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Regular paper

Promoting action of long non-coding RNA small nucleolar RNA host gene 4 in ovarian cancer

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Objective: Long non-coding RNA (LncRNA) small nucleolar RNA host gene 4 (SNHG4) has been shown to be aberrantly expressed in a variety of cancers and involved in cancer development, but its role in ovarian cancer (OC) is unclear. The purpose of this study was to explore the biological function of SNHG4 in OC and reveal its potential downstream molecular targets. Methods: OC tumor tissue and normal tissue were collected; normal human ovarian epithelial cell line (IOSE80) and human ovarian cancer cell line (A2780, SKOV-3, OV-90 and CAOV3) were selected. RT-qPCR was used to detect SNHG4, miR-98-5p, and TMED5, while western blot was used to detect the protein expression levels of TMED5, Ki67, MMP-9, Bcl-2, Bax, Gsk3β, Wnt3a, and β-catenin. The subcellular localization of SNHG4 was assessed by nucleocytoplasmic separation assay. CCK-8, colony formation assay, flow cytometry, and Transwell were used to assess the biological behavior of OC cells. The targeting relationship between SNHG4, miR-98-5p and TMED5 was verified by dual luciferase reporter assay and RIP assay. Results: In OC, SNHG4 and TMED5 were highly expressed, and miR-98-5p was underexpressed. Knockdown of SNHG4 inhibited OC cell proliferation, migration and invasion, promoted apoptosis, and prevented Wnt/β-catenin pathway activation. The effect of knockdown of SNHG4 was reversed by knockdown of miR-98-5p or overexpression of TMED5. Mechanistically, SNHG4 competitively adsorbed miR-98-5p to mediate TMED5 expression, thereby activating the Wnt/β-catenin pathway. Conclusion: SNHG4 accelerates OC development *via* mediating the miR-98-5p/TMED5 axis and activating the Wnt/β-Catenin pathway. SNHG4 gene silencing might be a novel option for OC treatment.

Keywords: ovarian cancer, Long non-coding RNA small nucleolar RNA host gene 4, MicroRNA-98-5p, Transmembrane emp24 protein transport domain containing 5, Wnt/β-Catenin signaling pathway

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Abbreviations: CC, cervical cancer; ceRNA, competitive endogenous RNA; LC, liver cancer; LncRNAs, Long non-coding RNA; OC, ovarian cancer; SNHG4, mall nucleolar RNA host gene 4

INTRODUCTION

Ovarian cancer (OC) is an extremely lethal gynecological tumor in women, taking up about 14,000 deaths per annum, which severely threatens women's health and safety around the world (Elsayed *et al*., 2021). OC's cause remains elusive, and presently, genetic and endocrine factors are regarded as the crucial reason (Gao *et al*., 2021). Presently, surgery, chemotherapy and radiotherapy are the crucial cure strategies for treating OC (Redondo *et al*., 2021). These methods have achieved a tremendous breakthroughs, the 5-year survival rate of OC patients is still inferior owing to the lack of elevated specificity of long-term imperative treatment and easy recurrence after recovery (Zhu *et al*., 2021; Opławski *et al*., 2021). Consequently, to improve OC patients' quality of life has been a hot topic to explore the molecular mechanism of OC occurrence and development and hunt for brand-new therapeutic targets.

Long non-coding RNA (LncRNAs), a group of ncRNA composed of more than 200 nucleotides transcribed *via* RNA polymerase II, have been testified in multiple cancer occurrence and development (Zhang *et al*., 2021). Accumulated evidence has illuminated that lncRNAs exert the regulatory role in OC biological functions *via* modulating genes, genomic stability, a competitive endogenous RNA (ceRNA) mechanism and epigenetics after transcription (Xu *et al*., 2021; Xie *et al*., 2021). For instance, it has been reported that LINC02323 boosts OC cell growth *via* targeting miR-1343-3p to stimulate TGF-β receptor 1 (Li *et al*., 2021). Yu and others (Yu *et al*., 2018) also maintain that lncRNA LUCAT1 accelerates OC malignant tumors *via* modulating the miR-612/HOXA13 pathway. LncRNA small nucleolar RNA host gene 4 (SNHG4) has been identified as a novel target for multiple cancers covering osteosarcoma (OS) (Xu *et al*, 2018), non-small cell lung cancer (NSCLC) (Li *et al*., 2021), liver cancer (LC) (Jiao *et al*., 2020), and cervical cancer (CC) (Ji *et al*., 2019) and a novel target in human diseases (Chu *et al*., 2021). Nevertheless, the role of SNHG4 in ovarian cancer is unclear.

In terms of mechanism, LncRNA-mediated ceRNA network is frequently explored (Xu *et al*., 2021). LncRNA prevents mRNA degradation *via* interacting with microRNA (miRNA) (Braga *et al*., 2020). Consequently, SNHG4-associated miRNA (miR-98-5p) and miRNA (TMED5) were studied. Studies have manifested that miR-98-5p is implicated in OC (Dong et *al*., 2020). Additionally, reports have clarified that miR-G-1 boosts CC cell nuclear autophagy and malignant behaviors by targeting LMNB1 and TMED5 in CC (Yang *et al*., 2019).

This research hypothesized that SNHG4 might be involved in the occurrence and development of OC. Through loss-of-function and gain-of-function experiments, the role of SNHG4 was identified as a tumor-promoting factor in OC. Through functional rescue experiments, it was confirmed that SNHG4 affects the biological behavior of OC by regulating the miR-98-5p/TMED5 axis.

MATERIALS AND METHODS

Clinical specimens

A total of 40 pairs of ovarian tumor tissues and adjacent normal tissues were collected from patients undergoing oophorectomy at Maternal and Child Health Hospital of Ningyang County from January 2017 to December 2018. Tissue specimens were histopathologically and clinically diagnosed by a pathologist and stored at −80°C. This study was approved by the Ethics Committee of Maternal and Child Health Hospital of Ningyang County (approval number: 20160811ER), and written informed consent was obtained from all participants. Clinical studies were conducted following the guidelines of the Declaration of Helsinki, and subjects were anonymized during data analysis.

