miR-1299/NOTCH3/TUG1 feedback loop contributes to the malignant proliferation of ovarian cancer

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Abstract. Recent studies have revealed the oncogenic role of notch reporter 3 (NOTCH3) in ovarian cancer (OC). However, the possible regulators and mechanisms underlying notch receptor 3 (NOTCH3)-mediated behaviors in OC remain to be completely investigated. In the present study, we aimed to identify regulators of NOTCH3 and their interactions underlying the pathogenesis of OC. Bioinformatics analysis and luciferase reporter assay were used to identify potential regulatory miRNAs and lncRNAs of NOTCH3 in OC. Several in vivo and in vitro assays were performed to evaluate their effects on the proliferative ability mediated by NOTCH3. We identified microRNA-1299 (miR-1299) as a novel negative regulator of NOTCH3. miR-1299 was downregulated in OC and was found to be considerably correlated with tumor differentia-tion. Upregulation of miR-1299 inhibited cell proliferation, colony formation, and 5-ethynyl-2'-deoxyuridine (EdU) incorporation, as well as induced cell cycle arrest in the G0G1 phase in OC cells. Overexpression of miR-1299 in xenograft mouse models suppressed tumor growth in vivo. The lncRNA taurine upregulated gene 1 (TUG1), acting as a sponge of miR-1299, was found to upregulate NOTCH3 expression and promote cell proliferation in OC through the competing endogenous RNA mechanism. In addition, TUG1 was found to be a potential downstream target of NOTCH3, forming a miR-1299/NOTCH3/TUG1 feedback loop in the development of OC. Collectively, our findings improve the understanding of NOTCH3-mediated regulation in OC pathogenesis and facilitate the development of miRNA- and lncRNA-directed diagnostics and therapeutics against this disease.

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Introduction

Ovarian cancer (OC) continues to be one of the most lethal malignant tumors among women worldwide. In the United States, OC accounts for only 2.5% of all malignancies among females but for 5% of cancer deaths, making it the fifth leading cause of cancer-related deaths in females (1,2). In China, the mortality rate of OC is ranked tenth among females throughout the country (3). The low survival rates in OC are largely driven by a late-stage diagnosis and a high rate of tumor recurrence. Although most OC patients have no evidence of disease after first treatment, approximately 19% of early OC patients and 60 to 85% of advanced OC patients experience a relapse, and recurrent OC after treatment is almost incurable (4-6). Current treatment for OC is not confined merely to surgery and clas-sical chemotherapy. Targeted therapy, such as polyADP ribose polymerase (PARP) inhibitors, as well as immunotherapy, are promising treatments (7-10).

The Notch signaling pathway is an important system that regulates cell proliferation, differentiation, and apoptosis. Integrated genomic analyses revealed that Notch is the main signaling pathway involved in the pathophysiology of OC (11,12). The expression of notch reporter 3 (NOTCH3) was found to be elevated in OC and closely correlated with the clinical stage, pathological grade, lymph node metastasis, drug-resistant recurrence, and survival rate of OC patients (13-15). NOTCH3 promotes the malignant progression of OC by enhancing the proliferation of tumor cells, maintaining their stemness, and resisting apoptosis (16,17). A better understanding of the tumor-specific regulation of NOTCH3 is crucial and may contribute to targeted therapy for OC. However, limited information regarding the molecules that regulate the expression of NOTCH3 is available.

Approximately 70% of the human genome can be transcribed, while less than 2% of the genome encodes proteins, generating thousands of non-coding transcripts (ncRNAs) (18,19). Among them, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are key regulators of gene expression at the post-transcriptional level. miRNAs are single-stranded non-coding small RNAs with a length of 17-22 nt. They are destabilizers and repressors of translation of mRNAs and regulate diverse biological functions, including proliferation, migration, apoptosis, angiogenesis, and metabolic progression in various cancers, including OC (20-24). lncRNAs are ncRNAs that are longer than 200 nt. They are implicated in multiple cancers and play critical roles in tumor initiation and progression (25-28). Recent studies have revealed that lncRNAs can act as competing endogenous RNA (ceRNA) or miRNA 'sponges' to modulate crucial genes in cancer (29-32), providing a feasible way to understand the regulatory mechanisms underlying cancer pathogenesis and identify novel diagnostic biomarkers or potential therapeutic candidates.

