in OC tissues and to detect the ability of H19 to influence OC cell migration and invasion *in vitro*.

Material and methods: We quantified the levels of H19 within the obtained cancerous and adjacent noncancerous tissues from 258 OC patients. H19 association with patient progression-free survival (PFS) was analyzed by a Kaplan-Meier plot. Expression levels of H19 were reduced by small interfering RNA transfection against H19 or restored by a H19 overexpression plasmid transfection in OC cells. H19 effects on OC cell migration and invasion *in vitro* were evaluated using wound-healing assay and transwell invasion assay. Wound healing assay and transwell invasion assay were used to evaluate the effects of H19 on OC cell migration and invasion *in vitro*.

Results : H19 is upregulated remarkably in primary OC tissues and human OC cell lines (OVCAR3, SKOV3, A2780, and Caov-3). We found that the median PFS was longer in patients with lower levels of H19 than in those with high levels, suggesting that overexpres- sion of H19 was linked to poor prognosis in OC patients. Intriguingly, the depletion of H19 expression induced by small interfering RNA inhibited the capability of migration and invasion of OC cell lines. Restoration of H19 in OC cell lines significantly increased cell migration and invasion.

Conclusions: The key finding of the present study suggests that overexpression of H19 may be associated with an unfavorable prognosis for OC and is likely to be a possible contributory force involved in OC cell migration and invasion. H19 may provide a new and attractive target for future prognostic and therapeutic intervention of OC patients.

Key words: ovarian cancer; long non-coding RNA; H19; prognosis; migration

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INTRODUCTION

The continuing increase in the incidence and prevalence of ovarian cancer (OC) is a cause for concern [1]. Up until now, OC represented the second most common gynecological malignancies, accounting for 5% of all cancers in females [2]. On an annual basis, an estimated 220,000 newly diagnosed OC cases worldwide [3]. Due to its late presentation and often being diagnosed at an advanced stage, most patients succumb to recurrence and wide metastasis, explaining the high mortality rate [4]. OC is heterogeneous in nature and encompasses a collection of distinct histologic types, all with characteristic differences, which is one of the major obstacles to improvement in this disease [5]. Early stage OC symptoms of peritoneal metastasis are generally nonspecific, which subsequently allows for frequent misdiagnoses as well as underdiagnoses. Expression patterns of a recently identified biomarker family, long non-coding RNA (lncRNA), seem to be characteristic of tumor type and developmental origin, including OC [6, 7].

Understanding of expression pattern and imprinting of H19 has progressed considerably in recent years. The function of H19 in cancers remains to be elucidated due to its dual roles acting either as a tumor suppressor or an oncogene. In a recent study, H19 is shown to inhibit cancer progression [8, 9]. However, increasing evidence showed that H19 expression was increased in several cancers such as breast cancer, lung cancer, gastric cancer, and bladder cancer [10–13], highlighting its oncogenic properties. In-

Corresponding author:
Xu Song
Hwamei Hospital, University Of Chinese Academy Of Sciences, 41 Xibei Street, 315000 Ning Bo, China
e-mail: xu.song@yandex.com

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triguingly, several gain- or loss-of-function studies demonstrated that H19 knockdown suppressed cell migration and invasion in clear cell renal carcinoma and glioma [14, 15]. Few data support the functional role of H19 in OC, and only a study reported by Zhu *et al.* proposed the contributory effect of H19 in OC [16]. However, measurement of H19 in 70 pairs of OC tissue samples and normal controls may limit the validity of results.

To investigate the expression and functional role of H19 in human ovarian cancer, in this study we quantified the levels of H19 within the obtained cancerous and adjacent noncancerous tissues from OC patients. Progression-free survival (PFS) was calculated by following up with the patients. In addition, functional assays were applied in OC cells. Our research may provide evidence for the diagnosis and treatment of ovarian cancer.

MATERIAL AND METHODS

Study subjects

We collected cancerous and adjacent noncancerous tissues from 258 patients with OC who were admitted into our hospital between January 2010 and January 2014. These patients had a mean age of 48.5 ± 6.3 (ranging from 29 to 68 years old). The inclusion criteria for patient enrollment: (1) an ovary biopsy with pathological and imaging diagnosis of OC; (2) an initial treatment in our hospital for OC; (3) no standard therapies, such as chemotherapy, surgery, or radiation therapy; (4) no previous history of the malignant tumors; (5) no distant metastases or second primary tumor. The study protocol was approved by the Institutional Ethics Committees at our hospital, and signed written informed consent was received from all patients prior to enrollment.

