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Lnc00908 promotes the development of ovarian cancer by regulating microRNA-495-5p

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether lnc00908 could affect the proliferative and migratory behaviors of ovarian cancer (OC) cells by regulating microRNA-495-5p, thus participating in the development of OC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR) was used to detect the expression levels of lnc00908 and microRNA-495-5p in OC tissues and normal ovarian tissues, as well as OC cell lines. The regulatory effects of lnc00908 and microRNA-495-5p on the proliferative and migration abilities of OC cells were detected by cell counting kit-8 (CCK-8) and transwell assay, respectively. The binding relationship between microRNA-495-5p and ANXA3, as well as miR-495-5p and lnc00908, was examined by luciferase reporter gene assay. Gain-of-function experiments were conducted to verify whether lnc00908 could affect the proliferative and migratory behaviors of OC cells by regulating microRNA-495-5p.

RESULTS: Lnc00908 was highly expressed in OC tissues, and its expression was positively correlated with tumor stage. Overexpression of lnc00908 markedly promoted the proliferative and migratory abilities of SKOV3 and OVCAR cells. Luciferase reporter gene assay showed that lnc00908 could bind to microRNA-495-5p. However, microRNA-495-5p was significantly downregulated in OC tissues. Overexpression of microRNA-495-5p reversed the enhanced abilities of proliferation and migration in SKOV3 and OVCAR3 cells by lnc00908 overexpression. ANXA3 was a target gene of microRNA-495-5p. Moreover, overexpression of ANXA3 attenuated the inhibitory effect of miR-495-5p on the proliferative and migratory behaviors of SKOV3 and OVCAR3 cells.

CONCLUSIONS: We found that the high expression of lnc00908 can promote the proliferation and migration abilities of OC cells through sponging microRNA-495-5p to regulate ANXA3 expression.

Key Words
Ovarian cancer (OC), Lnc00908, MicroRNA-495-5p, ANXA3.

Introduction

Ovarian epithelial cancer (ovarian cancer or OC for short) is one of the most common gynecologic malignancies in women. OC has extremely high mortality, rapid metastasis, strong invasion, and high recurrence. The five-year survival of advanced OC is less than 30%[1]. Specific and sensitive diagnostic methods of OC are lacked; meanwhile, symptoms of early-stage OC are not obvious. Due to the advanced stage at the first time of diagnosis, more than 70% of OC patients have already lost the optimal surgical opportunity[2,3]. Therefore, it is urgent to develop early diagnostic methods for OC.

Long non-coding RNA (LncRNA) is a newly discovered RNA that constitutes a major component of the human transcriptome[4]. They cannot encode proteins due to the lack of an open reading frame (ORF) and have a transcript with over 200 nt. Accumulating evidence has suggested the biological functions of lncRNAs at epigenetic, transcriptional and post-transcriptional levels through regulating RNA-protein, RNA-DNA, and RNA-RNA interactions[5]. LncRNAs are involved in the occurrence and development of malignant tumors, thereby affecting tumor cell proliferation and infiltration. Previous studies have shown that lncRNA

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H19 expression is higher in OC tissues than adjacent normal tissues. H19 knockdown markedly inhibits the proliferation whereas induced apoptosis of OC cells[6]. LncRNA HOST2 is highly expressed in OC. Meanwhile, its deficiency suppresses the proliferative, invasive, and metastatic behaviors of OC cell line OVCAR-3[7]. The above researches all indicate the potential role of lncRNA in OC.

Regulatory effects of lncRNA and miRNA on downstream genes are closely related to tumorigenesis[8]. LncRNA serves as a competitive endogenous RNA (ceRNA) that competitively binds to miRNAs, thus regulating the protein level of encoded genes and altering cellular behaviors[9,10]. LncRNA H19 promotes tumor invasion and metastasis through sponging and inhibiting miR-let-7 expression[11]. In addition, lncRNA HOST2 is considered as a molecular sponge that directly binds to miR-let-7b. Furthermore, lncRNA HOST2 inhibits miR-let-7b function in OC, leading to the upregulation of let-7b-regulated c-myc, HMGA2, Dicer, and Imp3. Finally, the proliferative and metastatic behaviors of OC cells are enhanced[12].

Through bioinformatics, lnc00908 exerts a potential binding site with microRNA-495-5p. We hypothesized that lnc00908 might serve as a sponge of miRNA that absorbed microRNA-495-5p, thus regulating its biological function.

Patients and Methods

Basic Information

Twenty-four cases of OC and normal ovarian tissues were harvested from surgical resection in the First Affiliated Hospital of Xiamen University. Clinical pathology data of all enrolled OC patients were collected during the follow-up period, including sex, age, tumor size, and number of tumors. None of OC patients received treatment before the study, and no one had a family history. Tissues were pathologically confirmed as OC. The investigation has been approved by the Ethics Committee of the hospital. Each subject signed the informed consent before the study.

