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LncRNA SNHG12 accelerates the progression of ovarian cancer via absorbing miRNA-129 to upregulate SOX4

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Abstract. – OBJECTIVE: To clarify whether long non-coding RNA (lncRNA) SNHG12 could regulate the proliferative and migratory abilities of ovarian cancer (OC) cells through mediating microRNA-129 (miRNA-129), thus influencing the progression of OC.

PATIENTS AND METHODS: The expression patterns of SNHG12 and miRNA-129 in OC tissues and adjacent normal tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, their expression levels in OC cell lines were also examined. Regulatory effects of SNHG12 and miRNA-129 on the proliferative and migratory abilities of OC cells were evaluated by cell counting kit-8 (CCK-8) and transwell assay, respectively. Through the dual-luciferase reporter gene assay, we explored the binding between miRNA-129 with SNHG12 and SOX4. A series of rescue experiments were conducted to clarify the role of SNHG12/miRNA-129/SOX4 regulatory loop in the progression of OC.

RESULTS: SNHG12 was upregulated in OC tissues relative to adjacent normal ones. Patients with metastatic OC or those in stage III-IV had a higher level of SNHG12 compared with non-metastatic or stage I-II patients. The 5-year survival was markedly worse in OC patients with high-level SNHG12 than those in the low-level group. Similarly, SNHG12 was highly expressed in OC cell lines. Overexpression of SNHG12 accelerated A2780 and HO8910 cells to proliferate and migrate. We observed the binding between SNHG12 and miRNA-129, and the latter was lowly expressed in OC. The miRNA-129 overexpression partially reversed the promotive effects of SNHG12 on proliferative and migratory abilities of OC cells. Subsequently, SOX4 was proved to be the target gene of miRNA-129. The SOX4 overexpression was further confirmed to reverse the inhibitory effects of miRNA-129 on proliferative and migratory abilities of OC cells.

CONCLUSIONS: SNHG12 accelerates the proliferative and migratory abilities of OC cells via sponging miRNA-129 to upregulate SOX4.

Key Words
Ovarian cancer, SNHG12, MiRNA-129, SOX4.

Introduction

Ovarian cancer (OC) is a common gynecologic malignancy worldwide[1]. Its incidence is on the rise in recent years. About 75% of OC cases are diagnosed as advanced stage owing to obscure early-stage symptoms and the lack of specific examination, thus resulting in the high mortality of OC[2,3]. Hence, OC is known as a silent killer in females. The diagnostic and therapeutic strategies have made great strides, including surgery, chemotherapy, targeted drugs, and biological therapy. Nevertheless, the 5-year survival of advanced OC patients still remains at 30%[3]. It is urgent to elucidate the pathogenesis of OC, thus developing novel diagnostic and therapeutic targets to improve the clinical outcomes.

Non-coding RNA is widely distributed in various organisms. It is divided into long non-coding RNA (lncRNA) and short-chain ncRNA (i.e., miRNA, siRNA, and piRNA) based on the length. LncRNA contains 200-1000 Kb and lacks the open reading frame[4]. Functionally, lncRNA could not encode proteins. LncRNA is formed by cleavage, folding, capping, and polyadenylation, which is similar to that of mRNA[5]. The diverse functions of lncRNA give them promising aspects in clinical researches. As a vital mediator, lncRNA participates in cellular behaviors, tumor progression, epigenetic regulation, genomic imprinting, etc.[6]. In particular, the specific effects of lncRNA on the occurrence and progression of tumors are well concerned. For example, lncRNA MALAT1 predicts the metastatic risk of NSCLC[7]. HOTAIR silences WIF1, the WNT inhibitor through the epigenetic pathway, thus enhancing the migratory ability of glioblastoma cells[8,9].