Cell culture and transfection

Normal human ovarian epithelial cell line (IOSE80) and human OC cell line (A2780, SKOV-3, OV-90 and CAOV3) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and identified by STR. Cell culture was done using Roswell Park Memorial Institute-1640 medium (Gibco) covering 10% fetal bovine serum, 2% sodium pyruvate and 1% streptomyces penicillin (Gibco) (Qiao *et al*., 2021).

Cells were harvested when SKOV-3 cells were subcultured for 3-4 passages and were 70–80% confluent. SKOV-3 cells were divided into 6 groups: si-negative control (NC), si-SNHG4, si-SNHG4 + in-NC, si-SNHG4 + in-miR-98-5p, si-SNHG4 + pcDNA3.1, si-SNHG4 + pcDNA-TMED5. Human TMED5 (Accession: NM_001167830.2) coding sequence (lack of 3'untranslated region (UTR)) was combined with BamH I and EcoR V sites. The recombined control sequences were synthesized by BGI (Shenzhen, China) and inserted into the pcDNA3.1 vector (Invitrogen). TMED5 overexpression plasmid (pcDNA-TMED5) and negative control (pcDNA) were generated with the BamH I and EcoR V sites opened (Zou, Chen, Liu, & Gan, 2021). SNHG4 small interfering RNA (siRNA) and control siRNA were generated (Invitrogen). miR-98-5p mimic (miR-98-5p), miR-98-5p inhibitor (in-miR-98-5p), NC mimic (miR-NC), and NC inhibitor (in-NC) were obtained (RIBOBIO, Guangzhou, China). Then transfection or co-transfection was implemented using Lipofectamine 2000 transfection reagent (Invitrogen). Wnt/β-catenin signal inhibitorXAV939 was purchased (Selleck Chemicals, Shanghai, China) (L. N. Gao *et al*., 2021). The transfection efficiency was verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) after 48 h.

Subcellular localization analysis

Cytoplasmic RNA and nuclear RNA of SKOV-3 cells were isolated using PARIS Kit (Ambion, Austin, TX, United States). SNHG4 expression was tested by RT-qPCR, taking U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as nuclear and cytoplasmic control, respectively.

RT-qPCR

Extraction of total RNA was done using Trizol reagent (Invitrogen). Reverse transcription for lncRNA/mRNA and miRNA was implemented *via* lnRcute lncRNA cDNA kit and Rcute Plus miRNA cDNA kit (TIANGEN, China), respectively. RT-qPCR was done using SYBR Green kit (Thermo Fisher Scientific) and Mx3005P QPCR system (Agilent Technologies, Santa Clara, CA, USA). U6 and GAPDH were loading controls for miRNA and mRNA/lncRNA, respectively. The primer sequence was manifested in Table 1. All primers were designed and validated through the primer-BLAST website.

Table 1. Primer sequences

Genes	Primer sequences (5′–3′)
SNHG4	F: 5′-GGCTAGAGTACAGTGGCTCG-3′
	R: 5′-GCAAATCGCAAGGTCAGG-3′
MiR-98-5p	F: 5′-GCGCGTGAGGTAGTAAGTTGT-3′
	R: 5′-GCAGGGTCCGAGGTATTC-3′
TMED5	F: 5′-CCCTCGATAGCGACTTCACC-3′
	R: 5′-TGTTGATGGATTCCAGGATGTCT-3′
U6	F: 5′-CTCGCTTCGGCAGCACA-3′
	R: 5′-AACGCTTCACGAATTTGCGT-3′
GAPDH	F: 5′-CACCCACTCCTCCACCTTTG-3′
	R: 5′-CCACCACCCTGTTGCTGTAG-3′

Note: F, forward; R, reverse.

Western blot

Total protein extraction was done with 500 μL Radio-Immunoprecipitation assay lysis buffer (Beyotime, China). Protein (20 μg) was loaded onto 8% sulfate polyacrylamide gel electro-pheresis gel (Solarbio), electroblotted onto a polyvinylidene fluoride membrane (Invitrogen), and blocked with 5% skim milk. Then incubation with primary antibodies and horseradish peroxidase conjugating with goat anti-rabbit secondary antibody Immunoglobulin G (IgG) (1:1000, ab181236, Abcam) was conducted. The signals were visualized using an enhanced chemiluminescence kit (34080, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Density analysis was done with ImageJ software. Primary antibodies were TMED5, Ki67, MMP-9, Bcl-2, Bax, Gsk3β, Wnt3a, and β-catenin (TMED5 and Gsk3β were from Millipore-Sigma; Others were from Cell Signaling Technology, Beverly, MA, USA, and the dilution was 1:1000) (T. Liu *et al*., 2021).

Cell Counting Kit-8 (CCK-8) detection

SKOV-3 cells (1×10^3 cells/well) were seeded on 96-well plates and added with 10 μL CCK-8 reagent (Dojindo, Kumamoto, Japan) at the designated time points (0, 24, 48 and 72 h). Ultimately, the absorbance was determined with a SPECTROstar nano spectrophotometer (Germany) at a wavelength of 450 nm.

Colony formation assay

SKOV-3 cells (1×10^3 cells/well) were seeded in a 6-well plate for 1 week. After, the cells were fixed with 4% formaldehyde and GIMSA staining was performed. Ultimately, colonies (≥100 μm) were counted with a microscope (Olympus, Tokyo, Japan).

Flow Cytometry

After transfection, SKOV-3 cells were detached with 0.25% trypsin, and mixed with 100 μL binding buffer to

prepare 1×10^6 cells/mL suspension. Then 5 μL Annexin V-fluorescein isothiocyanate and propidium iodide were added in sequence, and cells were loaded on a CytoFLE S flow cytometer for data analysis (Xu *et al*., 2021).