Follow-up

The follow-up was scheduled at discharge, ranging from 3 to 36 months and consisting of a telephone interview, a medical record review, or a hospital visit. The follow-up period ended on December 2016. Among 258 cases, 6 cases were censored. A follow-up rate of 93.0% was achieved. The primary endpoint was progression-free survival (PFS) was defined as the time from diagnosis to either disease progression or relapse, or to death as a result of any cause.

Cell preparation

Human normal ovarian surface epithelial cell lines were purchased from Shanghai Huiying Bio-technology Co., Ltd., China and human OC cell lines, OVCAR3, SKOV3, A2780, and Caov-3, from Cell Bank of Chinese Academy of Sciences, Shanghai, China. SKOV3 cells were cultured with McCoy's 5A Medium Modified (Sigma, St. Louis, MO, USA), in addition,RPMI-1640 medium (Gibco Company, Grand Island, NY, USA) for OVCAR3 and A2780 cells, and DMEM-H (Dulbecco's Modified Eagle's Medium, High Glucose; Hyclone Laboratories, Logan, Utah, USA) for Caov-3 cells. All of the media were supplemented with 10% fetal bovine serum (FBS, Gibco Company, Grand Island, NY, USA), and all cells were incubated with 5% CO_2 at 37°C. In order to evaluate the regulatory effects of H19 on OC cells, SKOV3 cells showing the highest expression level of H19 were treated by small interfering RNA (siRNA) against H19 and a H19 overexpression plasmid, respectively. An ineffective scramble of siRNA, a siRNA against H19, and a H19 overexpression plasmid was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) was used to transfection operations according to the manufacturer's instructions.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from tissues was isolated by TRIzol kits (Invitrogen, Carlsbad, CA, USA). High-molecular-weight RNA was identified on a denaturing formaldehyde gel, then l μg of RNA was reversely transcripted into cDNA using AMV-reverse transcriptase. Primers were obtained from Invitrogen Inc., Carlsbad, CA, USA (Tab. 1). GAPDH was used as a loading control. The PCR cycling reaction conditions: pre-denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were then subject to agarose gel electrophoresis and analyzed by Opticon Monitor™ version 3.0 software (Bio-Rad, Inc., Hercules, CA, USA). The Opticon monitor software (MJ Research, San Francisco, CA, USA) was used to set the cycle threshold or Ct line manually. Data were analyzed by $2^{-\Delta\Delta Ct}$ method. ΔCt = Ct (target gene)-Ct (loading control), $\Delta\Delta Ct = \Delta Ct$ (experimental group)-ΔCt (control group). The experiment was repeated three times independently, with the average obtained.

Transwell assay

Forty eight hours after transfection, 1×10^5 cells were counted and inoculated in Matrigel-coated (80 μl of at a ratio of 1:8) transwell chambers containing 100 μl serum-free DMEM medium. The Matrigel and cells that remained on the upper side were wiped off following 24 hours incubation. Then, the passed cells were fixed in 4% paraformaldehyde for 15 min and subsequently stained with 0.2% crystal violet for 10 min. Lastly, an inverted light microscope (Olympus IX70, Tokyo, Japan, at × 200 magnification) was applied to count the number of invading cells in five predetermined fields to evaluate cell invasion. All experiments were independently performed at least three times.

Table 1. primer sequences of OPN for reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Gene	Sequences
H19	F: 5′-GTCCGGCCTTCCTGAACACCTT-3′
	R: 5′-GCTTCACCTTCCAGAGCCGAT-3′
GAPDH	F: 5′-GACAACTTTGGCArCGTGGA-3′
	R: 5′-ATGCAGGGATGATGTTCTGG-3′

Scratch test

On the back of the 6-well plate, use a marker pen to scratch an even with an interval width of 0.8 cm. Each well was required to be crossed by more than five lines and added with 5×10^5 wells. When the cells grew to 100%, a uniform scratch was made in the center of the well using a sterile micropipette tip, followed by washing with phosphate-buffered saline. After 58 hours incubation with 5% CO_2 at 37°C, the wound healing was photographed for the record. The rate of cell migration was assessed by the wound closure assay. All experiment was also independently performed at least three times.

