LncRNA SNHG14 Promotes Progression of Ovarian Cancer by Regulating miR-206 Expression

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Objective: This study aimed to probe into the effect of LncRNA SNHG14 on ovarian cancer progression by regulating miR-206.Methods: Fifty-seven ovarian cancer (OC) patients who were treated in our hospital from December 2017 to December 2019 were collected as the research objects. During the operation, OC tissues and paracancerous tissues of patients were collected, and the effect of SNHG14 on OC tumor growth in nude mice was detected, and SNHG14 inhibitor was transfected into OC cells. The relative expression of SNHG14 in tissues and cells was detected by qRT-PCR, cell proliferation was testedvia CCK8, migration and invasion were detected through Transwell, apoptosis was assessedvia flow cytometry, and the targeted relationship between SNHG14 and miR-206 was detected by dual luciferase reporter gene.Results: SNHG14 is highly expressed in OC tissues, cells and nude mice. Down-regulating it can inhibit the biological ability of OC cells and inhibit the growth of nude mice tumors. It can directly target miR-206 to regulate CCND1 expression and promote OC progression.Conclusion: LncRNA SNHG14 can act as miR-206 sponge to regulate CCND1 expression downstream of miR-206 and promote OC progression.

Keywords: LncRNA SNHG14, miR-206, ovarian cancer, biological function, CCND1
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INTRODUCTION

OC is a common female reproductive system tumor clinically. According to statistics, in 2018, there were 22,240 OC patients, and 14,070 patients died [1], which became the main cause of gynecological cancer death in the world [2]. The main reason for the high mortality of OC is that early diagnosis is difficult, and the disease has developed to the middle and late stage when patients are admitted to hospital, and they miss the best treatment time [3]. About 70% of cases were diagnosed at advanced stage with poor prognosis, while advanced OC patients were usually incurable [4]. Although great progress has been made in OC treatment in recent years, the proportion of patients who get overall remission after treatment is less than 10%, and nearly 70% of patients relapse [5, 6]. Therefore, it is particularly important to explore the molecular mechanism of OC and seek new therapeutic targets for improving the prognosis of OC patients.

LncRNA is a non-coding RNA with a length of more than 200nt, which can not directly act on protein and is considered as metabolic waste in transcription process [7]. However, with the deepening of research, it has been found that lncRNA plays a regulatory role in reproductive diseases, providing a new perspective for diagnosis, prevention and treatment [8], and it has abnormal expression in various cancers and is relevant to cancer development [9]. Recently, many studies have shown that lncRNA can act as microRNA (miR) sponge to regulate the downstream target genes of miR, including transcription, translation and protein modification [10]. miR is a non-coding short-chain RNA with a length of about 22nt, which can completely or incompletely combine with the downstream target gene to influence the stability of mRNA or inhibit its translation, and it regulates protein expression [11-13]. In prostate cancer, LncRNA XIST can competitively combine with miR-23a to regulate the RKIP expression, thus regulating the proliferation and migration of prostate cancer cells [14]. In gastric cancer, lncRNA regulates the progression of gastric cancer by completely combining with miRNA to produce

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ceRNA or promoting transcription, so it can be used as a new anti-cancer target [15]. Li et al. [16] reported that LncRNA SNHG14 promoted the progression of non-small cell lung cancer (NSCLC) through miR-206/G6PD pathway. However, there are few studies on the mechanism of LncRNA SNHG14 and miR-206 in OC, so this study will discuss this and provide a new direction for OC treatment.

MATERIALS AND METHODS

Experimental Materials

Tissue samples

Fifty-seven OC patients who were treated in our hospital from December 2017 to December 2019 were collected as the research objects. OC tissues and paracancerous tissues were collected during the operation, and the collected samples were stored in liquid nitrogen and kept at -80℃. Patients did not receive chemotherapy before operation, and according to WHO classification, those samples were diagnosed as OC by pathological examination. This study was approved by the Medical Ethics Committee of our hospital and it conformed to Helsinki Declaration. Patients and their families signed an informed consent form.

Cell lines

Human OC cells CAOV3 (Guangzhou Saiku Biotechnology Co., Ltd.), TOV-112D, OV-90, HO-8910, SK-OV-3 cells (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.), and human normal ovarian epithelial cells IOSE80 (Shanghai Huiying Biotechnology Co., Ltd.) were put into use.