Transwell analysis

SKOV-3 cell invasion and migration were assessed by Transwell chamber (8 μm well, Corning Inc. Corning, NY, USA). The upper chamber was covered with 6×10^3 cells in 0.1 mL serum-free medium, and the lower chamber with a medium covering 20% fetal calf serum. Matrigel (356234, Millipore, Burlington, MA, USA) was coated only for invasion. After 12 h, 1% crystal violet staining (Sigma-Aldrich, St. Louis, MO, USA) was performed on the cells in the lower chamber, followed by cell counting under a light microscope (Yu *et al*., 2021).

The luciferase activity assay

SNHG4 and TMED5 wild-type sequences (SNHG4/TMED5-WT) and corresponding mutant sequences (SNHG4/TMED5-MUT) containing the miR-98-5p binding site were synthesized by RiboBio. These sequences were inserted into the psiCHECK2 reporter vector (Promega, WI, USA). The SNHG4-WT/MUT reporter vector was co-transfected with in-miR-98-5p or in-NC into

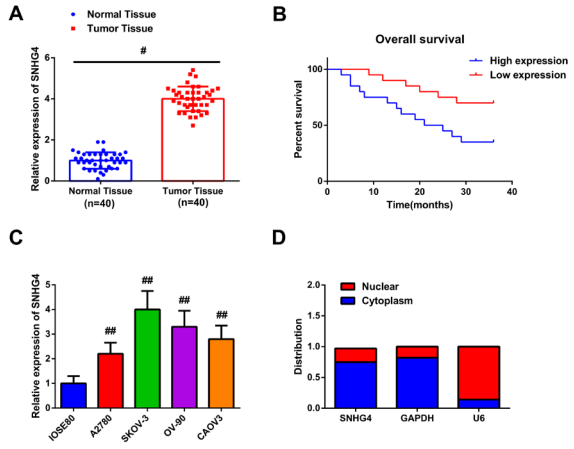

Figure 1. SNHG4 is elevated in OC and is linked with unpleasing clinical features
(**A**) RT-qPCR detection of SNHG4 in normal and tumor tissues; (**B**) Kaplan-Meier analysis of OC patients' survival prognosis; (**C**) RT-qPCR test of SNHG4 in OC cells; (**D**) SNHG4 localization in SKOV-3 cells; Measurement data were in the form of mean ± S.D.; $^{\#}$*vs*. the Normal Tissue, n=40, $P<0.05$; $^{\#\#}$*vs*. the IOSE80, N=3, $P<0.05$.

Table 2. Association of SNHG4 with clinicopathological characteristics in OC patients

Characteristic	Cases	SNHG4		P
	n = 40	Reduction (n = 20)	Elevation (n = 20)	
Age (year)				
60 or less	19	10	9	0.915
More than 60	21	10	11	
Tumor size				
Less than 3 cm	21	16	5	0.005*
3 cm or more	19	4	15	
FIGO[a] stage				
I/II	15	11	4	0.003*
II/IV	25	9	16	
Pathologic type				
Serous	31	18	13	0.268
Mucous and others[b]	9	2	7	
Lymph node metastasis				
Positive	29	11	18	0.001*
Negative	11	9	2	
Distant metastasis				
Positive	14	9	5	0.158
Negative	26	11	15	

FIGO[a]: Federation Internationale of Gynecologie and Obstetrigue. Others[b]: Endometrioid carcinoma, clear cell carcinoma and undifferentiated OC.

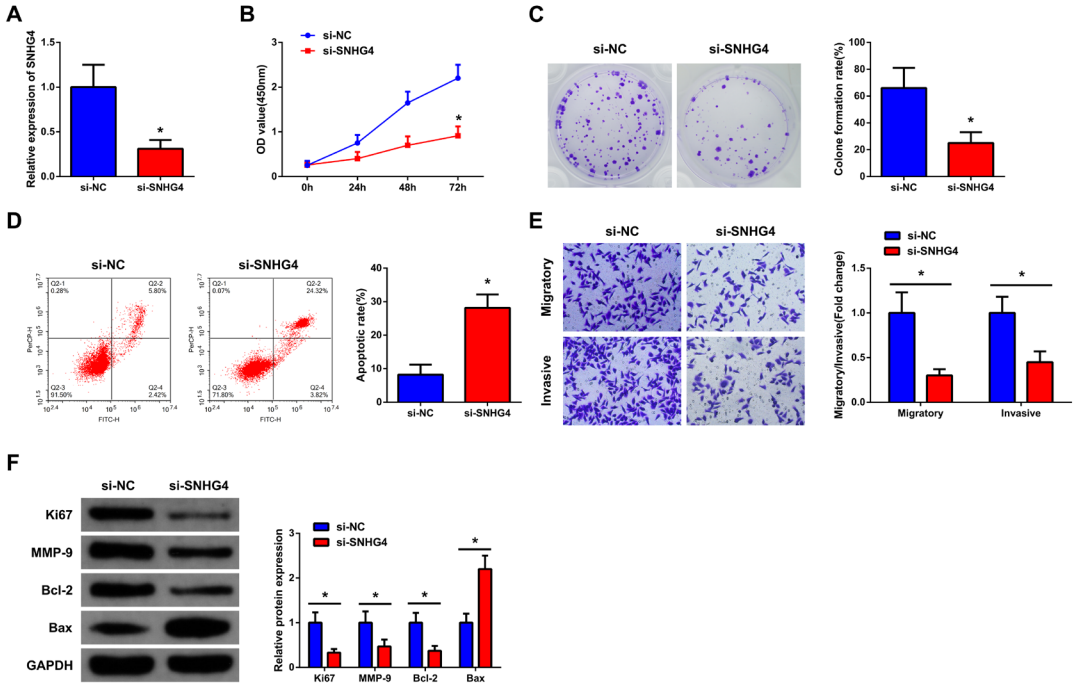

Figure 2. Silenced SNHG4 gene is available to constrain OC's deterioration
(**A**) RT-qPCR test of transfection efficiency in SKOV-3 cells; (**B–C**) CCK-8 method and plate cloning experiment examination of cell proliferation ability; (**D**) Flow cytometry detection of cell apoptosis rate; (**E**) Transwell test of cell migration and invasion; (**F**) Western blot examination of Ki67 (proliferation marker), MMP-9 (invasion marker), Bcl-2 (anti-apoptotic molecule) and Bax (pro-apoptotic molecule). Measurement data were in the form of mean ± S.D.*vs.* the si-NC, N=3, $P<0.05$.