LncRNA SNHG12 (small nucleolar RNA host gene 12) locates on chromosome 1p35.3. The current study identified the high abundance of SNHG12 in multiple types of tumors. In NSCLC, SNHG12 accelerates the tumor progression by targeting miR-181a. Additionally, SNHG12 is up-

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regulated in gastric cancer and colorectal cancer, and its abnormal expression is closely correlated to the tumor prognosis as a promising biological hallmark[10,11]. The potential function of SNHG12 in the progression of OC, however, has not been fully elucidated. In this work, we aim to clarify the role of SNHG12 in regulating the malignant progression of OC and its underlying mechanism.

Patients and Methods

Basic Data

A total of 24 paired OC tissues and adjacent normal tissues were surgically resected from OC patients. Samples were immediately transferred into liquid nitrogen and preserved at -80°C. OC patients were followed-up and their clinical data were recorded. None of the enrolled OC patients received preoperative therapy and they denied any family history. Patients and/or their families have been fully informed and signed the informed consent. This investigation was approved by the Ethics Committee of Hanchuan People's Hospital.

Cell Culture

Human normal ovarian epithelial cell line (IOSE80) and OC cell lines (SKOV3, OVCAR3, A2780, and HO8910) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Rockville, MD, USA) [containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 0.1 mg/mL streptomycin] in a 5% CO_2 and 37°C incubator.

Transfection

Until 70-80% of confluence, cells were transfected with relative plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 4 hours, fresh medium was replaced for another 24 h incubation.

RNA Extraction

Tissues (50 mg) or cells (5×10^6/mL) were fully lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance at room temperature for 5 min, we added 200 μL of chloroform, mixed and stand at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12,000 rpm for 15 min. Isopropanol with the same volume of the supernatant was added for harvesting RNA precipitate by centrifugation. The extracted RNA was air dried, quantified and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA samples were preserved at -80°C.

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

Qualified RNA was subjected to reverse transcription and prepared for PCR solution accompanied by SYBR Green pre-loaded buffer, complementary deoxyribose nucleic acid (cDNA), reverse/forward primers, and DEPC water. QRT-PCR was conducted at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, for a total of 40 cycles, and finally extended at 72°C for 7 min. Primer sequences were as follows: miRNA-129, F: 5′-ACCCAGTGCGATTTGTCA-3′ R: 5′-ACTGTACTGGAAGATGGACC-3′; U6, F: 5′-CTCGCTTCGGCAGCAGCACATATA-3′, R:AAATATGGAACGCTTCACGA-3′; SNHG12, F: 5′-TCTGGTGATCGAGGACTTCC-3′, R: 5′-ACCTCCTCAGTATCACACACT-3′; SOX4, F: 5′-GGCCTCGAGCTGGGAATCGC-3′, R: 5′-GCCCACTCGGGGTCTTGCAC-3′; GAPDH, F: 5′-CTAAGGCCAACCGTGAAAAG-3′, R: 5′-ACCAGAGGCATACAGGGACA-3′.

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type luciferase vectors of SNHG12/SOX4 were constructed, namely SNHG12/SOX4 WT and SNHG12/SOX4 MUT, respectively. Cells were co-transfected with SNHG12/SOX4 WT or SNHG12/SOX4 MUT and miRNA-129 mimics or NC for 24 h. Cells were then fully lysed, centrifuged at 10,000 g for 5 min, and 100 μL of supernatant was harvested for determining the luciferase activity.

Transwell Assay

Transfected cells were adjusted to 1.0×10^5/mL. 200 μL/well suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA). In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 10-15 min, dyed with 0.5% crystal violet for 20 min and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plate with 1×10^4 cells per well, with 5 replicates each group. Absorbance (A) at 450 nm was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). 50 μg protein sample was loaded for electrophoresis at 80 V for 40 min and then 120 V for 60-80 min. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup difference satisfying normality was analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

Upregulation of SNHG12 in OC

QRT-PCR data revealed that SNHG12 was upregulated in OC tissues relative to controls (Figure 1A). Moreover, SNHG12 level was higher in patients with metastatic OC compared with those of non-metastatic ones (Figure 1B). Based on different tumor stages, it is identified that SNHG12 was highly enriched in OC with stage III-IV than those with stage I-II (Figure 1C). Survival analysis demonstrated a worse prognosis in OC patients with high-level SNHG12 compared to those with low-level SNHG12 (Figure 1D). The above data illustrated that SNHG12 could serve as an oncogene in the progression of OC.