Given the significant oncogenic role of NOTCH3 in OC, we aimed to determine the functional significance of NOTCH3-mediated regulatory ncRNAs in OC. In this study, we found that microRNA-1299 (miR-1299) was a novel negative regulator of NOTCH3 expression in OC with clinical significance. Downregulation of miR-1299 promoted the malignant progression of OC by tumor cell proliferation. The lncRNA taurine upregulated gene 1 (TUG1) was found to function as a ceRNA, regulating NOTCH3 expression by sponging miR-1299 in OC. TUG1 was also identified as a potential downstream target of NOTCH3 and formed a miR-1299/NOTCH3/TUG1 feedback loop. Our results elucidate the regulatory mechanism underlying NOTCH3 expression in OC and provide potential therapeutic targets for the anticancer therapy of OC.

Materials and methods

Cell lines/cell culture. The human OC cell lines A2780, CAOV3, and SKOV3 were purchased from the National Infrastructure of Cell Line Resources in China (Beijing, China). Cells were cultured in DMEM or RPMI-1640 medium with 10% FBS (Thermo Fisher Scientific, Inc.) and incubated at 37°C with 5% CO_2. To block Notch signaling in OC cells, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT, #HY-13027; MedChem Express) dissolved in DMSO was added in the medium.

Clinical samples. Thirty-five epithelial serous OC and 16 pathologically confirmed normal ovary tissues were collected from patients (medium age 54 years; range 30-72 years and medium age 51 years; range 35-64 years, respectively) undergoing surgery at the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China) from July 2018 to June 2019. All OC cases were diagnosed by pathological evaluation and had complete clinical information. All tissue samples were immediately frozen in liquid nitrogen after resection from patients and stored at -80°C for RNA extraction. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committee of Peking Union Medical College Cancer Hospital (Beijing, China) (grant no: NCC2017G-115). Written informed consent was obtained from all of the subjects.

The OC samples were divided into high and low expression of miR-1299 and NOTCH3 groups by the cut-off value, which was defined as the cohort median.

Oligonucleotide transfection, plasmid construction, and lentiviral infection. miR-1299 mimics, inhibitors, and scramble miRNA controls were purchased from GenePharma (Shanghai, China). The lentivirus-containing short hairpin RNA (shRNA) targeting TUG1 or NOTCH3, and the plasmid expressing the active intracellular domain of NOTCH3 (NICD3) were purchased from GeneChem (Shanghai, China). The wild-type and mutated 3'UTR sequence of NOTCH3 was cloned into the pmirGLO vector (Promega) to construct recombinant plasmids named pmirGLO-NOTCH3-WT and pmirGLO-NOTCH3-MUT. Sequences of lncRNA TUG1 and XIST containing the predictive binding sites of miR-1299 and containing point mutations at the site region were cloned into the pmirGLO vector and were named pmirGLO-TUG1-WT, pmirGLO-XIST-WT, and pmirGLO-TUG1-MUT. OC cells were transfected with oligonucleotides using Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific, Inc.) and plasmids using TurboFect™ transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

RNA extraction and RT-qPCR. Total RNA was extracted from OC tissues and cultured cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. mRNA and lncRNA levels were quantified with RT-qPCR using the SYBR Premix Ex Taq reverse transcription PCR kit (Takara, China), and β-actin was used as an internal control. miRNA was quantified with RT-qPCR using the hairpin-it™ microRNA and U6 snRNA normalization RT-PCR quantitation kit (GenePharma). Each assay was carried out in triplicates in a Light Cycler 480 Instrument (Roche), and the relative expression of mRNA and miRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (33). The primers for RT-qPCR are shown in Table SI.