Statistical analysis

Statistical analysis was done using SPSS software (IBM SPSS Statistics, version 21.0, Armonk, NY, USA). Measurement data were expressed as the mean ± standard deviation (SD); Fisher's least significant difference (LSD) was conducted for pairwise comparisons, one-way analysis of variance (ANOVA) test for comparisons among multiple groups, and *t*-test for comparisons between two groups when demonstrating normal distribution. The association with survival was analyzed initially by Kaplan-Meier plot and log-rank test. Differences were accepted as significant if p-values less than 0.05.

RESULTS

Increased expression levels of H19 in OC primary tissues

Firstly, in order to evaluate the expression pattern of H19 in OC, we quantified the expression levels of H19 within the obtained cancerous and adjacent noncancerous tissues from 258 OC patients using RT-qPCR. We found that the expression levels of H19 in cancer tissues (5.82 ± 0.67) were higher than those in adjacent noncancerous tissues (2.46 ± 0.29, $P < 0.01$).

Increased expression levels of H19 associated with poor survival of OC patients

Next, we classified 258 OC patients into low- and high-level groups in terms of expression levels of H19 in OC primary tissues to evaluate the association between the H19 expression levels and patient survival. We regarded patient PFS as the end point event. The Kaplan Meier survival analysis (Fig. 1) showed the median PFS was longer in OC tissues with low expression levels of H19 than in those with high expression levels (30.00 months *vs.* 24 months, $P < 0.01$). The data reveal that increased expression levels of H19 associated with poor survival of OC patients.

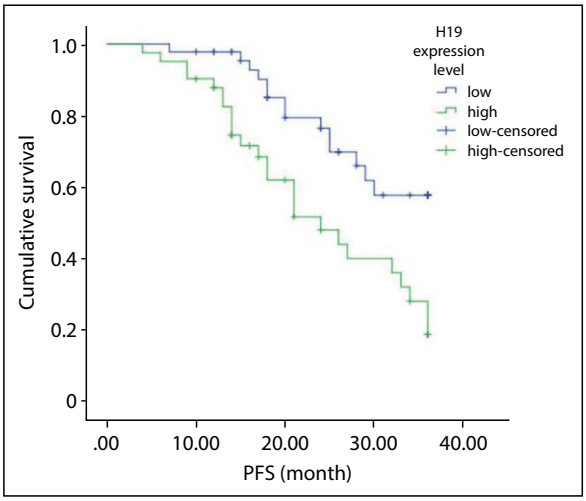

Figure 1. The Kaplan-Meier plot showed that increased expression levels of H19 are associated with poor survival of OC patients

Increased expression levels of H19 in OC cell lines

In this study, we chose OC cell lines that have been widely used in OC research, especially in functional studies. We examined the expression levels of H19 in OVCAR3, SKOV3, A2780, and Caov-3 cell lines by RT-qPCR. The result showed that H19 was increased in these four OC cell lines ($P < 0.05$, Fig. 2) in which SKOV3 cell lines were highest.

Silencing or restoring H19 in OC cells

Furthermore, we treated SKOV3 cells with an ineffective scramble of siRNA, siRNA against H19, and a H19 overexpression plasmid, respectively. We quantified the expression levels of H19 in SKOV3 cells after different treatments by RT-qPCR. Indeed, H19 expression was restored by H19 overexpression plasmids, while H19 expression was suppressed by siRNA against H19 ($P < 0.05$). There was no significant difference in H19 expression levels when untreated SKOV3 cells were compared to SKOV3 cells treated with ineffective scramble of siRNA. The data are shown in Figure 3.

The contributory effects of H19 on OC cell invasion and migration in vitro

To know the function of H19 in OC, we tested the effects of H19 on cell invasion and migration *in vitro* by transwell invasion assay and wound-healingassay. To determine the

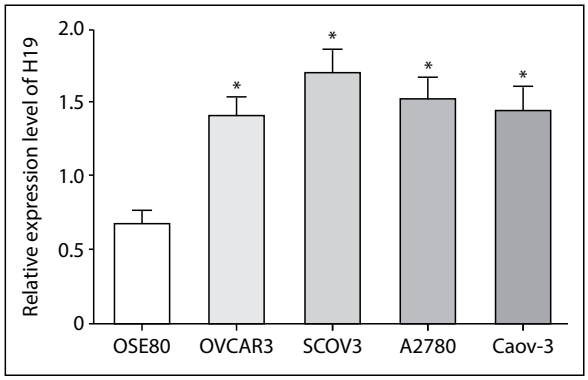

Figure 2. Increased expression levels of H19 in OC cell lines, determined by RT-qPCR. Compared with IOSE80 cells, OVCAR3, SKOV3, A2780, and Caov-3 cells showed higher expression levels of H19 (* — $P < 0.05$). Among these four OC cell lines, SKOV3 cell lines exhibited the highest expression levels of H19