Nude mice

Ten female nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.), weighing 18-20 grams and aged 4 weeks, were reared in an environment with a temperature of 23-28℃ and a relative humidity of 45%-70%. The alternation of diurnal rhythm was normal, and they could eat and drink freely. The experimental process conforms to the standards of the Experimental Animal Ethics Committee of our hospital.

Main experimental materials

DMEM medium (Gibco, USA), Lipofectamine™ 2000 kit (Invitrogen, USA), fetal bovine serum (FBS), si-SNHG14, NC, miR-206-mimics, miR-206-inhibitor (GenePharma, Shanghai, China), trypsin (Hyclone, Logan, USA), TaqMan™ Reverse reverse transcription kit and Trizol reagent (Invitrogen, USA), cell counting kit 8 (CCK8) (Dojindo Molecular Technologies, Gaithersburg, MD), multi-functional microplate reader spectrum MaxM5 (Molecular Devices, San Jose, CA), Transwell chamber (Corning Costar, Corning, NY), CAnnexin V-FITC/PI apoptosis kit (Shanghai Best Biotechnology Co., Ltd.), flow cytometry CytoFLEX LX (Beckman,Brea, California, USA).

Methods

Cell culture and transfection

Cell culture: All the above five kinds of cells were cultured in DMEM medium added with 10%FBS and 1% penicillin-streptomycin. When the cell adhesion growth and fusion reached 80%, they were digested with 0.25% trypsin and continued to be cultured to complete passage. Cell transfection: NC, si-SNHG14, miR-NC, miR-206-mimics and miR-206-inhibitor were transfected into the cells to be infected by Lipofectamine™ 2000 kit, and the operation steps were strictly in accordance with the instructions of the kit.

qRT-PCR

Total RNA in tissues and cells was extracted with Trizol reagent, and the purity and concentration were detected by ultraviolet spectrophotometer, and then 5 μg was taken from each group for cDNA reverse transcription in view of the instructions of the kit. The reaction parameters were: 37℃ for 15 min, 42℃ for 35 min, 70℃ for 5 min. Afterwards, it was amplified by PCR. The PCR reaction conditions were as follows:pre-denaturation at 94 ℃ for 45 s, denaturation at 94℃ for 10 s, annealing extension at 60℃ for 45 s, a total of 40 cycles. It was repeated three times. U6 was used as internal reference, and data were analyzed by $2^{-\Delta\Delta Ct}$.

Western blot (WB)

After total protein of cells was extracted by RIPA lysis method, the concentration was detected by BCA kit and it was then adjusted to 4 μg/μL. Next, it was transferred to PVDF membrane by 12%SDS-PAGE electrophoresis and sealed for 2 h. The membrane was washed, CCND1 (1: 500) and GAPDH (1: 500) were added and sealed at 4 ℃ all night. Primary antibody was cleaned away and goat anti-rabbit secondary antibody (1:5000) was added, and the membrane was washed again. Finally, it developed using enhanced chemiluminescence (ECL).

CCK8

Cell activity was detected by CCK8 kit: The cells were inoculated in a 96-well plate with 5×10^3 cells/well. Three repeated wells were set up, and this experiment followed the instructions: CCK8 solution was added to the culture medium, and it was then incubated 2 h in 5% CO_2 at 37℃. At the specified time points (24, 48, 72, 96 h), the absorbance was measured with a microplate reader at 450 nm.

Transwell

Cell migration and invasion were detected through Transwell test. Migration experiment:

cells (1×10^4) were resuspended in 200 μL serum-free DMEM medium, the medium containing cells was inoculated into the upper chamber, and 500 uL DMEM medium containing 10% serum was added into the lower chamber to culture for 24 h. The upper chamber cells were removed, and the lower layer cells were immobilized with 4% paraformaldehyde and stained with crystal violet. After the cell membrane was washed with PBS, we took photos and counted them under microscope. Invasion experiment: 50 uL mixed medium (Matrigel glue: serum-free medium =9:1) was evenly added into the upper chamber and dried. Other operations were the same as migration experiment.

Flow cytometry

The cells were washed twice with PBS and added with 100 μL binding buffer to prepare a suspension of 1×10^6 cells /mL. Then, AnnexinV-FITC and PI were added successively, cells were incubated for 5 min at indoor temperature in the dark and detected by flow cytometry. To get the average value, the experiment was repeated three times.