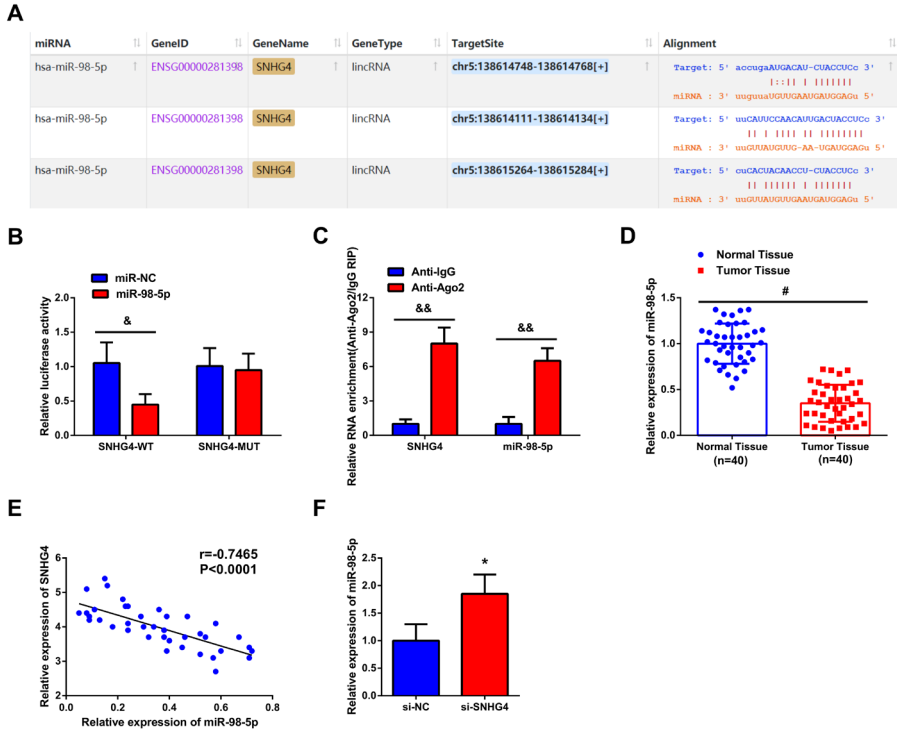

Figure 3. SNHG4 performs like a sponge for miR-98-5p
(**A**) The bioinformatics website prediction of the binding site of SNHG4 with miR-98-5p; (**B–C**) The luciferase activity assay and RIP experiment evaluation of the interaction of SNHG4 with miR-98-5p; (**D**) RT-qPCR detection of miR-98-5p in each tissue; (**E**) Pearson association analysis assessment of the relevance of miR-98-5p with SNHG4; (**F**) RT-qPCR test of miR-98-5p in SKOV-3 cells. Measurement data were in the form of mean ± S.D.; $^{\#}$*vs.* the Normal Tissue, n=40, $P<0.05$; $^{\&}$*vs.* the miR-NC, $^{\&\&}$*vs.* the Anti-IgG, *vs. the si-NC, N=3, $P<0.05$.

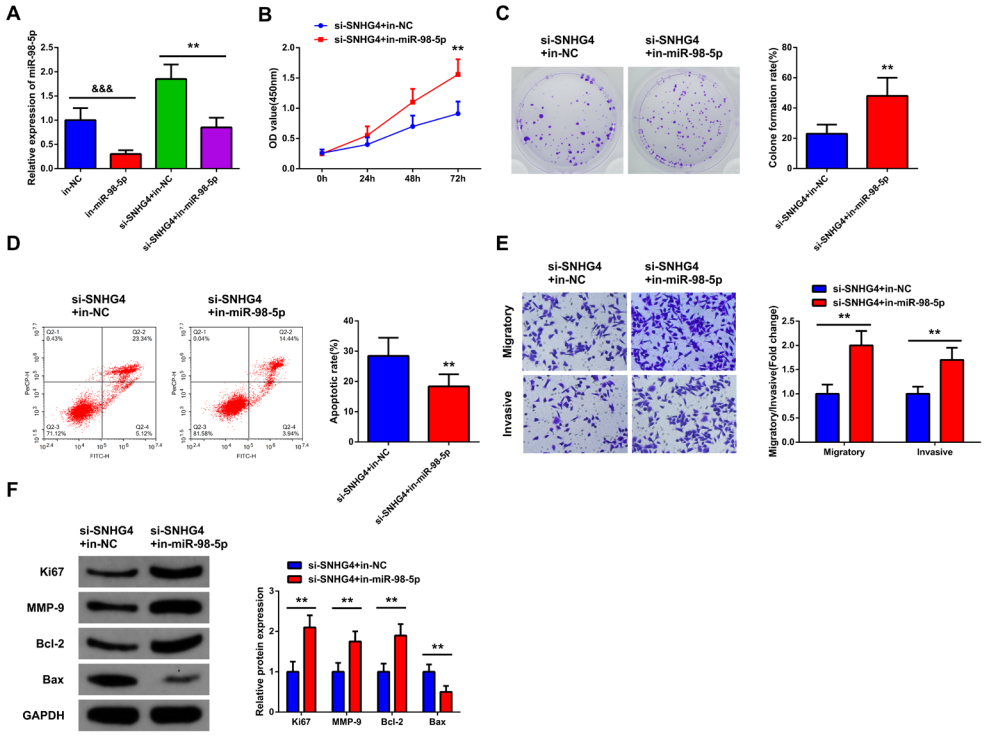

Figure 4. SNHG4 impacts OC cell progression via targeting miR-98-5p
(**A**) RT-qPCR examination of different transfections' efficiency in SKOV-3 cells; (**B–C**) CCK-8 and plate cloning experiments tested cell proliferation ability; (**D**) Flow cytometry detection of cell apoptosis rate; (**E**) Transwell examination of cell migration and invasion; (**F**) Western blot test of Ki67 (proliferation marker), MMP-9 (invasion marker), Bcl-2 (anti-apoptotic molecule) and Bax (pro-apoptotic molecule). The measurement data were in the form of mean ± S.D. &&&*vs*. the in-NC, ********vs*. the si-SNHG4 + in-NC, N=3, $P<0.05$.