Cell Culture

Normal ovarian cell line (HOSEpiC) and OC cell lines (C13K, 3AO, SKOV3, and OVCAR3) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and placed in a 37°C, 5% CO_2 incubator.

Cell Transfection

When the confluence of OC cells was up to 70-80%, they were transfected with microRNA-495-5p mimics, pcDNA-lnc00908, pcDNA-ANXA3 or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 24 hours later. 48 hours after the transfection, the cells were harvested for the following experiments.

Total RNA Extraction

Total RNA in cells (5×10^6) or tissues (50 mg) was first extracted using 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) reagent. The aqueous phase was transferred to a new tube. RNA in the aqueous phase was precipitated using 0.2 ml of chloroform, followed by centrifugation at 4°C, 12,000 rpm/min for 10 min. The supernatant was transferred to a new tube and mixed with isopropanol. After centrifugation at 4°C, 12,000 rpm/min for 10 min, the precipitant was washed with 75% ethanol. Extracted RNA was air dried, diluted in diethyl pyrocarbonate (DEPC) water, quantified using a spectrophotometer and preserved at -80°C.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNA was reversely transcribed into cDNA with U6 as the loading control. QRT-PCR was performed in strict accordance with SYBR® Green Master Mix (Tiangen, Beijing, China). Specific reaction conditions were as follows: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 1 min, 60°C for 1 min, and extension at 72°C for 1 min, for a total of 40 cycles. Primers were as follows: MicroRNA-495-5p, F: 5'-ACACTCCAGCTGGG GAAGTTGCCCATGTTA-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAUAAAAGC-3'; U6, F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'; Lnc00908, F: 5'-AAGTGGACGTAGAACTCCCC-3', R: 5'-TCCCTCTCCAATCCCCAAAC-3'.

Luciferase Reporter Gene Assay

The transcript 3'UTR sequence of ANXA3 was cloned into the vector pGL3 containing the luciferase reporter gene, which was the ANXA3 WT group. By mutating the core region of miRNA

3'UTR into a null binding sequence using a site-directed mutagenesis kit, ANXA3 MUT group was constructed. Lnc00908 WT group and lnc00908 MUT group were constructed in the same way. Renilla luciferase internal reference plasmid and microRNA-495-5p mimics were transfected into cells in each group. After 24 hours of transfection, the cells were completely lysed and centrifuged for harvesting the supernatant. RLUs values obtained by firefly luciferase and renilla luciferase measurement were recorded.

Cell Migration Assay

A total of 1×10^5 transfected OC cells were seeded into the upper chamber. Meanwhile, medium containing 10% FBS was added as a chemotactic agent to the lower chamber. Then, the cells were maintained in a 37°C, 5% CO_2 incubator for 48 h. Cells migrating to the lower chamber were fixed in 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. The number of cells migrating to the lower chamber was counted under an inverted microscope. 5 fields were randomly selected for each sample, and this experiment was repeated for three times.

Cell Proliferation Assay

OC cells were inoculated into 96-well plates at a density of 1×10^4 cells/well. After culturing for 6 h, 24 h, 48 h, 72 h, and 96 h, respectively, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) solution was added to each well, followed by incubation at 37°C for 1 h in the dark. The absorbance of each well at 450 nm was recorded by a microplate reader.

Western Blot

Total protein in OC cells was lysed with radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein expressions of genes in OC cells were detected in strict accordance with standard protocols of Western blot.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SD (x±s). The experimental results were analyzed with the standard *t*-test analysis. $p<0.05$ was considered statistically significant.

Results

Lnc00908 Was Highly Expressed in OC

We first detected lnc00908 expression in normal ovarian tissues (n=24) and OC tissues (n=24). Lnc00908 was highly expressed in OC tissues (Figure 1A), and its expression was significantly higher in OC tissues with stage III-IV than those with stage I-II (Figure 1B). Consistently, markedly higher expression of lnc00908 was observed in OC cell lines when compared with normal ovarian epithelial cells (Figure 1C). These results suggested the potential role of lnc00908 in the progression of OC. Survival analysis indicated that OC patients with higher expression of lnc00908 might experience worse survival (AUROC=0.8984, cut-off value=2.687, Figure 1D). Besides, the 5-year survival of OC patients with higher expression of lnc00908 was significantly lower (Figure 1E). By selecting OVCAR3 and SKOV3 cells, *in vitro* function of lnc00908 was subsequently explored. The CCK-8 assay showed that the proliferative potential of both OVCAR3 and SKOV3 cells was remarkably promoted after lnc00908 overexpression (Figure 1F and 1G), as well as it enhanced migratory ability (Figure 1H). The above findings suggested that lnc00908 might participate in the progression of OC by promoting the proliferative and migratory behaviors of OC cells.

