Abstract. Potential function of LINC00460 in the progression of ovarian cancer (OC) and its underlying mechanism were studied. LINC00460 level in OC tissues and normal ovarian tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Correlation between LINC00460 level with tumor stage, tumor size and pathological subtypes of OC was analyzed. Potential influence of LINC00460 on proliferative ability and cell cycle progression was evaluated. In vivo tumorigenesis model was conducted by administration of A2780 cells transfected with sh-NC or sh-LINC00460 in nude mice. Predicted through JASPAR database, ZNF703 was screened out as the transcriptional factor binding to LINC00460 promoter region. Chromatin immunoprecipitation (ChIP) assay was performed to verify the binding relationship between ZNF703 and LINC00460. The potential role of ZNF703 in LINC00460-mediated OC progression was examined. LINC00460 was upregulated in OC tissues and cell lines. Its level increased with the deterioration of tumor stage and enlargement of tumor size. LINC00460 was highly expressed in serous ovarian cancer relative to other subtypes of OC. Knockdown of LINC00460 attenuated proliferative ability and arrested cell cycle of A2780 and HO8910 cells. ZNF703 was upregulated in OC tissues. ChIP assay showed pronounced enrichment of LINC00460 in ZNF703. Rescue experiments revealed that ZNF703 overexpression reversed the regulatory effects of LINC00460 on cellular behavior of OC cells. LINC00460 is upregulated in OC tissues and cell lines, which is closely related to tumor progression. It accelerates proliferative ability and cell cycle progression of OC cells via interacting with ZNF703.

Introduction

Ovarian cancer (OC) is a prevalent malignancy of the female reproductive system. Its incidence rate is second only to cervical cancer and endometrial cancer (1). Epithelial cancer is the most common subtype in OC, followed by malignant germ cell tumor. The mortality of ovarian epithelial cancer ranks first of the various gynecological tumors, posing a serious threat to females (2). Due to the deep location, small volume and atypical symptoms, OC is hard to diagnose at early stage (3). Non-metastatic ovarian epithelial cancer only accounts for 30% of all OC cases (4). It is prone to metastasize, especially in the pelvic and abdominal organs. Sufficient diagnosis of OC at early stage is urgently needed.

As a subtype of non-coding RNAs, IncRNAs are long-chain RNAs over 200 nucleotides in length (5). They exert crucial roles in biological processes, and have been extensively explored in genetic researches (6). Accumulating evidence has shown the vital functions of IncRNAs in the regulation of cellular behavior (7). Several IncRNAs are reported to participate in the occurrence and progression of OC through interacting with other proteins, microRNAs or mRNAs. These IncRNAs may serve as diagnostic and therapeutic targets of OC (8).

It is reported that LINC00460 is involved in regulation of malignant phenotypes of tumors (9). LINC00460 is abnormally upregulated in multiple types of malignant tumors (10-16). Its role in OC, however, has not been fully explored. This study aimed to uncover the potential function of LINC00460 in the progression of OC and the underlying mechanism to provide new directions for developing treatment strategies for OC patients by targeting LINC00460.

Patients and methods

Sample collection and ethical statements. OC tissues were harvested from 40 OC patients undergoing radical surgery in Yuncheng County Hospital of Traditional Chinese Medicine (Heze, China). Normal ovarian tissues were collected from healthy controls during the same period. Samples were
immediately placed in liquid nitrogen and preserved at -80°C. Patients and their families of this study were fully informed. This study was approved by Ethics Committee of Yuncheng County Hospital of Traditional Chinese Medicine. Signed informed consents were obtained from the patients and/or guardians.

**Cell culture and transfection.** Epithelial ovarian cell line (IOSE-386) and OC cell lines (HO8910, SKOV-3, A2780 and ES-2) were obtained from American Type Culture Collection (ATCC). Cell culture was conducted in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone) with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO₂ at 37°C. For cell transfection, 1.5 ml of serum-free medium and 0.5 ml of Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) containing transfection vectors were applied to each well of a 6-well plate. Fresh medium was replaced 4-6 h later. Cells transfected for 24-48 h were collected for determination.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR).** Cells were lysed and tissues were homogenized using TRIzol method (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted RNAs were qualified using spectrometer and reverse transcribed into cDNAs using PrimeScript RT reagent Kit (Takara). QRT-PCR was carried out at 94°C for 5 min and 40 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec following the protocols of SYBR Premix Ex Taq™ (Takara). The relative gene expression was calculated using 2^-ΔΔCt method.

**Cell counting kit-8 (CCK-8) assay.** Cells (1x10⁵/ml) were pre-incubated in a 96-well plate. At the appointed times, each well was replaced with 10 µl of CCK-8 solution (Dojindo). The absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories).