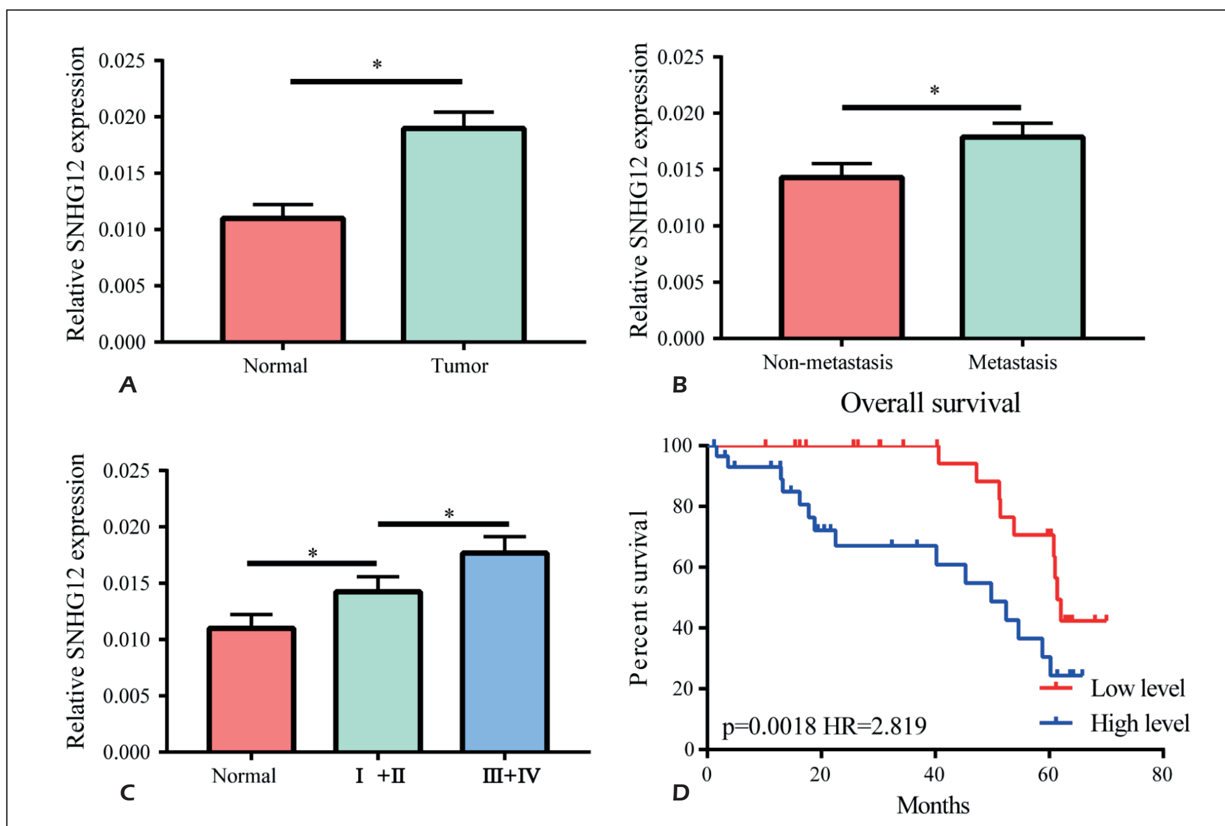

Figure 1. Upregulation of SNHG12 in OC. **A**, SNHG12 was upregulated in OC tissues relative to controls determined by qRT-PCR. **B**, SNHG12 level was higher in patients with metastatic OC compared with those of non-metastatic ones determined by qRT-PCR. **C**, SNHG12 level was higher in OC patients with stage III-IV compared with those with stage I-II determined by qRT-PCR. **D**, The survival analysis demonstrated a worse prognosis in OC patients with high-level SNHG12 relative to those with low-level SNHG12 (p=0.0018, HR=2.819). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