Lnc00908 Sponged MicroRNA-495-5p

Previous studies have shown that lncRNA exerts its biological function by targeting corresponding miRNAs. Bioinformatics predicted that microRNA-495-5p was a potential target gene for lnc00908, with a potential binding site between the two molecules (Figure 2A). Luciferase reporter gene assay further verified the binding condition between microRNA-495-5p and lnc00908 in both SKOV3 and OVCAR3 cells (Figure 2B and 2C). Subsequently, results showed that microRNA-495-5p was significantly downregulated in SKOV3 and OVCAR3 cells after lnc00908 overexpression, showing a negative regulation in OC cells (Figure 2D and 2E). We further detected the expression of microRNA-495-5p in OC tissues and cell lines. Results showed that microRNA-495-5p was highly expressed in OC (Figure 2F and 2G). Further analysis found a negative correlation between lnc00908 and microRNA-495-5p in OC tissues (Figure 2H). To verify whether lnc00908 could sponge microRNA-495-5p as a ceRNA, RIP assay was conducted. Higher abundances of microRNA-495-5p and lnc00908 binding with Ago2

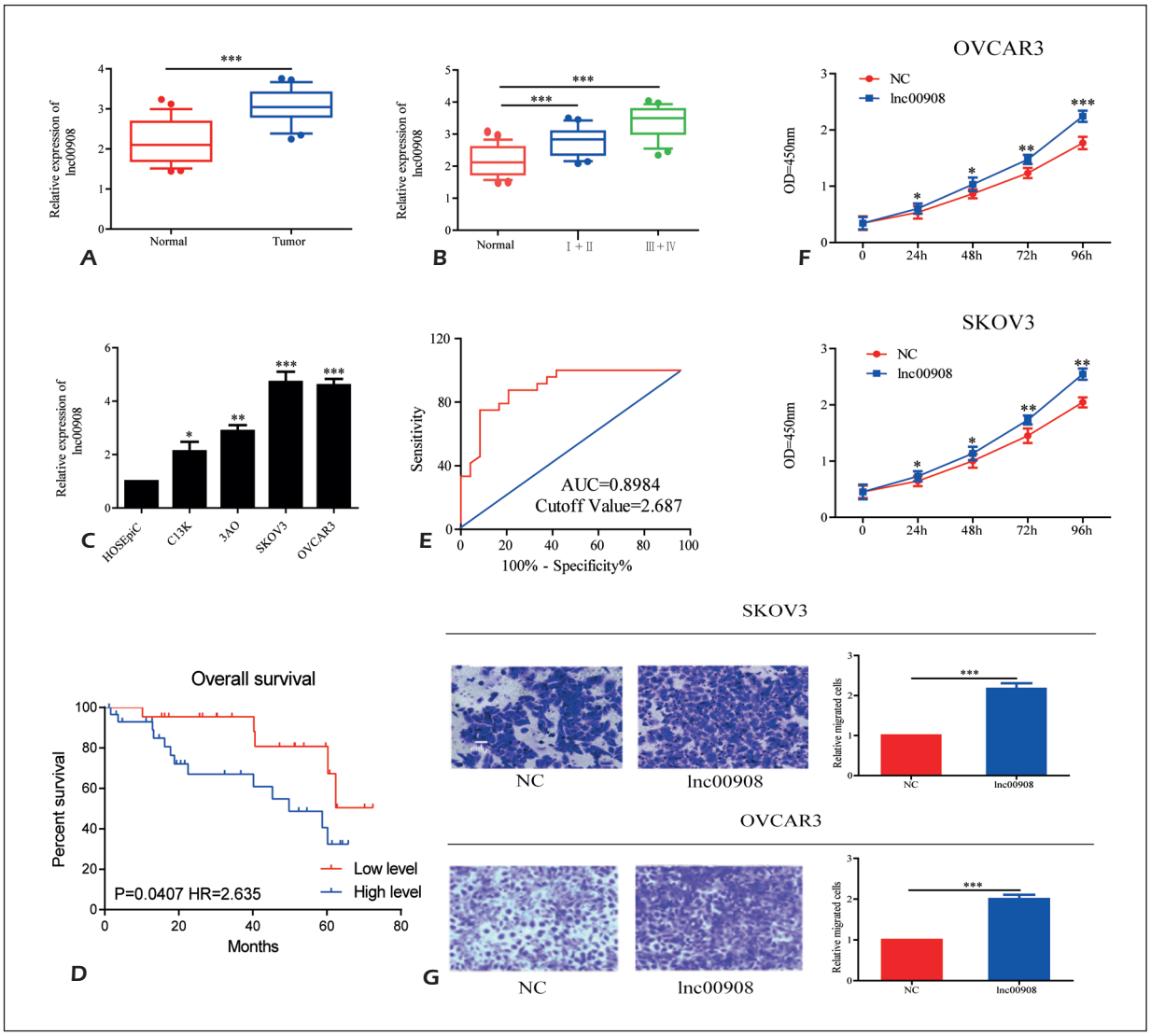

Figure 1. Lnc00908 was highly expressed in OC. **A**, Lnc00908 was highly expressed in OC tissues compared with normal ovarian tissue. **B**, Lnc00908 expression was significantly higher in OC with stage III-IV than in stage I-II. **C**, Lnc00908 was highly expressed in OC cell lines. **D**, ROC curve of lnc00908 expression and OC patients. E, Correlation between five-year survival rate of OC patients and lnc00908 expression. **F-G**, Overexpression of lnc00908 significantly promoted the proliferation of SKOV3 and OVCAR3 cell lines. H, Overexpression of lnc00908 significantly promoted the migration of SKOV3 and OVCAR3 cell lines. *p<0.05, **p<0.01, ***p<0.001.

were observed when compared with those of IgG, indicating the formation of a silencing complex (RISC) as the mechanism of ceRNA (Figure 2I).

Lnc00908 Exerted its Biological Function Through Targeting MicroRNA-495-5p

Since lnc00908 could target microRNA-495-5p, we hypothesized that lnc00908 functions might be dependent on microRNA-495-5p. To confirm our hypothesis, we co-overexpressed lnc00908 and microRNA-495-5p in SKOV3 and OVCAR3 cells. The results showed that overexpression of microRNA-495-5p reversed the promotive effect of lnc00908 on the proliferation of OC cells (Figure 3A and 3B). Similarly, the promoted migratory ability of OC cells was also reversed by overexpression of microRNA-495-5p (Figure 3C and 3D).

ANXA3 was the Potential Target Gene of MicroRNA-495-5p

It is well known that miRNAs exert their biological function through targeting downstream