Tumor formation in nude mice

In order to detect the effect of SNHG14 in vivo, cells infected with si-SNHG14, pcDNA3.1-SNHG14 or negative control lentiviral vector were injected subcutaneously into the buttocks of nude mice. The tumor size was measured at 7 days intervals and the tumor volume was calculated: V (mm^3) = $width^2\times length/2$. On the 28th day, the mice were killed. Tumor tissues of nude mice were collected to calculate tumor mass and detect the expression in tumor tissues. All studies have been approved by the Animal Protection and Utilization Committee of our hospital.

Dual luciferase report

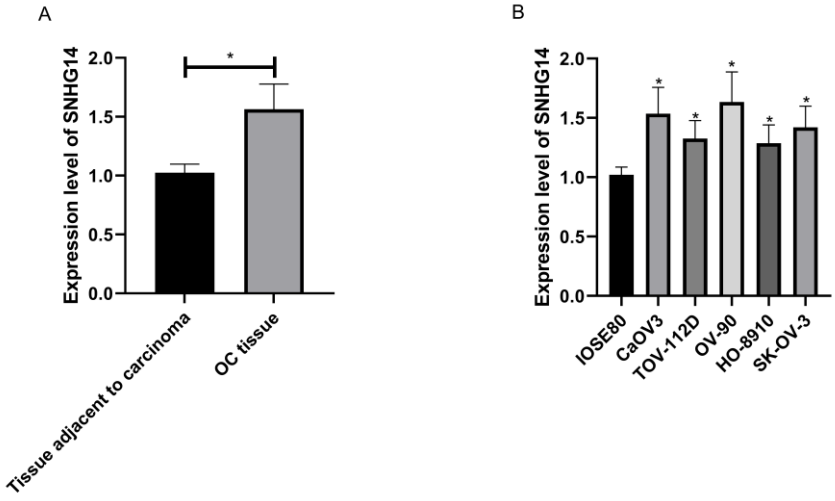
Complementary DNA fragments containing wild-type SNHG14 (SNHG14-WT) or mutant (SNHG14-MUT) fragments were sub-cloned into the downstream of luciferase gene in psi-CHECK2 luciferase reporter vector. Transfection reagents were used to transfect miR-206-mimics and miR-NC into HEK-293T cells. Luciferase activity was measured 48 h after transfection.

RESULTS

SNHG14 Expression in OC

qRT-PCR results showed that the SNHG14expression in OC tissues was markedly higher than that in paracancerous tissues ($P<0.05$);the expression in OC cell lines was dramatically higher than that in IOSE80 cells ($P<0.05$), and the highest level was found in CaOV3 and OV-90 cells, so these two cells were selected for follow-up study, as shown in Figure 1.