SNHG12 Accelerated OC Cells to Proliferate and Migrate

The cellular level of SNHG12 was determined by qRT-PCR as well. Upregulation of SNHG12 was observed in OC cell lines relative to controls (Figure 2A). In this study, we have chosen A2780 and HO8910 cell lines for subsequent experiments. First of all, the transfection efficacy of OE-SNHG12 was verified in OC cell lines (Figure 2B, C). As CCK-8 assay revealed, the viability markedly increased in A2780 and HO8910 cells overexpressing SNHG12 (Figure 2D, 2E, left). The transwell assay revealed that the overexpression of SNHG12 could elevate the number of migratory cells of A2780 and HO8910 cells (Figure 2D, 2E, right).

SNHG12 Exerted Its Function Through Downregulating MiRNA-129

The binding sequences between SNHG12 and miRNA-129 were predicted by bioinformatics methods (Figure 3A). Based on the predicted sequences, we constructed SNHG12 WT and SNHG12 MUT luciferase vector. The relative luciferase activity markedly decreased in OC cells co-transfected with SNHG12 WT luciferase vector and miRNA-129 mimics, suggesting the direct binding between SNHG12 and miRNA-129 (Figure 3B, 3C). Moreover, miRNA-129 expression was downregulated by SNHG12 overexpression in OC cells (Figure 3D, 3E). To evaluate the involvement of miRNA-129 in SNHG12-mediated progression of OC, cells were transfected with