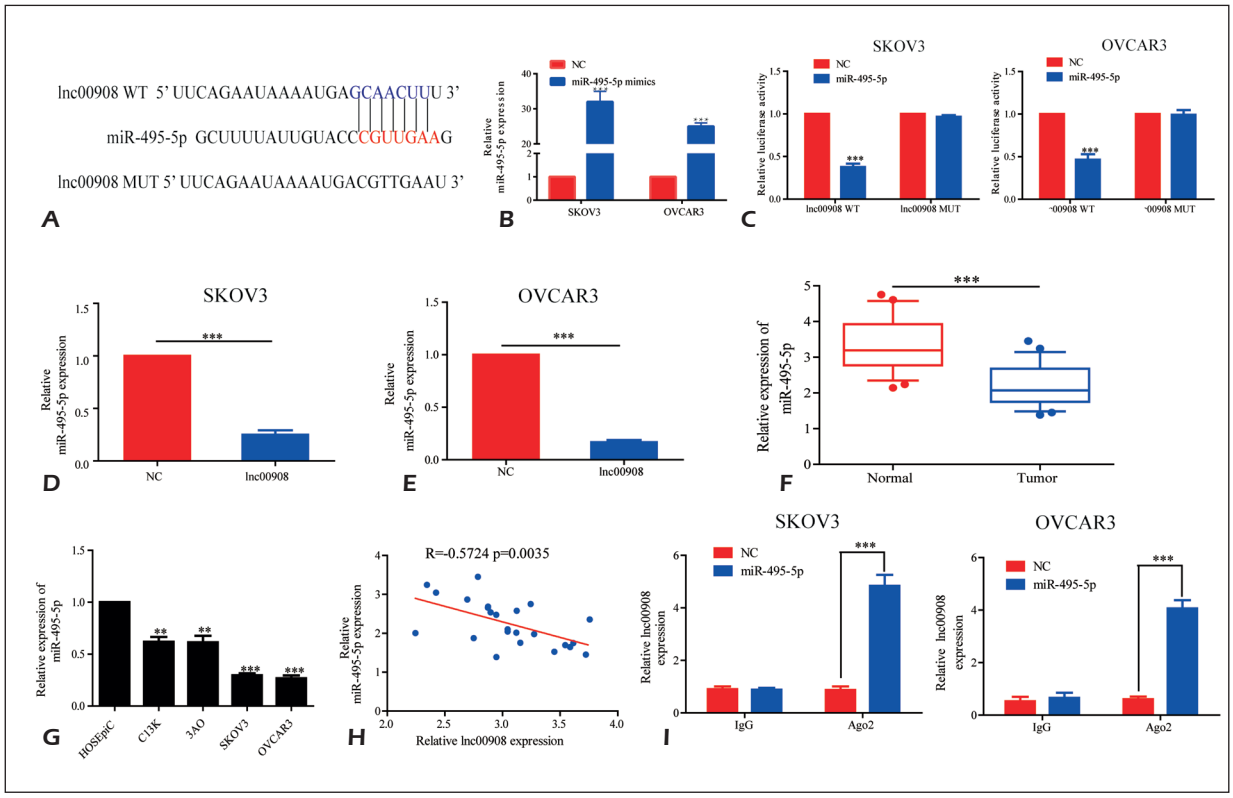

Figure 2. Lnc00908 sponged microRNA-495-5p. **A**, A potential binding site between lnc00908 and microRNA-495-5p. **B-C**, Luciferase reporter gene assay indicated that lnc00908 could bind to microRNA-495-5p in SKOV3 and OVCAR3 cell lines. **D-E**, Overexpression of lnc00908 significantly inhibited the expression of microRNA-495-5p in SKOV3 and OVCAR3 cell lines. **F**, MicroRNA-495-5p was lowly expressed in OC tissues compared with normal tissues. **G**, MicroRNA-495-5p was lowly expressed in OC cell lines. **H**, Lnc00908 was negatively correlated with microRNA-495-5p in OC. **I**, RIP test results showed that the abundances of microRNA-495-5p and lnc00908 binding with Ago2 were significantly higher than those of IgG. *p<0.05, **p<0.01, ***p<0.001.