**Western blot analysis.** Cells were lysed by radioimmunoprecipitation assay (RIPA) (Beyotime Institute of Biotechnology) to extract the total protein and under went gel electrophoresis. Protein samples were separated and incubated with primary antibody (Cell Signaling Technology) overnight at 4°C. Subsequently, membranes were incubated with corresponding secondary antibodies. Then, protein band was exposed and captured by the Tanon detection system using electrochemiluminescence (ECL) reagent (Thermo Fisher Scientific). The absorbance was measured using an enhanced chemiluminescence detection system (FACSCalibur; BD Biosciences). The expression level of LINC00460 was quantified using corresponding secondary antibodies. The absorbance was measured using an enhanced chemiluminescence detection system (FACSCalibur; BD Biosciences).

**Chromatin immunoprecipitation (ChIP).** Cells were subjected to cross-link with 1% formaldehyde for 10 min at room temperature. Subsequently, the cross-linked cells were lysed and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with antibodies and IgG.

**Colony formation assay.** Cells were seeded in a 6-well plate with 500 cells per well and cultured for 2 weeks. Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 30-min staining in 0.1% violet crystal. After removing the staining solution, colonies were air dried and observed under a microscope.

**Cell cycle detection.** Cells were digested for preparation of cell suspension and washed with pre-cooled PBS twice. After pre-cooled 75% alcohol was added, cells were placed at 4°C in a refrigerator for overnight fixation. Then the ethanol was discarded, and cells were washed with 1X phosphate-buffered saline (PBS). After centrifugation at 4°C, 950 x g for 5 min, cells were incubated with 100 µl propidium iodide and 100 µl RNA enzyme. Cell cycle was detected using flow cytometry (FACSCalibur; BD Biosciences).

**In vivo tumorigenesis in nude mice.** Female, 5-week-old, nude mice were provided by Shandong University Experimental Animal Center, and habituated in Specific Pathogen Free (SPF) level according to protocols approved by the Animal Care and Use Committee. A2780 cells were transfected with sh-NC or sh-LINC00460. Transfected cells (1x10⁷) were subcutaneously implanted in the right armpit of nude mice (n=6). Tumor size was recorded every week. Four weeks later, mice were sacrificed for collecting tumor tissues and weighing. This study was approved by the Animal Ethics Committee of Yuncheng County Hospital of Traditional Chinese Medicine Animal Center.

**Statistical analysis.** Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM Corp.) was used for data analysis. Data were expressed as mean ± standard deviation (mean ± SD). Intergroup data were compared using the t-test. P<0.05 indicates the difference is statistically significant.

**Results**

**LINC00460 is upregulated in OC tissues and cell lines.** Expression level of LINC00460 was higher in OC tissues relative to normal ovarian tissues (Fig. 1A). In particular, LINC00460 level remained higher in OC patients with stage III-IV than those with stage I-II (Fig. 1B). OC ≥5 cm in tumor size presented higher abundance of LINC00460 compared with those smaller than 5 cm (Fig. 1C). Taking into consideration pathological subtypes of OC, serous OC expressed higher level of LINC00460 relative to other subtypes (Fig. 1D). LINC00460 was upregulated in OC cell lines compared with that of epithelial ovarian cell line (Fig. 1E). It is suggested that LINC00460 was involved in the progression of OC.

**Knockdown of LINC00460 suppresses proliferative ability and arrests cell cycle of OC cells.** Among the four detected OC cell lines, HO8910 and A2780 cells expressed high abundance of LINC00460. Transfection of si-LINC00460 in these two cell lines markedly downregulated LINC00460 level, showing a pronounced transfection efficacy (Fig. 2A). After transfection of si-LINC00460, the viability in A2780 and HO8910 markedly decreased (Fig. 2B and C). Knockdown of LINC00460 decreased the relative colony number in OC cells (Fig. 2D and E). Flow cytometry revealed elevated cell ratio in G0/G1 phase after transfection of si-LINC00460 in OC cells, suggesting arrested cell cycle progression (Fig. 2F and G).

**Knockdown of LINC00460 alleviates in vivo growth of OC.** To further analyze the in vivo role of LINC00460 in the
progression of OC, A2780 cells were transfected with sh-NC or sh-LINC00460. After the sacrifice of mice for harvesting tumor samples, tumor weight was found to be markedly lower in mice administered with A2780 cells transfected with sh-LINC00460 relative to controls (Fig. 3A). Transfected cells were subcutaneously implanted in the right armpit of nude mice. Tumor growth was regularly observed. During the experimental period, tumor size was smaller in nude mice implanted with A2780 cells with downregulated level of LINC00460 (Fig. 3B). Transfection of sh-LINC00460 stably downregulated LINC00460 level in A2780 cells (Fig. 3C). It is believed that knockdown of LINC00460 alleviated tumor growth in OC bearing nude mice.