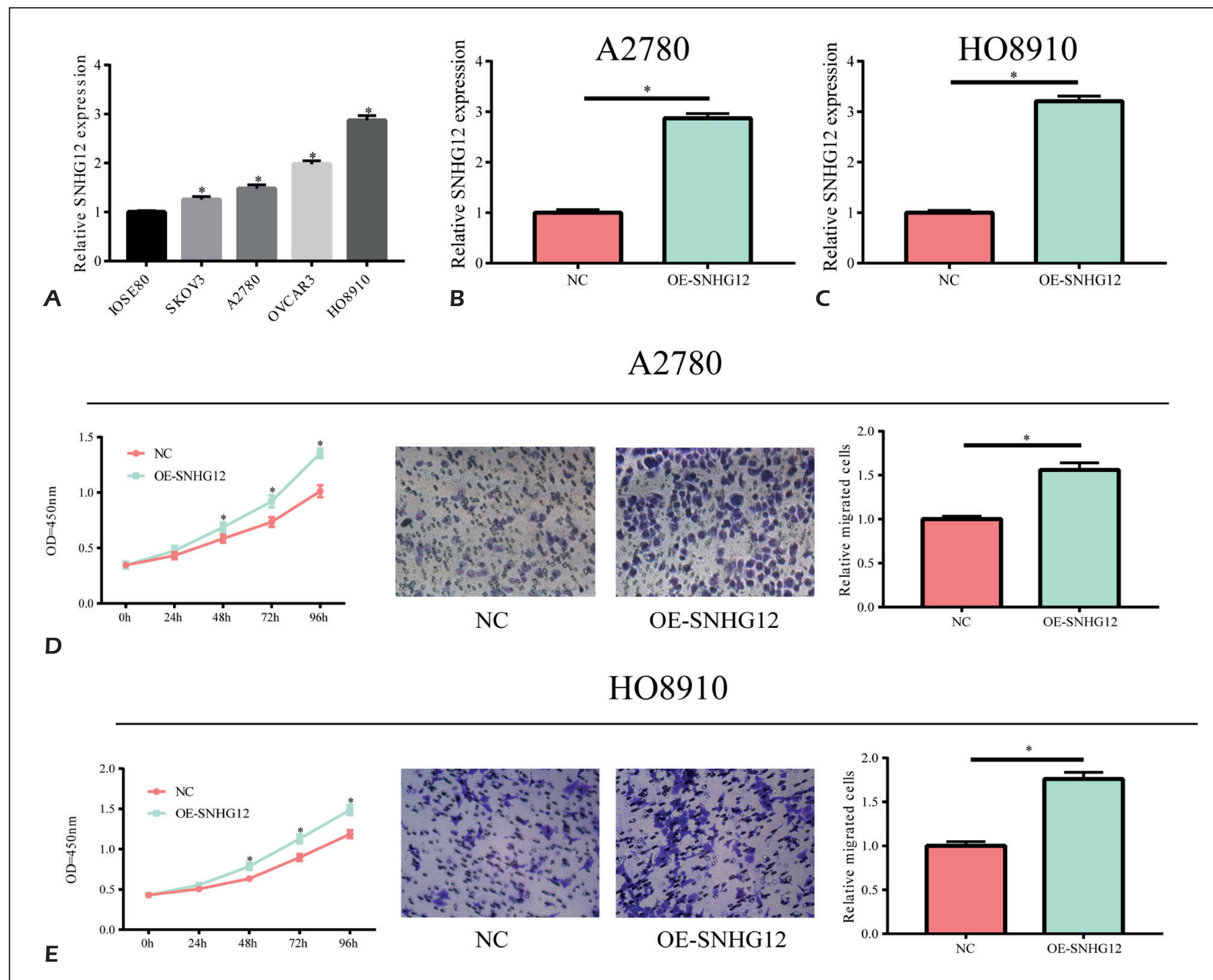

Figure 2. SNHG12 accelerated OC cells to proliferate and migrate. **A**, SNHG12 was upregulated in OC cell lines relative to controls determined by qRT-PCR. **B-C**, Transfection efficacy of OE-SNHG12 in A2780 and HO8910 cells determined by qRT-PCR. **D-E**, The CCK-8 and transwell assay showed increased viability and migration in A2780 and HO8910 cells transfected with OE-SNHG12. *p<0.05, **p<0.01, ***p<0.001.

NC, OE-SNHG12 or OE-SNHG12+miRNA-129 mimics, respectively. Enhanced viability in OC cells overexpressing SNHG12 was partially reversed by miRNA-129 overexpression (Figure 3F, 3G). Meanwhile, the miRNA-129 overexpression also reversed the elevated migratory abilities in OC cells transfected with OE-SNHG12 (Figure 3H, 3I). It is suggested that SNHG12 promoted the proliferative and migratory abilities of OC cells *via* downregulating miRNA-129.

SOX4 was the Target Gene of MiRNA-129

Current researches have demonstrated that miRNAs exert their biological functions through degrading the downstream. Here, SOX4 was predicted to be the potential target of miRNA-129 (Figure 4A). In a similar way, we proved the binding between miRNA-129 and SOX4 through the dual-luciferase reporter gene assay (Figure 4B, 4C). By transfection of miRNA-129 mimics,