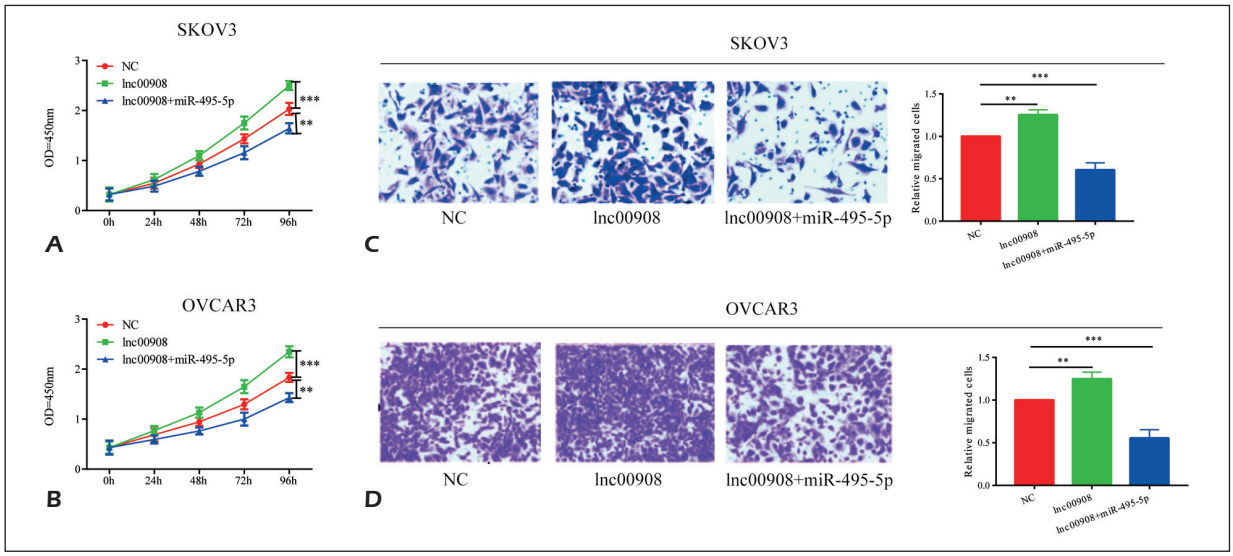

Figure 3. Lnc00908 exerted its biological function through targeting microRNA-495-5p. **A**, Overexpression of microRNA-495-5p reversed the promotive effect of lnc00908 on the proliferation of SKOV3 cells. **B**, Overexpression of microRNA-495-5p reversed the promotive effect of lnc00908 on the proliferation of OVCAR3 cells. **C**, Overexpression of microRNA-495-5p reversed the promotive effect of lnc00908 on the migration of SKOV3 cells. **D**, Overexpression of microRNA-495-5p reversed the promotive effect of lnc00908 on the migration of OVCAR3 cells. *p<0.05, **p<0.01, ***p<0.001.