Cell cycle, proliferation, and colony formation assays. For cell cycle analysis, cells were collected and adjusted to $1x10^6$/ml 48 h after transfection. Ethanol (70%) was used to fix the cells, and RNase A (100 μg/ml) was used to remove RNAs. Finally,

cells were stained with propidium iodide (PI) (50 μg/ml) at room temperature for 30 min and analyzed using LSRII flow cytometry (BD Biosciences). Cell viability was detected using the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were seeded in 96-well plates at 3,000 cells/well. The proliferative ability of cells was determined at 0, 24, 48, 72 and 96 h by measuring the absorbance values at a wavelength of 450 nm. For colony formation assay, OC cells (800 cells per well) were seeded into 6-well plates 24 h after transfection. After 10 days of incubation, the cells were fixed in methanol and stained with 0.1% crystal violet. The colonies were counted using a GBOX F3 gel documentation system (Syngene, Cambridge, UK).

Immunofluorescence (IF) assay. A total of 10,000 cells were seeded on glass coverslips in 24-well plates and grown overnight. For the EdU incorporation assay, 10 μM EdU was used to treat the cells, and an EdU cell proliferation kit (Sangon) was used for measurement. Cellular nuclei were stained using 5 μg/ml Hoechst 33342 at 26°C for 30 min. Images were observed under a DMI 4000 confocal laser scanning microscope at x200 magnification (Leica, Frankfurt, Germany).

Luciferase reporter assay. Cells were seeded in 96-well plates at 1.5x10^4 cells/well. When the cells reached 60% confluence, vectors containing the wild-type or mutant 3'UTR of NOTCH3 and wild-type or mutant binding site sequence of TUG1 were co-transfected with miR-1299 mimics and scramble miRNA using Turbofect (Thermo Fisher Scientific, Inc.). Forty-eight hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega) and expressed as the ratio of firefly and *Renilla* luciferase activities.

Western blotting. Proteins were extracted from cells or tissues using RIPA lysis buffer (Solarbio) with protease inhibitors and phosphatase inhibitors. A total of 30 μg proteins were loaded per lane, separated on 10% SDS-PAGE gels, and blotted on polyvinylidene difluoride membrane. After being blocked with 5% skim milk for 2 h at room temperature, the membranes were incubated with primary antibodies (dilution 1:1,000) overnight at 4°C. The primary antibodies used in this study were NOTCH3 antibody (product code ab23426; Abcam) and GAPDH (cat. no. 2118; Cell Signaling Technology, Inc.). Anti-mouse IgG, peroxidase-linked antibody was used as secondary antibody (dilution 1:2,500; cat. no. 5174; Cell Signaling Technology, Inc.) and was incubated at room temperature for 1 h. The blots were detected using a chemiluminescence kit (cat. no. 34577; Thermo Fisher Scientific, Inc.) and imaged using MiniChemi 610 system (Sage Creation Science, Co., Ltd.).

In vivo animal experiments. Eight four-week-old female BALB/c nude mice (body weight range, 17.1-18.2 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and randomly divided into two groups. The mice were housed with filtered air, 12 h light/dark cycle, constant temperature (25°C) and had free access to sterilized food and water. After 24-h transfection, 2x10^6 cells containing miR-1299 or NC agomir were infected into the right armpit of the mice. miR-1299 agomir or NC agomir (RiboBio) was directly injected into the implanted tumor at the dose of 2 nmol/30 μl phosphate-buffered saline (PBS) per mouse every 6 days for 6 times. Tumor growth was monitored by measuring the tumor volume (V) every 6 days with a Vernier caliper and calculated as: V=length x width^2/2. All the mice were anesthetized with sodium pentobarbital (500 mg/kg, intraperitoneally) and sacrificed by cervical vertebra dislocation on day 42 or when the tumor volume reached the threshold of 1,500 mm^3, and tumors were weighed and snap-frozen for protein and RNA extraction.

Animal experiments were conducted with the approval of the Animal Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing (ACC2019A060) and in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health.