Figure 1
SNHG14 expression in OC



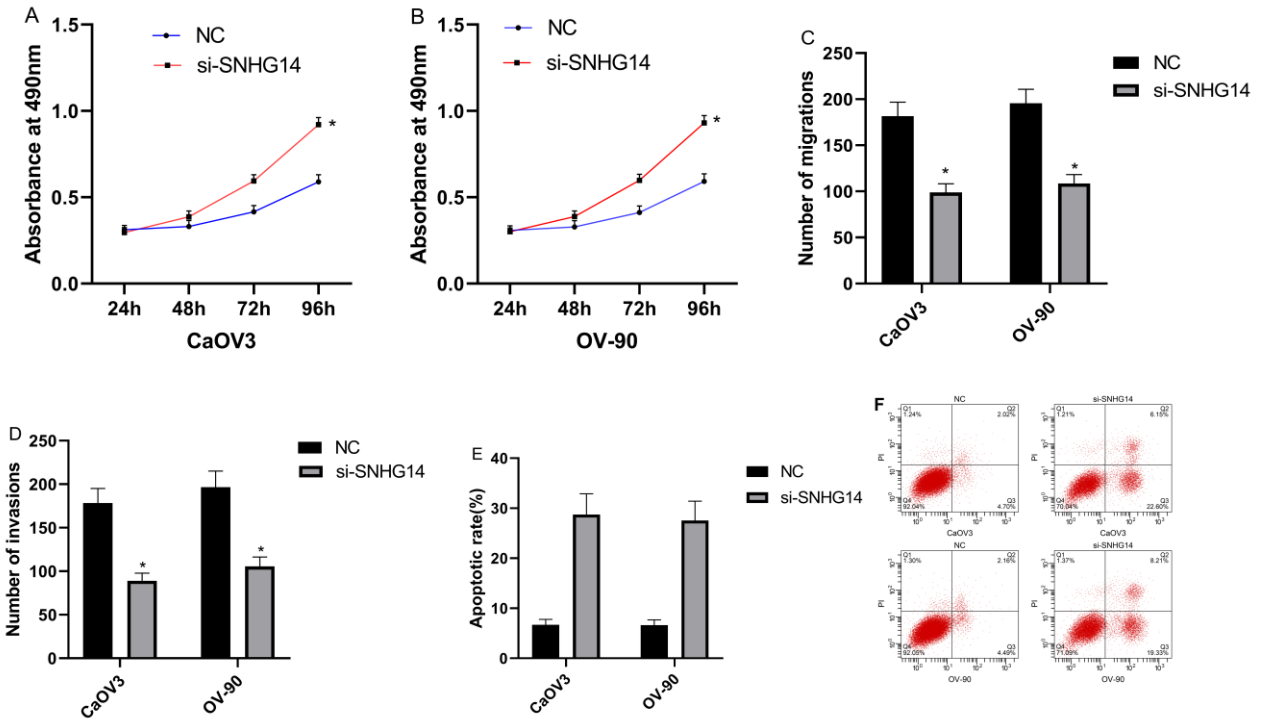
A. The LncRNA SNHG14 expressionin OC tissues is markedly higher than that in paracancerous tissues; B. The SNHG14 expression in OC cell lines is markedly higher than that in IOSE80 cells. * means $P<0.05$.

Effect of SNHG14 on Biological Ability of OC Cells

After transfecting NC, si-SNHG14 to CaOV3 and OV-90 cells, we discovered that the relative expression of SNHG14 in CaOV3 and OV-90 cells in the si-SNHG14 group was obviously down-regulated compared with the NC group ($P<0.05$). While detecting the biological ability of CaOV3 and OV-90 cells, we found that

transfecting si-SNHG14 could dramatically reduce the proliferation, migration and invasion of OC cell lines (P<0.05), and increase the apoptosis rate (P<0.05), as shown in Figure 2.