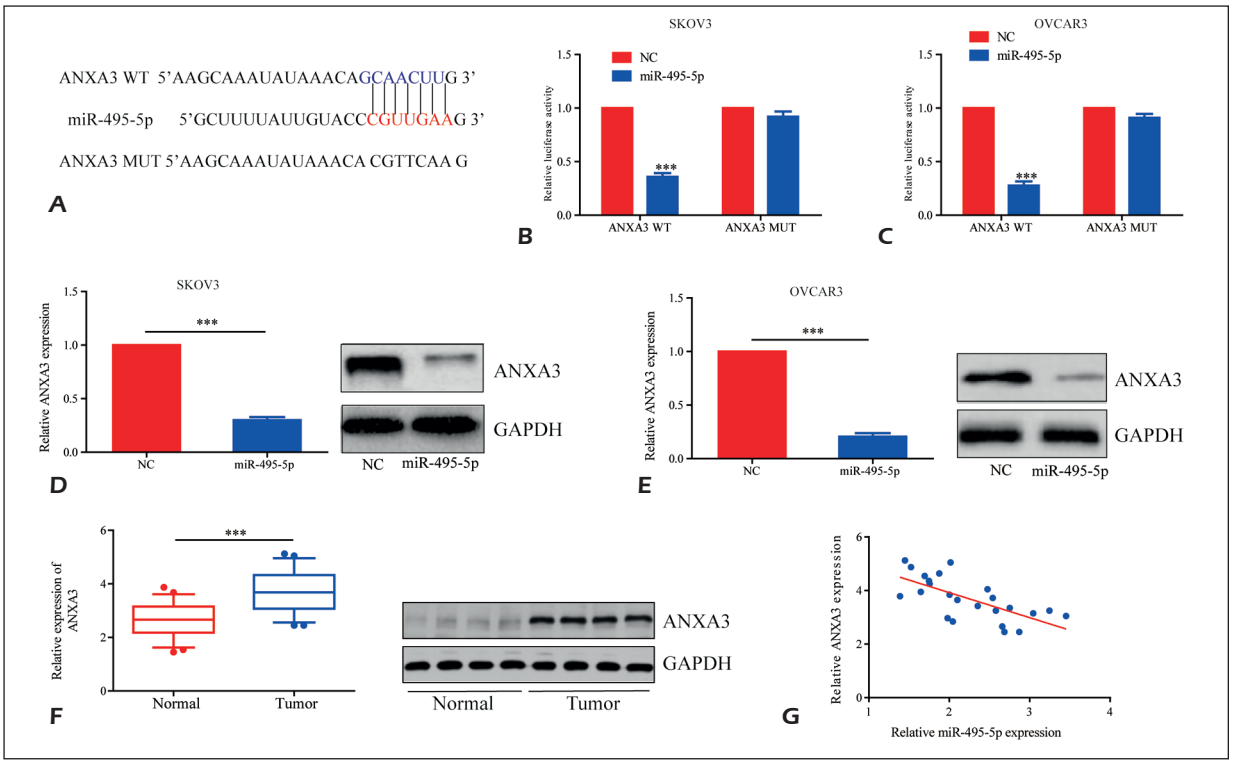

Figure 4. ANXA3 was the potential target gene of microRNA-495-5p. **A**, A potential binding site between microRNA-495-5p and ANXA3. **B**-**C**, Luciferase reporter gene results indicated that microRNA-495-5p could bind to ANXA3. **D**-**E**, Overexpression of microRNA-495-5p significantly inhibited the mRNA and protein levels of ANXA3 in SKOV3 and OVCAR3 cells. **F**, ANXA3 was highly expressed in OC tissues. **G**, MicroRNA-495-5p was negatively correlated with ANXA3 in OC. **p*<0.05, ***p*<0.01, ****p*<0.001.

target genes. In the present study, we predicted that ANXA3 was a potential target gene for microRNA-495-5p (Figure 4A).

Subsequently, the luciferase reporter gene assay validated the binding relationship between microRNA-495-5p and ANXA3 (Figure 4B and 4C). Both the mRNA and protein levels of ANXA3 were inhibited by microRNA-495-5p overexpression in SKOV3 and OVCAR3 cells. This indicated that microRNA-495 -5p negatively regulated the expression level of ANXA3 (Figure 4D and 4E). Based on these findings, we speculated that ANXA3 was highly expressed in OC. QRT-PCR results further confirmed our speculation (Figure 4F). Moreover, a negative correlation was found between microRNA-495-5p and ANXA3 in OC tissues (Figure 4G).