Statistical analysis. SPSS software version 25.0 (IBM Corp.) and GraphPad Prism 8 (GraphPad Software, Inc.) were used for statistical analysis. Each experiment was performed at least in triplicate, and numerical data are expressed as means ± SD. The difference in clinicopathological features between two groups was determined by independent-sample Student's t-test or Mann-Whitney U test for continuous variables and the Chi-square test or Fisher exact test for categorical variables. One-way ANOVA analysis with Tukey post hoc test was used for comparisons among multiple groups. Pearson's correlation analysis was used to examine the relationship between two gene expression levels. A value of $P<0.05$ was indicative of statistical significance.

Results

miR-1299 is a negative regulator of NOTCH3 in OC with clinical significance. To investigate the potential miRNAs that regulate NOTCH3, we firstly performed a bioinformatics analysis using the miRWalk database (34), which integrated predicted gene-miRNA target information from 13 databases and compared the results with miRNAs reported to be significantly downregulated in previously published miRNA profiles (NCBI/GEO/GSE47841) (35) (Table SII). The two screening methods overlapped on only 11 miRNAs among which miR-1299 had the highest score in miRWalk. Therefore, we focused our subsequent analysis on miR-1299 as a putative regulator of NOTCH3 in OC. We measured the level of mature miR-1299 and NOTCH3 in 35 fresh OC tissues and 16 normal ovarian tissues, as well as in four OC cell lines. miR-1299 expression was significantly downregulated in the OC tissues compared with that in the normal tissue ($P<0.05$, Fig. 1A). There was an inverse correlation between the level of miR-1299 and NOTCH3 both in OC tissues (Fig. 1B) and in OC cell lines (Fig. 1C).