ZNF703 participates in LINC0046-mediated progression of OC. ZNF703 was upregulated in OC tissues relative to controls (Fig. 4A). To further explore its potential function, pcDNA-ZNF703 was constructed. Transfection of pcDNA-ZNF703 remarkably upregulated ZNF703 at both mRNA and protein levels in OC cells (Fig. 4B). Overexpression of ZNF703 greatly upregulated LINC00460 level in OC cells, indicating a potential correlation between these two genes (Fig. 4C). ChIP assay demonstrated the pronounced enrichment of LINC00460 in ZNF703 relative to control IgG, suggesting the interaction between LINC00460 and ZNF703 (Fig. 4D and E). Interestingly, the inhibited viability in OC cells transfected with si-LINC00460 was reversed by co-transfection of pcDNA-ZNF703 (Fig. 4F and G). Hence, LINC00460 regulated the proliferative ability of OC cells via interacting with ZNF703.

Discussion

Pathological subtypes of OC are diverse owing to the histological features of the ovaries (17). The specific pathogenesis of OC remains unclear, and genetic and endocrine factors are considered to be involved (18). Ovarian epithelial cancer is more common in postmenopausal women, and malignant germ cell tumor is prevalent in adolescents or young women (19). Obvious symptoms of early-stage OC are lacking. Approximately 70% of OC patients are diagnosed in advanced stage, manifesting as abdominal distension, abdominal pain, and weight loss. According to the International Harmonized Classification developed by the World Health Organization (WHO), the main histological type of OC is epithelial-derived tumors. Pathological subtypes of OC include serous tumors, mucinous tumors, endometrioid tumors, clear cell tumors, fibro-epitheliomas and mixed type of epitheliomas (20). Although therapeutic approaches for OC have been advanced, tumor metastasis and chemotherapy-resistance are treatment difficulties that severely restrict the clinical outcome of OC (21). Generally speaking, the occurrence and progression of OC are multi-step processes, where genomic alterations and genetic mutations are involved (22).

Dysregulated IncRNAs are crucial in the pathogenesis of OC (23,24). It is reported that IncRNA NEAT1 stimulates paclitaxel-resistance in OC cells through interacting with miR-194 to regulate ZEB1 level (25). IncRNA MNX1-AS1 is upregulated in EOC, posing a positive relationship with tumor staging, grade, distant metastasis, and poor prognosis (26). IncRNA FAM83H-AS1 promotes radioresistance,
proliferation and metastasis of OC by stabilizing HuR (27). HOXD-AS1 promotes proliferative, invasive abilities and epithelial-mesenchymal transition (EMT) of EOC by activating Wnt/β-catenin pathway and sponging miR-133a-3p (28).
lncRNA As-SLC7A11 inhibits proliferative, migratory and invasive abilities, and induces apoptosis of EOC cells through targeting SLC7A11 (29). Through targeting miR-186-5p and PIK3R3, HOXD-AS1 promotes EMT of EOC cells (30).

Located on 13q33.2, LINC00460 is upregulated and exerts a carcinogenic role in many tumors (31,32). Relative level, regulatory characteristic and clinical significance of LINC00460 in OC, have not been fully elucidated. This study showed that LINC00460 was upregulated in OC tissues and cell lines. LINC00460 level was positively correlated to tumor staging and tumor size of OC. In vitro experiments proved that knockdown of LINC00460 attenuated proliferative ability and arrested cell cycle of OC cells. Furthermore, in vivo function of LINC00460 was identified. LINC00460 stimulated tumor enlargement and growth rate in OC bearing nude mice.

Studies have confirmed that transcriptional factors are able to activate lncRNAs and thus upregulate their expression levels. The interaction between transcriptional factors and lncRNAs participates in various biological progresses, including tumorigenesis and tumor progression. Herein, potential binding sequences between ZNF703 and LINC00460 were predicted in JASPAR database. Furthermore, ChIP confirmed the interaction between ZNF703 and LINC00460. Notably, ZNF703 overexpression reversed the inhibited viability in OC cells with LINC00460 knockdown.

Collectively, this study is the first to demonstrate the carcinogenic role of LINC00460 in the progression of OC. The biological function of LINC00460 in OC was closely regulated to the transcriptional factor ZNF703. LINC00460 shows promise as a diagnostic and therapeutic target for OC.

In conclusion, LINC00460 is upregulated in OC tissues and cell lines, which is closely related to tumor progression. It accelerates proliferative ability and cell cycle progression of OC cells via interacting with ZNF703.

Acknowledgements

Not applicable.
Funding
Not applicable.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
XW, XG and WZ designed the study and performed the experiments. XW, and CL collected the data. XG and CL analyzed the data. XW, XG and WZ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Yuncheng County Hospital of Traditional Chinese Medicine (Heze, China). Signed informed consents were obtained from the patients and/or guardians. This study was approved by the Animal Ethics Committee of Yuncheng County Hospital of Traditional Chinese Medicine Animal Center.

Patients consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

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