MicroRNA-495-5p Exerted its Biological Function Through Targeting ANXA3

Since ANXA3 was the target gene for microRNA-495-5p, we speculated that microRNA-495-5p might exert its function in OC cells through targeting ANXA3. To confirm our hypothesis, we first overexpressed microRNA-495-5p in SKOV3 cells. Results showed that microRNA-495-5p significantly inhibited the proliferative and migratory behaviors of the SKOV3 cell (Figure 5A and 5B). Overexpression of microRNA-495-5p in OVCAR3 cells obtained the identical results (Figure 5C and 5D). Subsequently, gain-of-function experiments were conducted by co-overexpression of ANXA3 and microRNA-495-5p. ANXA3 overexpression reversed the regulatory effects of microRNA-495-5p on the proliferative and migratory behaviors of SKOV3 cells (Figure 5E and 5F). Similarly, ANXA3 overexpression reversed microRNA-495-5p mimics-induced abilities of OVCAR3 cell proliferation and migration (Figure 5G and 5H). Combined with the previous results, we found that lnc00908 might attenuate the regulatory effect of microRNA-495-5p on its target gene ANXA3 by adsorbing microRNA-495-5p, thus promoting the development of OC.

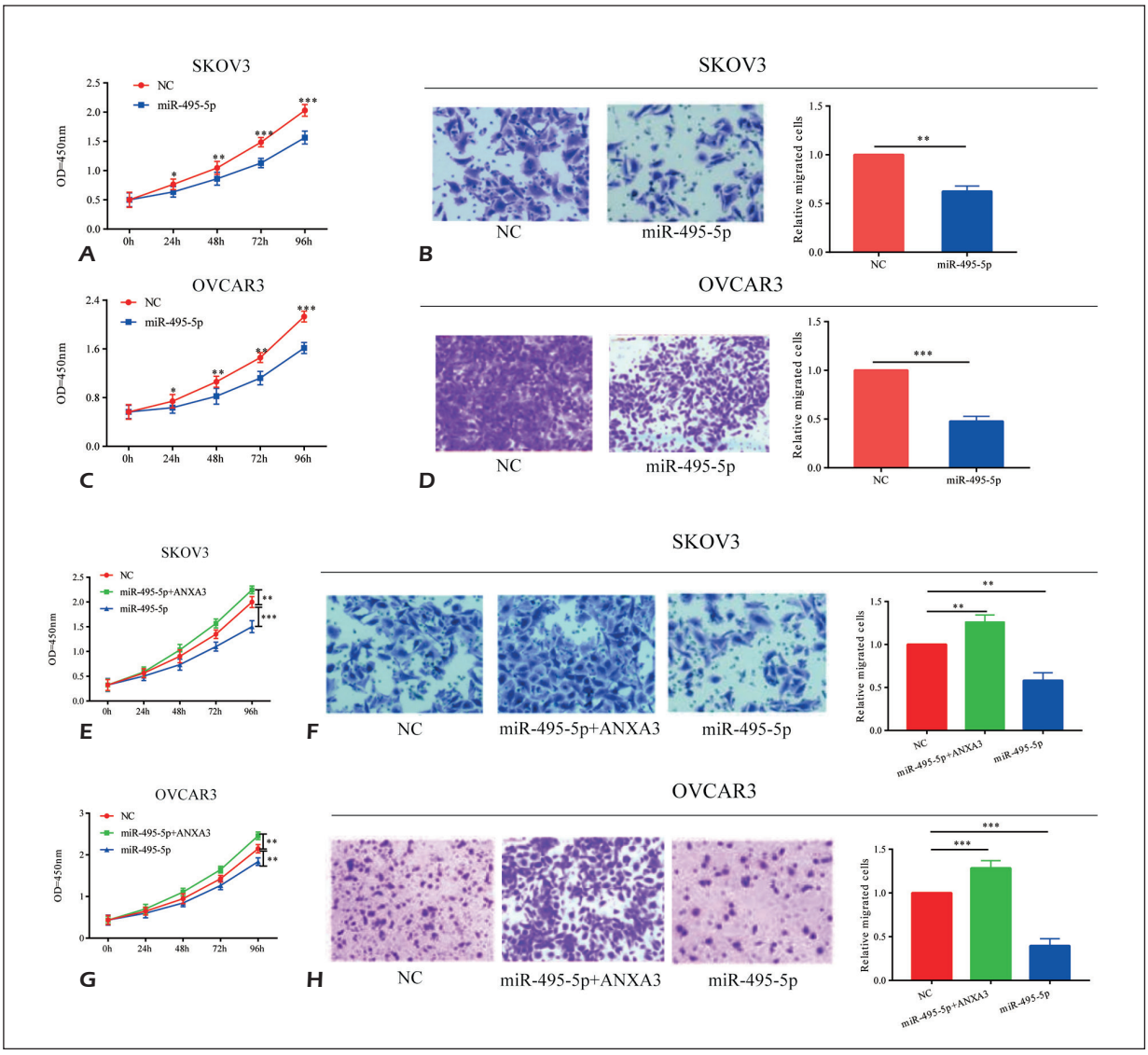

Figure 5. MicroRNA-495-5p exerted its biological function through targeting ANXA3. **A-B**, Overexpression of microRNA-495-5p inhibited the proliferation and migration of SKOV3 cells. **C-D**, Overexpression of microRNA-495-5p significantly inhibited the proliferation and migration of OVCAR3 cells. **E-F**, Overexpression of ANXA3 reversed the effect of microRNA-495-5p on the proliferation and migration of SKOV3 cells. **F-H**, Overexpression of ANXA3 reversed the effect of microRNA-495-5p on the proliferation and migration of OVCAR3 cells. *p<0.05, **p<0.01, ***p<0.001.

Discussion

OC is one of the most serious diseases in gynecological malignancies. Many OC patients have already experienced the advanced stage accompanied by extensive metastasis when diagnosed. Nearly half of OC patients may relapse within 16 months, and the 5-year survival is lower than 50%[13]. Meanwhile, drug resistance in OC treatment limits the traditional therapeutic efficacy. Hence, it is necessary to study the pathogenesis of OC, thereby providing new therapeutic targets.

Current researches have indicated that lncRNA is involved in the progression of a variety of tumors and serves as a new molecular target. For example, lncRNA H19 is highly expressed in gastric cancer tissues. Overexpression of H19 remarkably promotes the growth of AGS cells, suggesting the potential role of H19 in the development of gastric cancer[14]. The expression level of HOTAIR in colon cancer tissues is positively correlated with its prognosis. Further researches have shown that high expression of HOTAIR in colon cancer can promote polycomb repressive complex 2 (PCR2), thus

enhancing the metastatic potential of colon cancer cells and promoting its progression[15]. In this work, we found that lnc00908 was highly expressed in OC tissues. Meanwhile, its expression was positively correlated with tumor stage. Further biological experiments showed that high expression of lnc00908 markedly promoted the proliferative and migratory abilities of SKOV3 and OVCAR cells, indicating that lnc00908 might play a role in the occurrence and development of OC.