To confirm the regulation of miR-1299 on NOTCH3 expression, we first transfected miR-1299 mimics and NC scramble into miR-1299 low-expressing cell A2780 and CAOV3. RT-qPCR results showed that NOTCH3 and its pathway genes were largely reduced in the A2780
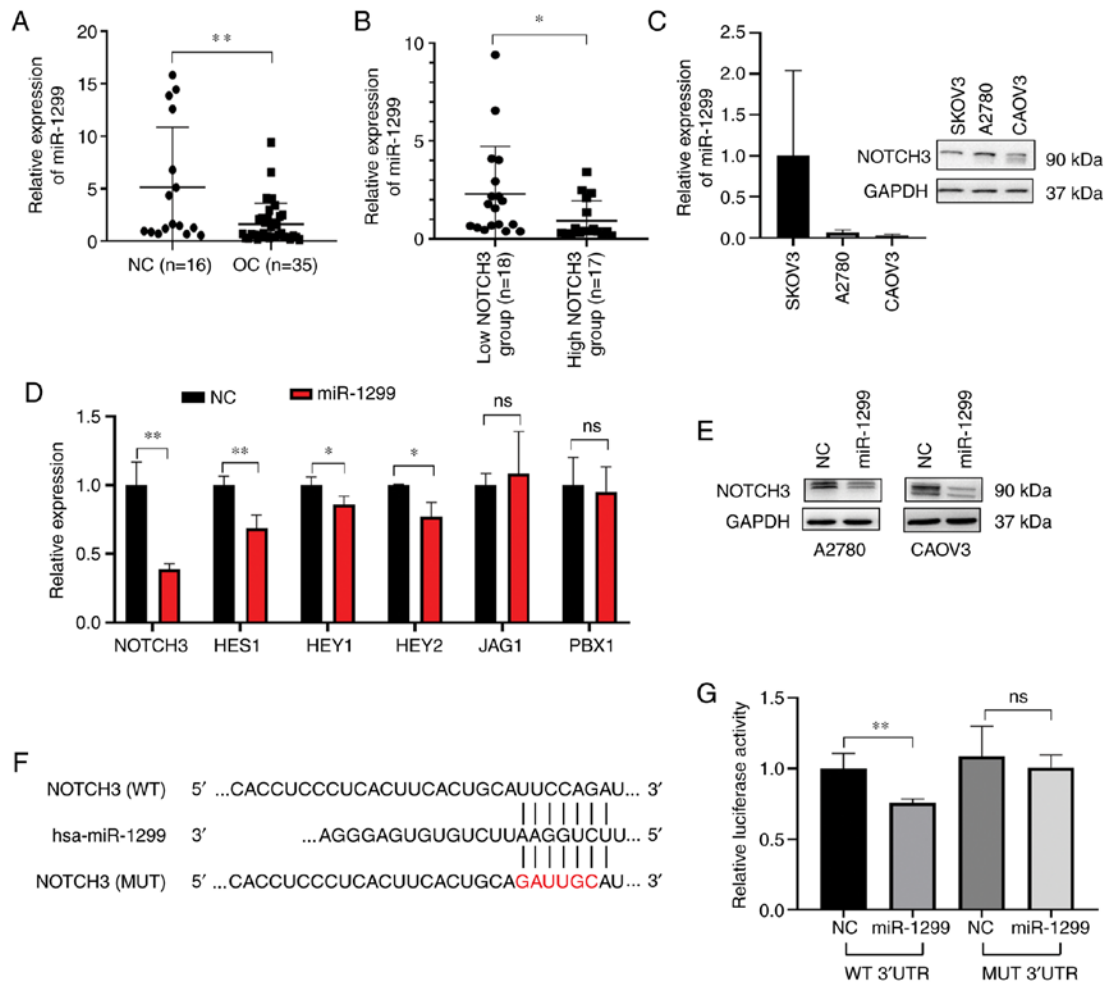
Figure 1. miR-1299 is downregulated in OC and acts as a negative regulator of NOTCH3. (A) Analysis of miR-1299 expression in OC and normal ovary tissues (NC) using RT-qPCR. (B) miR-1299 expression was significantly lower in OC tissues with high NOTCH3 expression compared to those with low NOTCH3 expression. (C) Correlation between miR-1299 and NOTCH3 levels in OC cell lines. (D) RT-qPCR analysis of NOTCH3 pathway genes after transfection of miR-1299 mimics in A2780 cells. (E) Western blot analysis of NOTCH3 after transfection of miR-1299 mimics in A2780 and CAOV3 cell lines. (F) Putative miR-1299 binding sequence in NOTCH3 3'UTR (untranslated region) (WT) and the mutated 3'UTR sequence (MUT). (G) Relative luciferase activity of reporter plasmids carrying wild-type (WT) or mutant (MUT) NOTCH3 3'UTR in A2780 cells co-transfected with NC or miR-1299 mimics. Means ± SD are shown. Statistical analysis was conducted using Student's t-test. *P<0.05 and **P<0.01; ns, not significant. OC, ovarian cancer; NOTCH3, notch receptor 3; HES1, hairy and enhancer of split-1; HEY1, Hes related family BHLH transcription factor with YRPW motif 1; HEY 2, Hes related family BHLH transcription factor with YRPW motif 2; JAG1, Jagged canonical Notch ligand 1; PBX1, Pre-B-cell leukemia transcription factor 1.

(Fig. 1D) and CAOV3 cells (Fig. S1) when transfected with miR-1299 mimics, and western blotting results confirmed the decreased protein level of NOTCH3 (Fig. 1E). In turn, when we knocked down NOTCH3 or overexpressed NICD3 in OC cells, we observed a subsequent increase or decrease in the miR-1299 level (Fig. S2). Furthermore, we cloned wild-type 3'UTR sequence of NOTCH3 and 3'UTR containing point mutations in the putative binding sites into a luciferase reporter plasmid (pmirGLO-NOTCH3-WT and pmirGLO-NOTCH3-MUT) (Fig. 1F). Both plasmids were co-transfected with miR-1299 mimics or NC mimics separately in A2780 cells. The results showed that overexpression of miR-1299 suppressed luciferase activity significantly in cells transfected with pmirGLO-NOTCH3-WT plasmid but not in those transfected with pmirGLO-NOTCH3-mutant vectors (Fig. 1G). These observations suggested that miR-1299 bound directly to the predicted binding sites in the NOTCH3 3'UTR region and negatively regulated NOTCH3 expression.