Figure 2
effect of SNHG14 on biological ability of OC cells

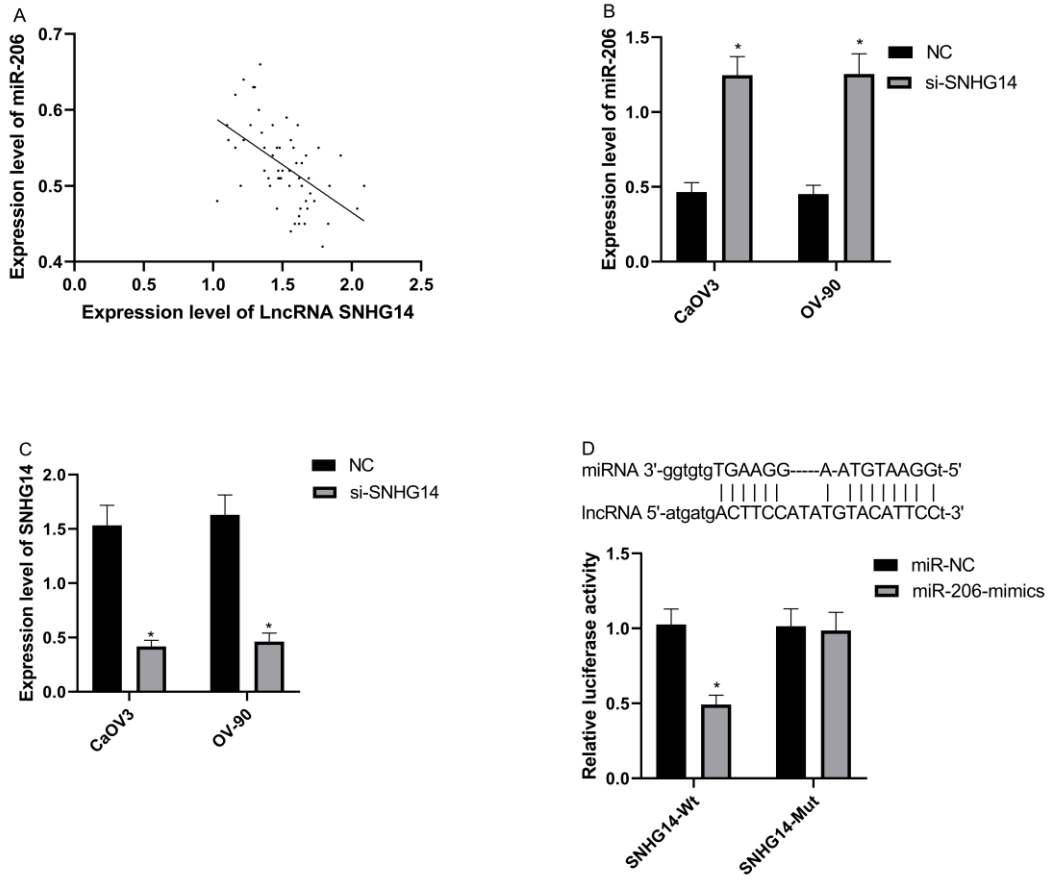


A/B. Transfecting si-SNHG14 can inhibit the re-growth ability of CaOV3/OV-90 cells; C. Transfecting si-SNHG14 can dramatically reduce the migration of CaOV3/OV-90 cells. D. Transfecting si-SNHG14 can dramatically reduce the invasion of CaOV3/OV-90 cells. E. Transfecting si-SNHG14 can dramatically increase the apoptosis rate of CaOV3/OV-90 cells. F. Flow cytometry. * means P<0.05.

Regulatory effect of SNHG14 on miR-206 in OC cells

To explore the correlation between the expression of SNHG14 and miR-206, the expression of the two in OC tissues was detected by qRT-PCR. Pearson test showed that there was a negative correlation between them (r=-0.527, P<0.001). After transfecting NC and si-SNHG14 into OC cell lines respectively, and detecting the miR-206 expression in OC cell lines, we found that the miR-206 expression in OC cell lines of the si-SNHG14 group was obviously higher than that of the NC group. To study the targeted relationship between SNHG14 and miR-206, miR-NC and miR-206-mimics were transfected into OC cell lines, which were verified by dual luciferase reporter gene assay. It showed that transfecting miR-206-mimics could decrease the luciferase activity of SNHG14-Wt in OC cells, but it did not affect the activity of SNHG14-Mut, as shown in Figure 3.

Figure 3
Regulation of SNHG14 on miR-206 in OC cells

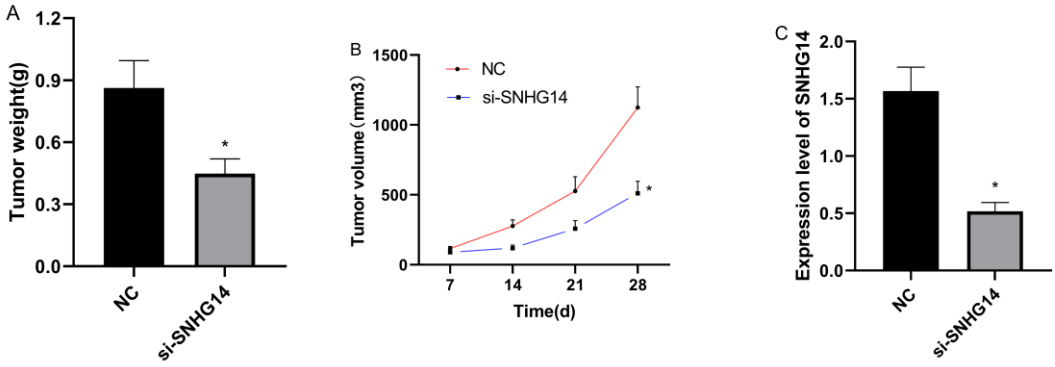


A. The expression of SNHG14 and miR-206 in OC tissues was negatively correlated (r=-0.527, P<0.001); B. Transfecting si-SNHG14 can dramatically increase the miR-206 expression in OC cell line; C. Transfecting si-SNHG14 can dramatically reduce the SNHG14 expression in OC cell line; D. Transfecting miR-206-mimics can decrease the luciferase activity of SNHG14-Wt in OC cells, but it has no effect on the activity of SNHG14-Mut. * means P<0.05.