SKOV-3 cells. After transfection of 48 h, an assessment of the luciferase activity was done using the luciferase reporter test kit (Promega) (Xing, An, & Chen, 2021).

RNA immunoprecipitation (RIP) detection

RIP was implemented using Magna RIP kit (Millipore). The cells were lysed with complete RIP lysis buffer and combined with magnetic beads. Subsequently, the magnetic beads were combined with human anti-ago2 or normal mouse immunoglobulin G (Millipore). After digestion with proteinase K, immunoprecipitated RNA was analyzed by RT-qPCR (Liu *et al.*, 2021).

Statistical analysis

The data were analyzed by SPSS 21.0 (SPSS, Inc, Chicago, IL, USA) statistical software. After Kolmogorov-Smirnov test, the data were normally distributed, and the results were manifested in the form of mean ± standard deviation (S.D.). Two-group comparison was done using *t*-test and multiple-group comparison was performed with one-way analysis of variance (ANOVA) and Fisher's least significant difference *t*-test. *P* was a two-sided test, and $P<0.05$ was accepted as indicative of distinct differences.

RESULTS

SNHG4 is elevated in OC and associated with adverse clinical characteristics

SNHG4 has been shown to be highly expressed in various cancers. The expression of SNHG4 was assessed in OC. As presented in Fig. 1A, SNHG4 expression in OC tissues was higher than that in normal tissues. Clinical analysis indicated that SNHG4 expression was linked with tumor size, lymph node metastasis and tumor node metastasis (TNM) staging, but had no link with age, distant metastasis and pathological type (Table 2). Additionally, survival analysis (Kaplan-Meier) elaborated that OC patients with high expression of SNHG4 had poor overall survival (OS) rates (Fig. 1B). SNHG4 expression in all human OC cell lines (A2780, SKOV-3, OV-90 and CAOV3) was higher in the normal human ovarian epithelial cell line (IOSE80) (Fig. 1C). Nucleocytoplasmic separation experiments also confirmed that SNHG4 was mainly expressed in the cytoplasm of SKOV-3 cells (Fig. 1D).

To sum up, SNHG4 was elevated in OC and might be implicated in OC progression.

Silenced SNHG4 gene is available to restrain OC's deterioration

Since SNHG4 has the highest expression level in SKOV-3 cells, the SKOV-3 cell line was selected for subsequent functional experiments. To explore the function of SNHG4 in ovarian cancer, an SNHG4 knockdown cell model was constructed. The transfection efficiency was affirmed by RT-qPCR, as manifested in Fig. 2A, si-SNHG4 restrained SNHG4 expression in SKOV-3 cells. Silenced SNHG4 constrained the SKOV-3 cell proliferation (Fig. 2B–C). After restraining SNHG4, SKOV-3 cell apoptosis was promoted and migration and invasion were suppressed (Fig. 2D, E). Meanwhile, knockdown of SNHG4 suppressed the expression of Ki67, MMP-9, Bcl-2 proteins and increased

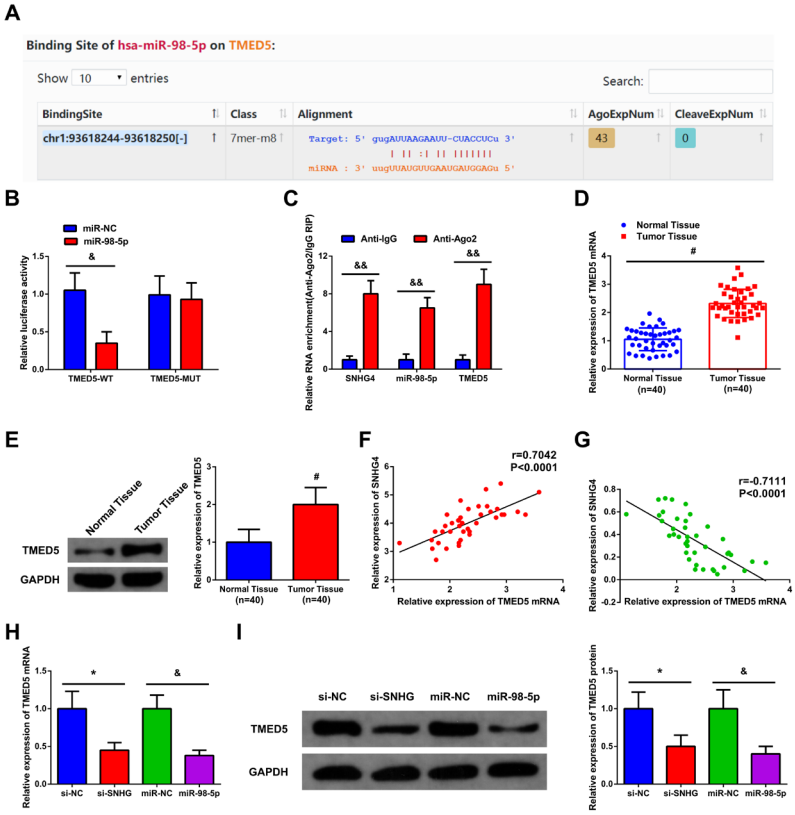

Figure 5. MiR-98-5p immediately targets TMED5
(**A**) The bioinformatics website predicted the binding site of TMED5 with miR-98-5p; (**B–C**) The luciferase activity assay and RIP experiment evaluation of the interaction of TMED5 with miR-98-5p; (**D–E**) RT-qPCR and Western blot test of TMED5 in OC tumor tissues; (**F–G**) Pearson association analysis of TMED5 with miR-98-5p and SNHG4; (**H–I**) RT-qPCR and Western blot detection of TMED5 in SKOV-3. Measurement data were in the form of mean ± S.D.; $^{\#}$*vs.* the Normal Tissue, n=40, $P<0.05$; $^{\&}$*vs.* the miR-NC, $^{\&\&}$*vs.* the Anti-IgG, **vs.* the si-NC, N=3, $P<0.05$.

the expression of Bax protein (Fig. 2F). These data suggest that knockdown of SNHG4 suppresses the biological behaviors of OC.