The association of miR-1299 level and clinicopathological features was also analyzed in OC patients (Table I). Low expression of miR-1299 was significantly correlated with low tumor differentiation. Decreased miR-1299 level was associated with younger age, advanced tumor stage, and necessity to receive neoadjuvant chemotherapy before surgery; however, the association was not statistically significant because of the limited number of cases. Thus, we considered miR-1299 as a regulator of NOTCH3 and hypothesized that decreased expression of miR-1299 promoted OC progression and development.

miR-1299 inhibits OC cell proliferation, colony formation, and cell cycle in vitro. Subsequently, we investigated the role of miR-1299 in OC by transfecting miR-1299 mimics in OC cell lines A2780 and CAOV3 (Fig. 2A), both of which had an endogenous low miR-1299 expression. Overexpression of miR-1299 in OC cells significantly reduced cell proliferation, as observed with a CCK-8 assay compared to cells transfected with scrambled miRNA (NC mimic) (Fig. 2B). Colony forma-

Table I. Association between miR-1299 levels and clinicopathological characteristics of the OC patients.

Characteristics	miR-1299 expression		P-value
	High expression (n=17)	Low expression (n=18)	
Mean age (years)	55.2±2.8	52.9±1.8	0.121
Tumor stage			
I-II	3	0	0.057
III	13	13	
IV	1	5	
Tumor differentiation			**0.045**
High/median	4	0	
Low	13	18	
Nodal metastasis			0.305
No	12	9	
Yes	5	9	
Neoadjuvant chemotherapy			0.060
No	15	10	
Yes	2	8	
Serum CA125 level at initial diagnosis	534.1 (108.0, 1,362.0)	1017.3 (197.4, 1,676.0)	0.211

High and low expression of miR-1299 was determined by the cut-off value for miR-1299, which was defined as the cohort median. OC, ovarian cancer; CA125, cancer antigen 125. Significant P-values (P<0.05) are indicated in bold print.

tion assay showed that treatment with miR-1299 mimics decreased the number of colonies formed by OC cell lines (Fig. 2C). EdU incorporation assay revealed that miR-1299 inhibited DNA synthesis in cell proliferation (Fig. 2D). Additionally, cell cycle analysis showed that transfection of miR-1299 mimics significantly blocked the cells in the G0/G1 phase, accompanied by a smaller population in S and G2 phases (Fig. 2E).

miR-1299 suppresses tumor growth in vivo. We further examined the impact of miR-1299 on OC in a xenograft nude mouse model. A2780 cells transfected with miR-1299 or NC mimics were implanted subcutaneously in BALB/c nude mice. Starting on day 8 post-implantation, miR-1299 or NC mimics were injected intratumorally every 6 days for 6 times. *In vivo* tumor growth was evaluated by measuring tumor volume and final weight. We observed that treatment with miR-1299 mimics significantly inhibited tumor growth *in vivo*. The tumor volume and weight were significantly lower in the miR-1299 mimic group than in the NC group (Fig. 3A-C), substantiating the tumor suppressor function of miR-1299 in OC tumorigenesis. Moreover, decreased NOTCH3 protein level in the xenograft tissues with overexpression of miR-1299 was further confirmed (Fig. 3D).

lncRNA TUG1 functions as a sponge of miR-1299 and promotes cell proliferation in OC. To investigate the potential regulators of NOTCH3 by ceRNA mechanism, we first performed a comprehensive bioinformatics analysis using predicted miR-1299-targeted lncRNAs from DIANA databases (36), and OC-relevant lncRNAs from Starbase

database (37) and lncRNA disease database (38) (Fig. 4A). The three-screening data overlapped on only two lncRNAs, TUG1 and XIST (X-inactive specific transcript). Therefore, we constructed luciferase reporter plasmids containing the two highest scoring putative miR-1299 binding sites in TUG1 (pmirGLO-TUG1-WT1 and pmirGLO-TUG1-WT2) and XIST (pmirGLO-XIST-WT1 and pmirGLO-XIST-WT2). After co-transfecting the plasmids with miR-1299 mimics or NC mimics in A2780, we found that overexpression of miR-1299 suppressed luciferase activity significantly in two wild-type plasmids of TUG1 (Fig. 4B and C) but not in those of XIST (Fig. S3). We also constructed plasmids carrying point mutations in either binding site of TUG1 (pmirGLO-TUG1-MUT1 and pmirGLO-TUG1-MUT2), and no significant changes in luciferase activity was observed in cells transfected with the two mutant plasmids (Fig. 4B and C). RT-qPCR further revealed that miR-1299 could be upregulated in OC cells after knockdown of TUG1 by lentiviral shRNA particles (Fig. 4D and E). Taken together, these results indicated that the lncRNA TUG1 was a direct sponge of miR-1299 in OC.