Effect of SNHG14 on Proliferation of OC Cells in Vivo

In order to explore the role of SNHG14 in vivo, we conducted tumor formation experiment in nude mice. The results showed that compared with the NC group, the weight and volume of tumor in the si-SNHG14 group reduced obviously. And qRT-PCR detection manifested that the SNHG14 expression in OC tissues of nude mice in the NC group was remarkably higher than that in the si-SNHG14 group, as shown in Figure 4.

Figure 4
effect of SNHG14 on proliferation of OC cells in vivo

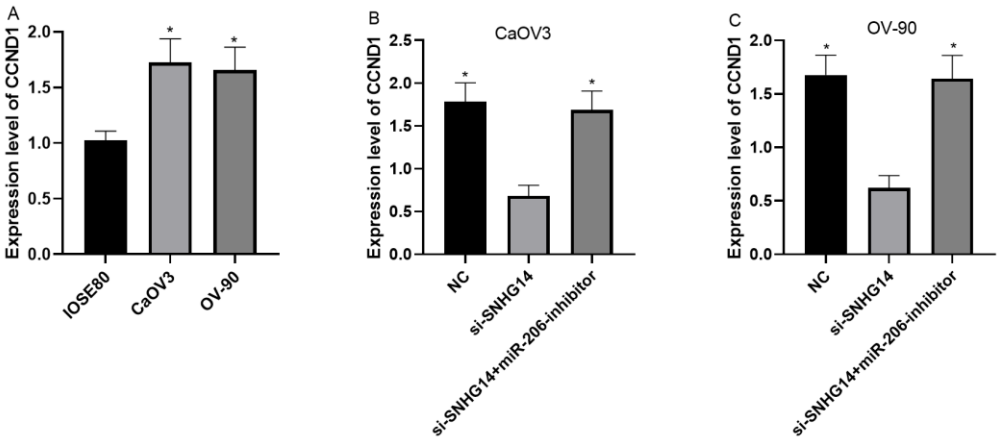


A. Transfecting si-SNHG14 can dramatically reduce the tumor weight in nude mice; B. Transfecting si-SNHG14 can dramatically reduce the tumor volume in nude mice; C. Transfecting si-SNHG14 can dramatically reduce the SNHG14 expression in OC tissues of nude mice. * means P<0.05.

SNHG14 May Combine Competitively with miR-206 to Regulate CCND1 Expression in OC Cells

Previous studies have shown that CCND1 may be the downstream target protein of miR-206, so we suspect that SNHG14 may regulate CCND1. Therefore, by detecting the CCND1 expression in OC and IOSE80 cells, we found that the expression in OC cells was obviously up-regulated. Then, we detected the expression in the NC group and the si-SNHG14 group, and found that it in the latter was obviously lower than that in the former. Besides, by transfecting miR-206-inhibitor, we found that the CCND1 expression in the si-SNHG14 group increased, and the inhibitory effect of SNHG14 knockdown on CCND1 was obviously offset, as shown in Figure 5.

Figure 5
SNHG14 in OC cells may competitively bind with miR-206 to regulate CCND1 expression



A. The CCND1 expression in CaOV3/OV-90 cells increases markedly; B/C. The CCND1 expression in the si-SNHG14 group is markedly lower than that in the NC group. After miR-206-inhibitor transfection, the CCND1 expression in the si-SNHG14 group increases, and the inhibitory effect of SNHG14 knockdown on CCND1 is obviously offset. * means P<0.05.

DISCUSSION

OC is a familiar disease of female reproductive system, and its poor prognosis has been widely concerned by people [17]. With the continuous development of medical level in recent ten years, although some progress has been made in OC treatment, the improvement of survival of OC

patients is still not satisfactory [18]. Recently, LncRNA is a research hotspot. Many studies have shown that LncRNA SNHG14 is differentially expressed in many diseases and is relevant to the occurrence and development of diseases. Ji et al. [19] pointed out that the relative expression of SNHG14 was obviously up-regulated in cervical cancer tissues and cells, which was related to the overall survival of CC patients. SNHG14 promoted the progression of cervical cancer by regulating miR-206/YWHAZ. Knocking out SNHG14 dramatically inhibited the proliferation, migration and invasion of cervical cancer cells, and promoted apoptosis. This indicates that SNHG14 may be the target of diagnosis and treatment. However, the mechanism of LncRNA SNHG14 in OC is unclear, so this study will discuss it.