A growing number of studies have shown that lncRNAs, as competing molecules for miRNAs and mRNAs, may have potential characteristics of ceRNAs[16]. In prostate cancer, lncRNA PTENP1 can abolish PTEN inhibition by sponging miR-19 and miR-20a, thereby inhibiting cell growth[17]. In addition, lncRNAs are involved in the regulation of cell cycle and cell death in malignancies, including gastric cancer and liver cancer[17-19]. Through affecting the invasion and metastasis of tumors, lncRNAs have shown its potential in the incidence and development of tumors[20]. We found a potential binding site between microRNA-495-5p and lnc00908 through bioinformatics prediction and further analysis. Moreover, we confirmed that lnc00908 could adsorb microRNA-495-5p and regulate its expression. To further validate the possible mechanism of lnc00908 in OC, we performed an Ago2 RIP assay. Results showed that the abundances of microRNA-495-5p and lnc00908 binding with Ago were significantly higher than those of IgG. Overexpression of microRNA-495-5p in SKOV3 and OVCAR3 cells revered the promotive effect of lnc00908 on the proliferation and migration of OC cells. The above results indicated that lnc00908 exerted its regulatory effect on OC through targeting microRNA-495-5p.

MiRNAs are a class of non-coding, endogenous, small RNAs with 19-25 nucleotides in length. They are characterized by high conservation, temporal expression specificity, and tissue expression specificity. MiRNAs regulate target genes and reduce the expressions of target genes through binding to their 3'-UTR. Through online prediction, we found that ANXA3 was a potential target gene for microRNA-495-5p. Luciferase reporter gene assay showed that ANXA3 could bind to microRNA-495-5p. Furthermore, overexpression of microRNA-495-5p in SKOV3 and OVCAR3 cells significantly inhibited ANXA3 expression at both mRNA and protein levels. To verify whether the regulatory function of microRNA-495-5p in OC cells was dependent on targeting ANXA3, the two molecules were co-overexpressed in OC cells. Results demonstrated that ANXA3 overexpression reversed the inhibitory effects of microRNA-495-5p on the proliferative and migratory behaviors of OC cells. These results verified our speculation.

Based on the above results, this study mainly elucidated the role of lnc00908 in OC and its possible mechanism. Our findings suggested that the lnc00908/microRNA-495-5p/ANXA3 axis might be significant in the development of OC. Furthermore, our results provide a new target for the prevention and treatment of OC.

Conclusions

We found that the high expression of lnc00908 can promote the proliferation and migration abilities of OC cells through sponging microRNA-495-5p to regulate ANXA3 expression.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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