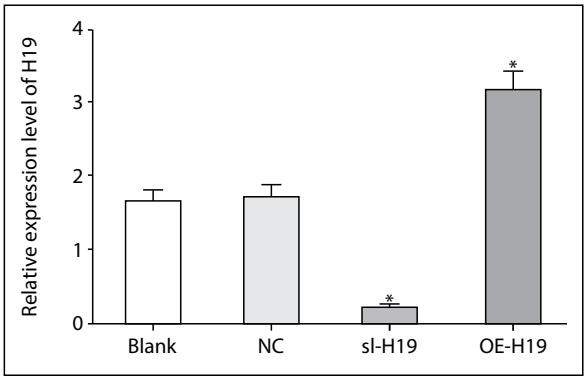

Figure 3. H19 expression was suppressed or restored in SKOV3 cells, verified by RT-qPCR; * — $P < 0.05$ compared with untreated SKOV3 cells

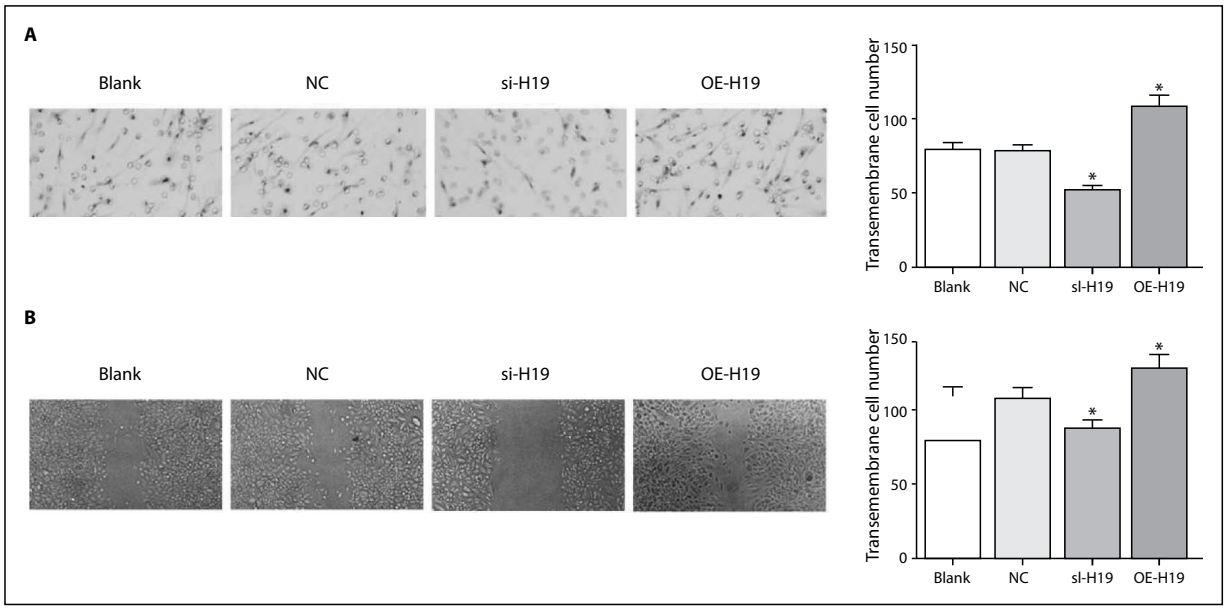

Figure 4. H19 could promote OC cell invasion and migration; **A.** wound-healing assay was used to determine the effect of H19 on the invasion potential of SKOV3 cells (× 200); **B.** transwell invasion assay was used to determine the impact of H19 on migration potential of SKOV3 cells (×200); * — $P < 0.05$ compared with untreated SKOV3 cells

effect of H19 on the invasion potential of SKOV3 cells, we first carried out Matrigel invasion assays (Fig. 4A). After harvesting for 48 h, we found fewer SKOV3 cells treated with siRNA against H19 passed through Matrigel, but more SKOV3 cells treated with H19 overexpression plasmids passed through Matrigel ($P < 0.05$). An ineffective scramble of siRNA did not significantly affect the invasion of SKOV3 cells ($P > 0.05$). Next, wound-healing assay was used to determine the effect of H19 on the migration potential of SKOV3 cells (Fig. 4B). After harvesting for 48 h, we found SKOV3 cells treated with siRNA against H19 were distinctively less migrated, but SKOV3 cells treated with H19 overexpression plasmids healed the wound area faster ($P < 0.05$). An ineffective scramble of siRNA did not significantly affect the migration of SKOV3 cells ($P > 0.05$). These findings indicated that H19 could promote OC cell invasion and migration *in vitro*.