In this study, the relative expression of SNHG14 in tumor tissues and normal tissues adjacent to OC patients was detected by qRT-PCR. The results showed that the expression in OC tissues increased markedly, which suggested that SNHG14 had the potential to diagnose OC. In addition, we also detected the SNHG14 expression in OC cell lines and normal ovarian epithelial cells, and found that it was also dramatically up-regulated in OC cells. It indicated that SNHG14 was differentially expressed in OC. But, the pathogenesis and mechanism of OC are still vague. Studies have reported that LncRNA can be used as a biological indicator of prognosis of OC patients; it regulates the mechanism of OC metastasis, recurrence and drug resistance and can be used as a personalized therapeutic target for OC [20]. Thus, we discussed the molecular mechanism of LncRNA SNHG14 in OC. By transfecting si-SNHG14 into OC cells and detecting the biological ability of the cells, we discovered that transfecting si-SNHG14 could inhibit the proliferation, migration and invasion of OC cells and promote their apoptosis. Zhao et al. [21] explained that LncRNA SNHG14 could promote the migration and invasion of ovarian cancer cells by up-regulating DGCR8 expression, similar to our research. The current research shows that LncRNA and miR have a close cooperative relationship in regulating transcription and post-transcriptional gene expression during tumorigenesis [22]. Sun et al. [23] found that MALAT1 could be used as an oncogene, which combined with miR-503-5p to induce cell proliferation and inhibit apoptosis of OC cells. Hence, we suspect that LncRNA SNHG14 competitively binds to miR-206, and influences the expression of related proteins by regulating the downstream target genes of miR-206, thus promoting OC progression. To verify this hypothesis, we discussed the interaction mechanism between the two. Pearson test analysis identified that there was a negative correlation between the expression of SNHG14 and miR-206 in OC, and the miR-206 expression increased dramatically by transfecting si-SNHG14 into OC cell lines, which suggested that these two indicators participated in OC progression through negative regulation. Then, through dual luciferase reporter gene assay, we found that SNHG14 had a targeted binding site with miR-206, which indicated that it could act as a sponge of miR-206 and promote OC proliferation and migration and inhibit apoptosis by inhibiting miR-206 expression, thus promoting OC progression. Similar studies have shown that [24] SNHG14 confers gefitinib resistance through the miR-206/ABCB1 axis in NSCLC, which is a vital molecular mechanism in NSCLC and can be used as a new therapeutic direction.

To explore the effects of SNHG14 and miR-206 on the proliferation of OC cells in vivo, we conducted the tumor formation experiment of OC nude mice. The results showed that the SNHG14 expression reduced markedly by transfecting si-SNHG14 into nude mice. We measured the weight and volume of OC tumors in nude mice after 28 days, and discovered that transfecting si-SNHG14 could dramatically reduce the weight and volume of tumors. It indicated that SNHG14 might act as an oncogene in vivo and inhibit its expression can inhibit tumor growth, so it can be used as a therapeutic target for OC. Previous studies have shown that CCND1 is a downstream target gene of miR-206, which can inhibit the development of pleural mesothelioma by targeting miR-206 [25]. It is an important protooncogene, which is amplified in many cancers and is related to the proliferation and apoptosis of OC cells [26]. Therefore, we suspect that LncRNA SNHG14 promotes OC progression by competitively binding with miR-206 and regulating CCND1 expression downstream of miR-206. To prove this conjecture, we detected the CCND1 expression in OC and IOSE80 cells, and found that the expression in OC cells was remarkably up-regulated. Transfecting si-SNHG14 markedly reduced the CCND1 expression in cells, while transfecting miR-206-inhibitor increased the expression, indicating that the inhibitory effect of SNHG14 knock-down on CCND1 was obviously offset. Similar to our research, Chang et al. [27] clarified that LncRNA HOTAIR could act as miR-206 sponge to regulate the expression of CCND1 and CCND2 and promote OC progression.

The above studies show that LncRNA SNHG14 can promote OC progression by competitively binding with miR-206 and regulating CCND1 expression downstream of miR-206. However, this study still has limitations. We have not detected the diagnostic value of LncRNA SNHG14 in OC patients, and it is not clear whether miR-206 has other active sites to promote OC progression. Thus, we will improve this study in the future.

To sum up, LncRNA SNHG14 can act as miR-206 sponge to regulate CCND1 expression downstream of miR-206 and promote OC progression.

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