SNHG4 performs like a sponge for miR-98-5p

To investigate the novel mechanism by which SNHG4 regulates OC progression, the bioinformatics website https://starbase.sysu.edu.cn was utilized to predict potential miRNAs of SNHG4. miR-98-5p was found to have a potential binding site for SNHG4 (Fig. 3A). The interaction between miR-98-5p and SNHG4 was verified by dual-luciferase reporter gene assay. As shown in Fig. 3B, overexpression of miR-98-5p significantly inhibited the luciferase activity of SNHG4-WT, but not SNHG4-MUT. RIP assay also further confirmed that both SNHG4 and miR-98-5p were enriched in Ago2 magnetic beads (Fig. 3C). Additionally, miR-98-5p expression was downregulated in OC tumor tissues (Fig. 3D) and was negatively linked with SNHG4 expression (Fig.3E). RT-qPCR data showed that inhibition of SNHG4 promoted the expression of miR-98-5p in SKOV-3 cells (JIP 3F). In short, SNHG4 targeted miR-98-5p.

SNHG4 impacts OC cell progression via targeting miR-98-5p

To explore whether miR-98-5p is involved in the regulation of SNHG4 in OC, a functional rescue experiment was performed. si-SNHG4 and miR-98-5p inhibitor were co-transfected into SKOV-3 cells. RT-qPCR showed that knockdown of SNHG4 promoted the expression of miR-98-5p, which was reversed by miR-98-5p inhibitor (Fig. 4A). Functional experiments showed that knockdown of SNHG4 inhibited cell proliferation, invasion and migration, and promoted apoptosis, but these effects were reversed by knockdown of miR-98-5p (Fig. 4B–F). In brief, SNHG4 impacts OC cell progression *via* targeting miR-98-5p.

MiR-98-5p immediately targets TMED5

Subsequently, potential downstream mRNAs of miR-98-5p were explored. First, miR-98-5p was found to have potential binding sites with TMED5 by starBase prediction (Fig. 5A). Meanwhile, dual-luciferase reporter assays revealed that miR-98-5p overexpression could attenuate the luciferase activity of TMED5-WT, while the luciferase activity of TMED5-MUT was not significantly changed (Fig. 5B). Additionally, SNHG4, miR-98-5p and TMED5 are all enriched in RNA-induced silencing complexes immunoprecipitated by Ago2 antibody (Fig. 5C). TMED5 expression in OC tumor tissues was higher than in normal tissues (Fig. 5D–E) and positively associated with SNHG4 while negatively linked with miR-98-5p (Fig. 5F–G). Additionally, in SKOV-3 cells depleted of SNHG4 or restored of miR-98-5p, TMED5 expression was suppressed (Fig. 5H–I). In general, TMED5 was a downstream gene of miR-98-5p.

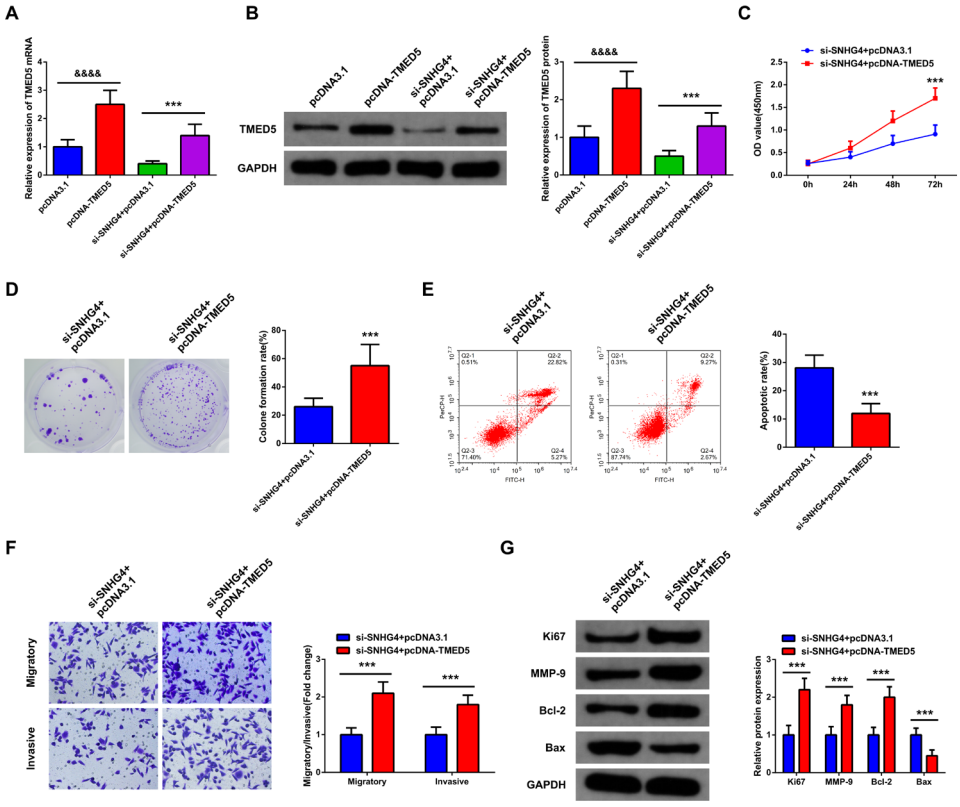

Figure 6. SNHG4 modulates OC's advancement *via* targeting miR-98-5p/TMED5 axis
(**A–B**) RT-qPCR and western blot test of transfection efficiency in SKOV-3 cells; (**C–D**) CCK-8 and plate cloning experiments detection of cell proliferation ability; (**E**) Flow cytometry examination of cell apoptosis; (**F**) Transwell detection of cell migration and invasion; (**G**) Western blot examination of Ki67 (proliferation marker), MMP-9 (invasion marker), Bcl-2 (anti-apoptotic molecule) and Bax (pro-apoptotic molecule). Measurement data were in the form of mean ± S.D.; &&&&*vs*. the pcDNA3.1, ****vs*. the si-SNHG4 + pcDNA-3.1, N=3, $P<0.05$.