We next evaluated the potential tumorigenicity of TUG1 in OC. As expected, downregulation of TUG1 by shRNA resulted in the inhibition of OC cells in terms of cell proliferation, colony formation, EdU incorporation, and cell cycle, which could be partially rescued by inhibition of miR-1299 (Fig. 4F-I). Moreover, knockdown of TUG1 reduced NOTCH3 expression in OC cells, and miR-1299 inhibitors partially rescued the NOTCH3 level (Fig. 4J). This suggested that TUG1, acting as a ceRNA, affects NOTCH3 expression and promotes cell proliferation by sponging miR-1299.
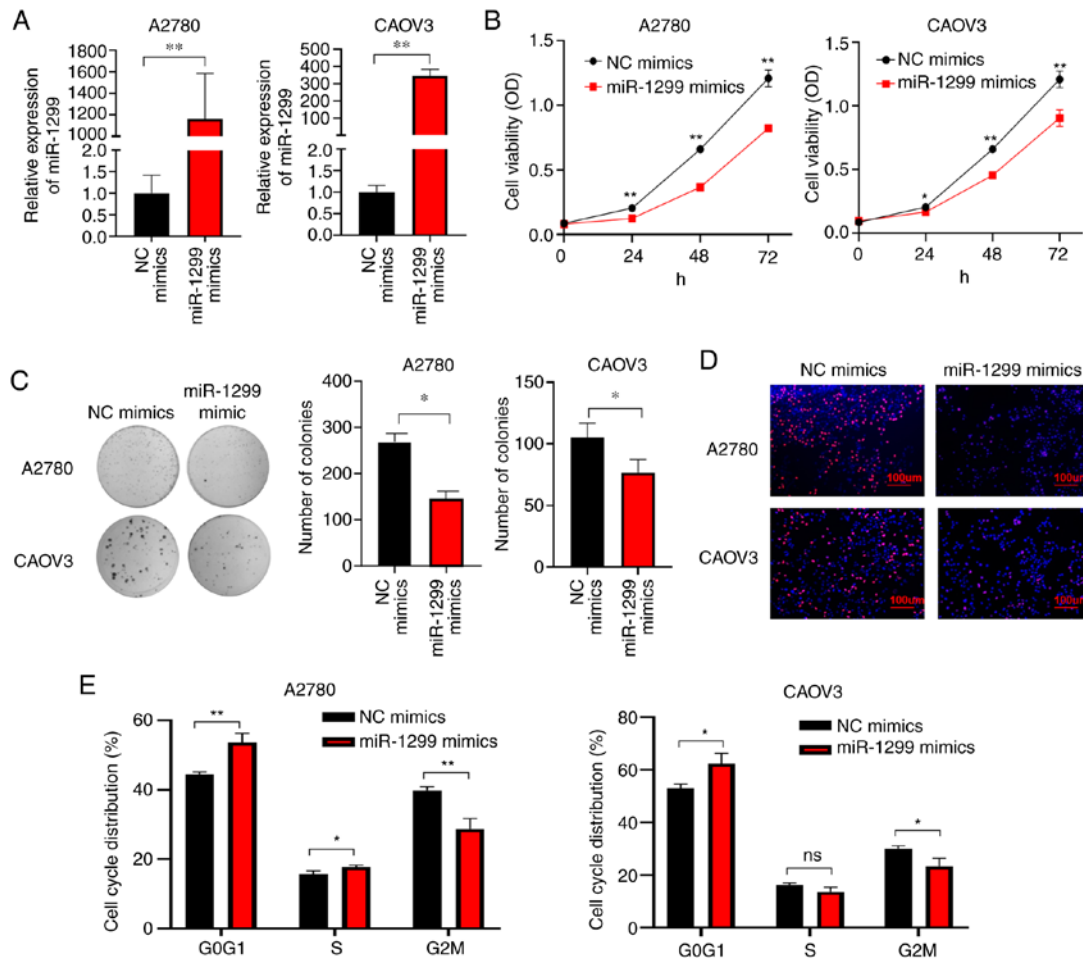
Figure 2. Upregulation of miR-1299 inhibits cell proliferation, colony formation, EdU incorporation, and cell cycle in OC A2780 and CAOV3 cell lines. (A) RT-qPCR analysis of miR-1299 expression level in OC cells after transfection of NC or miR-1299 mimics for 48 h. (B) Cell proliferation of OC cells after transfection of NC or miR-1299 mimics by CCK-8 assay. (C) Representative photographs and quantifications of