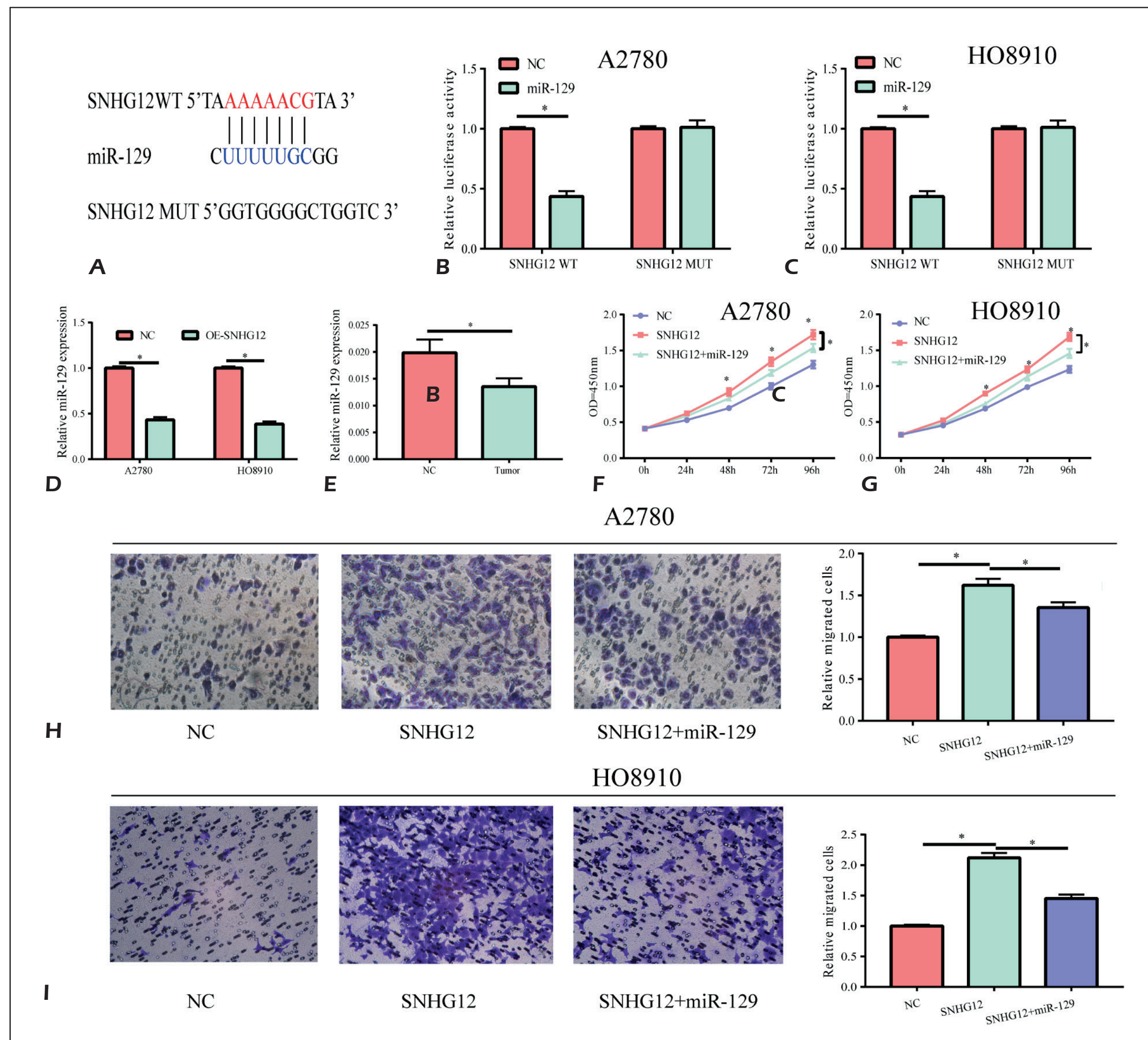

Figure 3. SNHG12 exerted its function through downregulating miR-129. **A**, The binding sequences between SNHG12 and miR-129 predicted by bioinformatics methods. **B-C**, The relative luciferase activity markedly decreased in A2780 and HO8910 cells co-transfected with SNHG12 WT luciferase vector and miR-129 mimics. **D-E**, The miR-129 expression was downregulated by transfection of OE-SNHG12 in A2780 and HO8910 cells. A2780 and HO8910 cells were transfected with NC, OE-SNHG12 or OE-SNHG12+miR-129 mimics, respectively. **F-G**, The CCK-8 assay showed viability in each group. **H-I**, The transwell assay showed migration in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

the SOX4 level was downregulated in OC cells at both mRNA and protein levels (Figure 4D, 4E). Subsequently, we determined the SOX4 level in OC tissues and adjacent normal tissues. Higher abundance of SOX4 was shown in OC tissues relative to controls (Figure 4F). We speculated that miRNA-129 regulated the progression of OC through degrading SOX4. Upregulation of SOX4 elevated viability of A2780 and HO8910 cells, which was partially reversed by miRNA-129 overexpression (Figure 4G, 4H, right). Identically, SOX4 overexpression accelerated the migratory ability of OC cells, and further reversed by miRNA-129 overexpression (Figure 4G, 4H, left). Therefore, miRNA-129 regulated cellular behaviors of OC cells *via* downregulating SOX4.