SNHG4 modulates OC progression *via* targeting the miR-98-5p/TMED5 axis

To assess whether SNHG4 is involved in ovarian cancer progression through miR-98-5p-regulated TMED5, a functional rescue assay was performed. In SKOV-3 cells, transfection of pcDNA-TMED5 elevated TMED5 expression, while TMED5 expression was partially upregulated after pcDNA-TMED5 blocked si-SNHG4 (Fig. 6A–B). As manifested in Fig. 6C–E, upregulation of TMED5 reversed the proliferation-inhibiting and apoptosis-promoting effects of SNHG4 silencing on SKOV-3 cells. At the same time, transfection with overexpressing TMED5 plasmid also reversed the inhibitory effect of si-SNHG4-induced migration and invasion of SKOV-3 cells (Fig. 6F). Additionally, Ki67, MMP-9 and Bcl-2 protein expression were augmented, while Bax protein expression was inhibited after pcDNA-TMED5 blocked si-SNHG4 (Fig. 6G). In brief, SNHG4 modulates OC progression *via* targeting the miR-98-5p/TMED5 axis.

Wnt/β-Catenin pathway participates in SNHG4/miR-98-5p/TMED5 axis-mediated OC cell growth

Wnt/β-Catenin pathway may lead to uncontrolled cell growth. Therefore, we further investigated whether SNHG4 regulates the downstream target of miR-98-5p, TMED5, to alter the Wnt/β-catenin signaling pathway in OC cells. Western blot results showed that the expression of Gsk3β was decreased, while the expressions of Wnt3a and β-catenin were upregulated in SKOV-3 cells transfected with pcDNA-TMED5 (Fig. 7A).

To determine whether the Wnt/β-catenin signaling pathway is involved in SNHG4-induced OC progression, the Wnt/β-catenin-specific inhibitor XAV939 (10 μM) was added to pcDNA-TMED5-treated SKOV-3 cells. XAV939 treatment increased Gsk3β expression in pcDNA-TMED5-treated SKOV-3 cells, while Wnt3a and β-catenin protein expression decreased (Fig. 7B). In addition, XAV939 also inhibited the effect of pcDNA-TMED5 on the promotion of proliferation and inhibition of apoptosis in SKOV-3 cells (Fig. 7C/D/E). Similarly, XAV939 inhibited the promoting effect of pcDNA-TMED5 on SKOV-3 cell migration and invasion (Fig. 7F). More importantly, Western blot results showed that the expression of Ki67, MMP-9, and Bcl-2 proteins decreased, while the expression of Bax protein increased (Fig. 7G). In short, SNHG4 activated the Wnt/β-Catenin pathway by targeting the miR-98-5p/TMED5 axis.

DISCUSSION

Some factors like late diagnosis, chemotherapy resistance rate and easy recurrence lead to unpleasing prognosis and high mortality rate in OC patients (Wu *et al.*, 2021). Novel evidence has illuminated that targeting aberrantly-expressed lncRNAs in OC may be latent biomarkers and curative targets for OC (Guo *et al.*, 2021). Additionally, the lncRNA-microRNA-mRNA regulatory