DISCUSSION

LncRNAs are becoming new candidates for diagnosing cancer disease, explaining the mechanism of the pathogenesis and development of malignant tumors, predicting prognosis and treating disease as targets in recent years [17]. In the present study, we demonstrate the role of H19 as an oncogene in OC. Significantly, H19 was found to be increased in primary OC tissues and cell lines compared with adjacent normal tissues and normal cell lines,

and overexpression of H19 was linked to poor prognosis in OC patients. In addition, in order to detect the ability of H19 to influence OC cell migration and invasion *in vitro*, the expression levels of H19 were reduced by small interfering RNA transfection against H19, or restored by a H19 overexpression plasmid transfection in OC cells. The contributory effects of this lncRNA on cell migration and invasion indicate that H19 promotes tumorigenesis in OC.

Recently, H19 is highlighted for its association and involvement with many cancers, as it plays an important role in regulating the expression of many genes that are essentials for numerous cellular processes [18]. Yang *et al.* reported that ectopic expression of H19 increased cell proliferation, and cell apoptosis was induced in gastric cancer cell lines while siRNA-mediated down-regulation of H19 [12]. They also found H19 affected the activity of p53, and that this effect was leading to partial p53 inactivation. Suppression of H19 induces invasion of serous borderline ovarian tumor cells *via* reducing PI3K/Akt-mediated inhibition E-cadherin [19]. He *et al.* reported the mechanism that overexpression of H19 was sufficient to increase the expression of E2F1 by which H19 promotes OC migration and invasion [14]. E2F1 exerts an anti-proliferative effect in OC cells, becoming a target for preventing OC [20]. Luo and his team found that upregulated H19 promotes bladder cancer cell migration associating with enhancer of zeste homolog 2 (EZH2), and that this association was leading to Wnt/β-catenin activation and subsequent inhibition of E-cadherin [13]. EZH2 is found to be upregulated in malignant tumors and is involved in metastasis, including OC, and overexpression of EZH2 facilitates OC cell invasion and migration [21]. An unexpected mode of action of H19 can antagonize the let-7 family of microRNAs [22]. An increase of microRNA let-7i expression induces OC cell apoptosis, which is the mechanism of propofol, can effectively inhibit proliferation and induce apoptosis in OC cells [23]. Inhibited let-7i expression remarkably reduced the resistance of OC cells to the chemotherapy drug, and decreased let-7i expression was associated with the shorter PFS of OC patients, which may explain the fact that overexpression of H19 was linked to poor prognosis in OC patients [24]. A high H19 expression contributes to poor overall survival and can be served as an independent predictor of the overall survival of gastric cancer patients [25], which may provide evidence for the prognostic role of H19 in OC, as reflected in our study.

CONCLUSIONS

Based on the key findings obtained from our study, we believe that H19 could potentially act as atherapeutic target. The reasons were as follows: (1) H19 expression levels are significantly increased in tissues and cell lines of OC; (2) H19 in relation to OC prognosis; (3) inhibiting H19 suppresses OC cell migration and invasion.

Taken together, our findings indicate that H19 plays a vital role in the development and progression of OC. The development of downregulation of this oncogenic lncRNAs based on H19-based therapeutic strategies may provide new and promising alternative therapeutics for future OC treatment. However, a larger sample size and longer follow-up period are required to confirm the correlation between H19 expression level and overall 5-year survival rate of OC patients. Meanwhile, more attention should be paid to exploring the mechanism of H19 and its interaction with oncogenes, and a new target should be discovered to cope with the highly migratory and invasive OC.

Ethical approval

The study protocol was approved by the Institutional Ethics Committees of Haimei Hospital, University Of Chinese Academy Of Sciences, and signed written informed consent was received from all patients prior to enrollment.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interests

None.

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