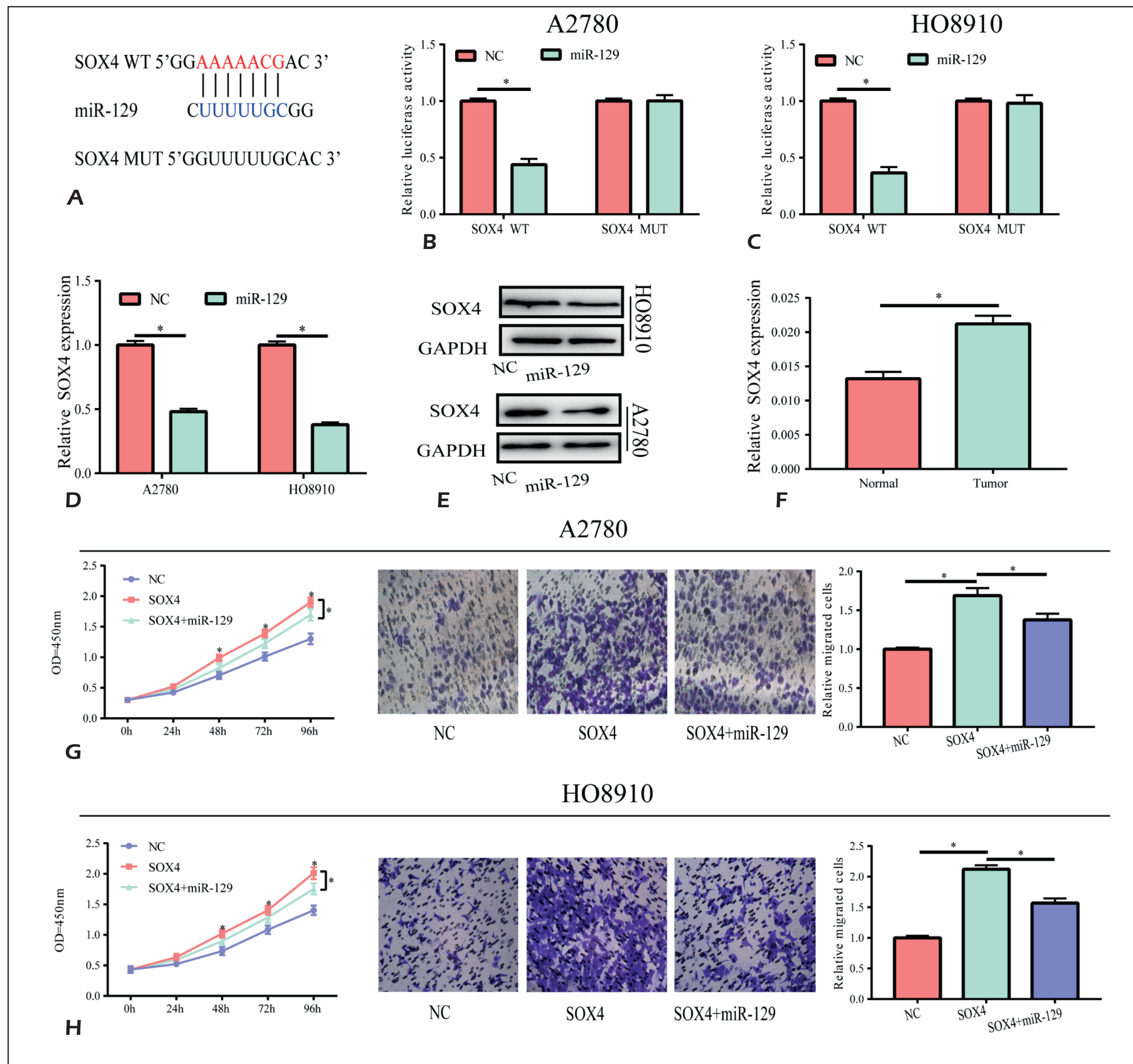

Figure 4. SOX4 was the target gene of miR-129. **A**, The binding sequences between miR-129 and SOX4 predicted by bioinformatics methods. **B-C**, The relative luciferase activity markedly decreased in A2780 and HO8910 cells co-transfected with SOX4 WT luciferase vector and miR-129 mimics. **D-E**, The SOX4 level was downregulated in A2780 and HO8910 cells transfected with miR-129 mimics determined by qRT-PCR and Western blot. **F**, SOX4 was upregulated in OC tissues relative to controls determined by qRT-PCR. A2780 and HO8910 cells were transfected with NC, pcDNA-SOX4 or pcDNA-SOX-4+miR-129 mimics, respectively. **G-H**, The CCK-8 and transwell assay showed viability and migration in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Discussion

MiRNA is highly conserved with 18-25 nt in length. It mediates gene expressions at a post-transcriptional level through suppressing protein translation or degrading downstream genes[12]. In the process of tumorigenesis and tumor progression, miRNA serves as an oncogene or tumor-suppressor gene based on its specific function[13,14].

In 2011, the ceRNA hypothesis proposed a novel regulatory network involving different types of transcriptome products, in which RNA transcripts constrain each other by competitively binding to the common MREs (miRNA response elements)[15]. ceRNA is not only a newly discovered RNA, but it also represents a brand-new regulatory mechanism in gene regulation to complement the traditional miRNA-RNA axis. Impaired ceRNA regulatory network may lead to tumorigenesis. LncRNA-miRNA-mRNA axis composes a typical ceRNA network, and its imbalance is believed to promote tumor progression[16]. For instance, lncRNA MEG3 mediates the occurrence and progression of gastric cancer by competitively binding to miR-181a[17]. In renal cell carcinoma, HOTAIR inhibits miR-217 expression to mediate the HIF-1α/AXL axis as a ceRNA, thus inducing the tumorigenesis[18]. RNAHNF1A-AS1 is capable of promoting proliferative ability and inhibiting apoptosis of bladder cancer cells through absorbing miR-30b-5p to upregulate Bcl-2[19].