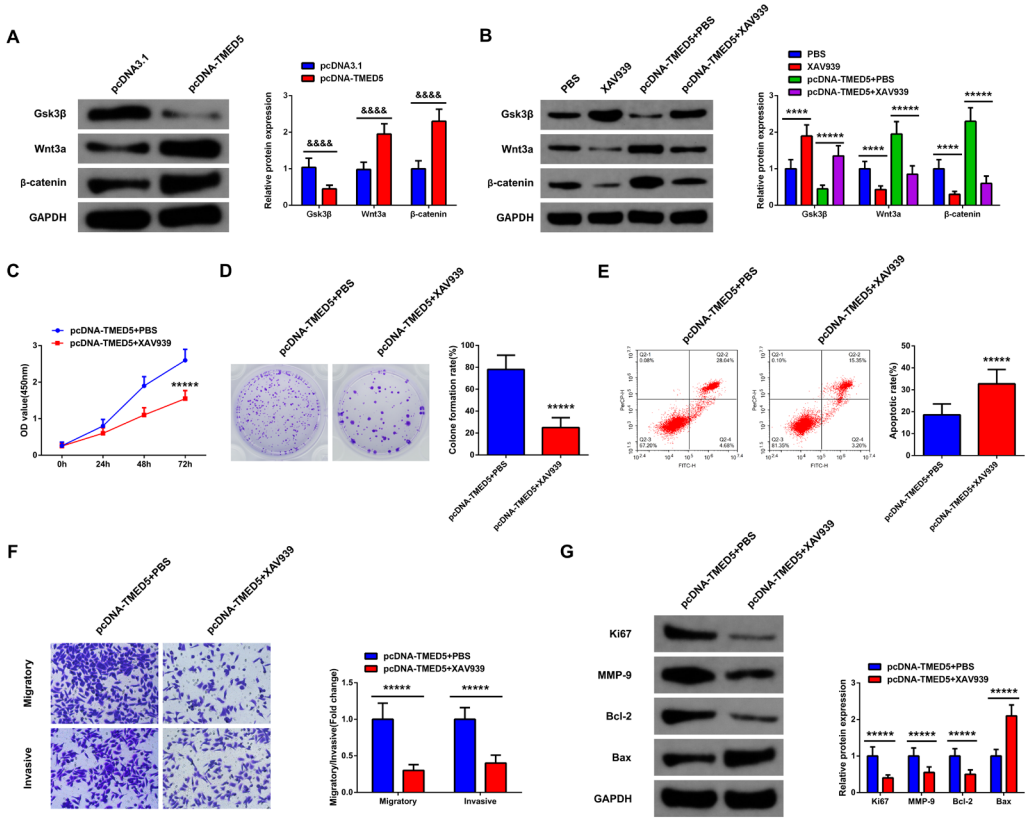

Figure 7. Wnt/β-Catenin pathway implicates in the SNHG4/miR-98-5p/TMED5 axis-mediated OC cells' deterioration
(**A–B**) Western blot test of Gsk3β, Wnt3a and β-catenin; (**C–D**) CCK-8 and plate cloning experiments examination of cell proliferation ability; (**E**) Flow cytometry test of cell apoptosis; (**F**) Transwell examination of cell migration and invasion; (**G**) Western blot test of Ki67 (proliferation marker), MMP-9 (invasion marker), Bcl-2 (anti-apoptotic molecule) and Bax (pro-apoptotic molecule). Measurement data were in the form of mean ± S.D.; &&&&*vs*. the pcDNA3.1, ****vs*. the PBS, *****vs*. the pcDNA-TMED5 + PBS, N=3, P<0.05.

network offers novel perspective for brand-new therapy strategies for OC (Lin *et al*., 2021).

As reported, SNHG4 is elevated in OS (Xu *et al*., 2018), NSCLC (Li *et al*., 2021), LC (Jiao *et al*., 2020), CC (Ji *et al*., 2019). SNHG4 has been considered a biomarker for cancer prognosis or diagnosis. Likewise, our work found the upregulated SNHG4 in OC tissues was associated with patients' pathological characteristics and prognosis. Accumulated evidence has elaborated that SNHG4 exerts a regulatory role in cancer cells with multiple biological functions like chemotherapy resistance (Chu *et al*., 2021). For instance, in colorectal cancer (CRC), suppression of SNHG4 constrains CRC cell growth with immune escape (Zhou *et al*., 2021). In CC, silenced SNHG4 distinctly restrains CC cell progression (Li *et al*., 2019). As expected, in this study, suppressing SNHG4 was available to constrain OC cell development (HeLa), clarifying that SNHG4 performed as an oncogene in OC.

In terms of mechanism, lncRNAs, as ceRNAs, elevate mRNA expression *via* interacting with miRNAs (Xu *et al*., 2021; Xie *et al*., 2021). It is speculated that SNHG4 may act as a ceRNA to regulate genes, thereby promoting the malignant phenotype of OC cells. In this research, miR-98-5p was confirmed as SNHG4's downstream miRNA in OC. As reported, miR-98-5p is negatively mediated by SNHG4 in LC (Tang *et al*., 2019). Additionally, a report has illuminated that miR-98-5p expression is reduced in OC, and its silencing is available to alle*via*te OC cell development with cisplatin resistance (Guo *et al*., 2021). Likewise, in this research, miR-98-5p was downregulated in OC tissues, and SNHG4 combined with miR-98-5p in OC cells. Besides, silencing miR-98-5p rescued the effects of silenced SNHG4 on OC cells.

In this research, miR-98-5p immediately targeted TMED5's 3'UTR in OC, leading to degradation of TMED5 and repression of its transcription. TMED5, appertaining to the TMED protein family, is implicated in vesicular transport of proteins. As reported, TMED5 expression is elevated in CC and boosts CC cell malignant behaviors (Zou *et al*., 2021). In this research, TMED5 was augmented in OC tissues, while SNHG4 was available to positively modulate TMED5 *via* miR-98-5p. Additionally, elevated TMED5 rescued silenced SNHG4-mediated repression on OC cell development.

Wnt/β-Catenin, the crucial oncogenic pathway, exerts in multiple cancer progression covering OC and OS (Gao *et al*., 2021; Guo *et al*., 2021). Numerous studies have clarified that the Wnt/β-Catenin pathway is linked with OC development with chemotherapy resistance (Vallée *et al*., 2021). For instance, it has been reported that miR-217 constrains the Wnt/β-Catenin pathway activation, thereby suppressing OC cell progression with drug resistance (Liu & Zhao, 2021). LncRNA HCP5 stimulates OC cell development with epithelial-mesenchymal transition (EMT) *via* the miR-525-5p/PRC1/Wnt/β-Catenin axis (Wang *et al*., 2020). TMED5 is available to activate the Wnt/β-Catenin pathway in CC (Yang *et al*., 2019). As expected, in this study, TMED5 declined Gsk3β expression, while enhanced Wnt3a and β-catenin

expression, illuminating that TMED5 activated the Wnt/β-Catenin pathway in OC. Additionally, SNHG4 activated the Wnt/β-Catenin pathway *via* targeting the miR-98-5p/TMED5 axis, thereby boosting OC cell development. We speculate that SNHG4/miR-98-5p/TMED5 may also be involved in the chemoresistance of OC, which may affect the drug resistance of OC by regulating the Wnt/β-catenin pathway. Notably, although we demonstrated *in vitro* that SNHG4 can regulate the biological behavior of the SKOV-3 cell line, more cell lines are needed to validate the role of SNHG4 in OC. In addition, it is unclear whether SNHG4 has similar effects in animal models of OC, which needs to be explored in follow-up studies. SNHG4 may target a variety of molecules in OC, including miRNA and mRNA, and it is necessary to further refine the regulatory network of SNHG4 in the future.

In conclusion, this work demonstrates for the first time that SNHG4 is highly expressed in OC and reveals the role of SNHG4 as a tumor-promoting factor in OC by regulating the miR-98-5p/TMED5 axis. The SNHG4/miR-98-5p/TMED5 axis may serve as a potential therapeutic target for OC in the future.

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