In this study, we determined the SNHG12 level in OC by qRT-PCR, which was highly expressed in OC tissues and cell lines. Furthermore, SNHG12 remained a higher level in metastatic OC or stage III-IV compared with that of non-metastatic or stage I-II. OC patients with a high level of SNHG12 presented a worse prognosis than those with a low level, suggesting the promotive role of SNHG12 in the malignant progression of OC. Through a series of functional experiments, SNHG12 was proved to be able to enhance proliferative and migratory abilities of OC.

We thereafter speculated whether SNHG12 exerted its function in OC through the ceRNA theory. Bioinformatics prediction identified that miRNA-129 was the potential downstream of SNHG12, which was further verified by the dual-luciferase reporter assay we performed. Besides, SNHG12 overexpression could downregulate miRNA-129 level in OC cells, further demonstrating that SNHG12 bound to miRNA-129 and regulated its level.

MiRNA-129 has been reported[20,21] as a tumor-suppressor gene in inhibiting the proliferative and invasive abilities of lung cancer and breast cancer. Here, we speculated the involvement of miRNA-129 in SNHG12-mediated progression of OC. As expected, miRNA-129 overexpression partially reversed the promotive effects of SNHG12 on proliferative and migratory abilities of OC cells.

SOX4 was then predicted to be the target of miRNA-129, which was confirmed to be regulated by miRNA-129 in OC cells. We designed the rescue experiments and revealed that SOX4 upregulation markedly promoted OC cells to proliferate and migrate, and of note, reversed the regulatory effects of miRNA-129 on OC cells.

Conclusions

We found that SNHG12 accelerates the proliferative and migratory abilities of OC cells *via* sponging miRNA-129 to upregulate SOX4. This study provides novel directions for preventing and treating OC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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