the colony formation assay after transfection of NC or miR-1299 mimics. (D) Representative photographs of EdU incorporation assay in OC cells after transfection of NC or miR-1299 mimics. (E) Cell cycle distribution of OC cells after transfection of NC or miR-1299 mimics by flow cytometry. Means ± SD are shown. Statistical analysis was conducted using Student's t-test. *P<0.05 and **P<0.01, miR-1299 mimic compared with the NC mimic; ns, not significant; OC, ovarian cancer.
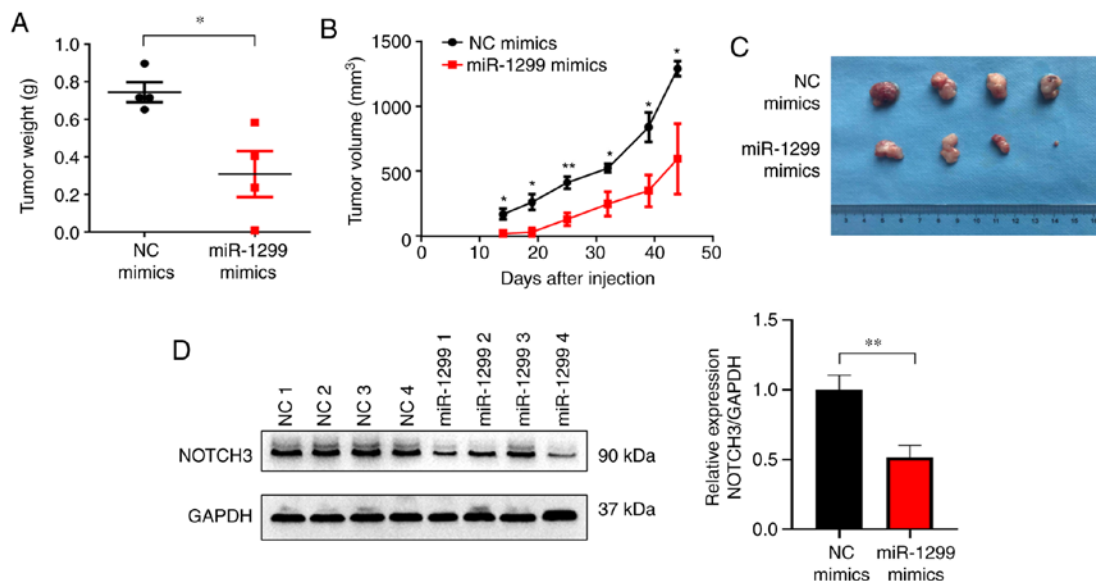

Figure 3. Ectopic miR-1299 suppresses OC tumor growth *in vivo*. (A) Tumor weight and (B) tumor volume in the xenograft mouse model. (C) Photographs of dissected tumors treated with NC or miR-1299 mimics. (D) Quantification of NOTCH3 protein in tumors treated with NC or miR-1299 mimics by western blotting. Means ± SD are shown. Statistical analysis was conducted using Student's t-test. *P<0.05 and **P<0.01, miR-1299 mimic compared with the NC mimic. OC, ovarian cancer; NOTCH3, notch receptor 3.
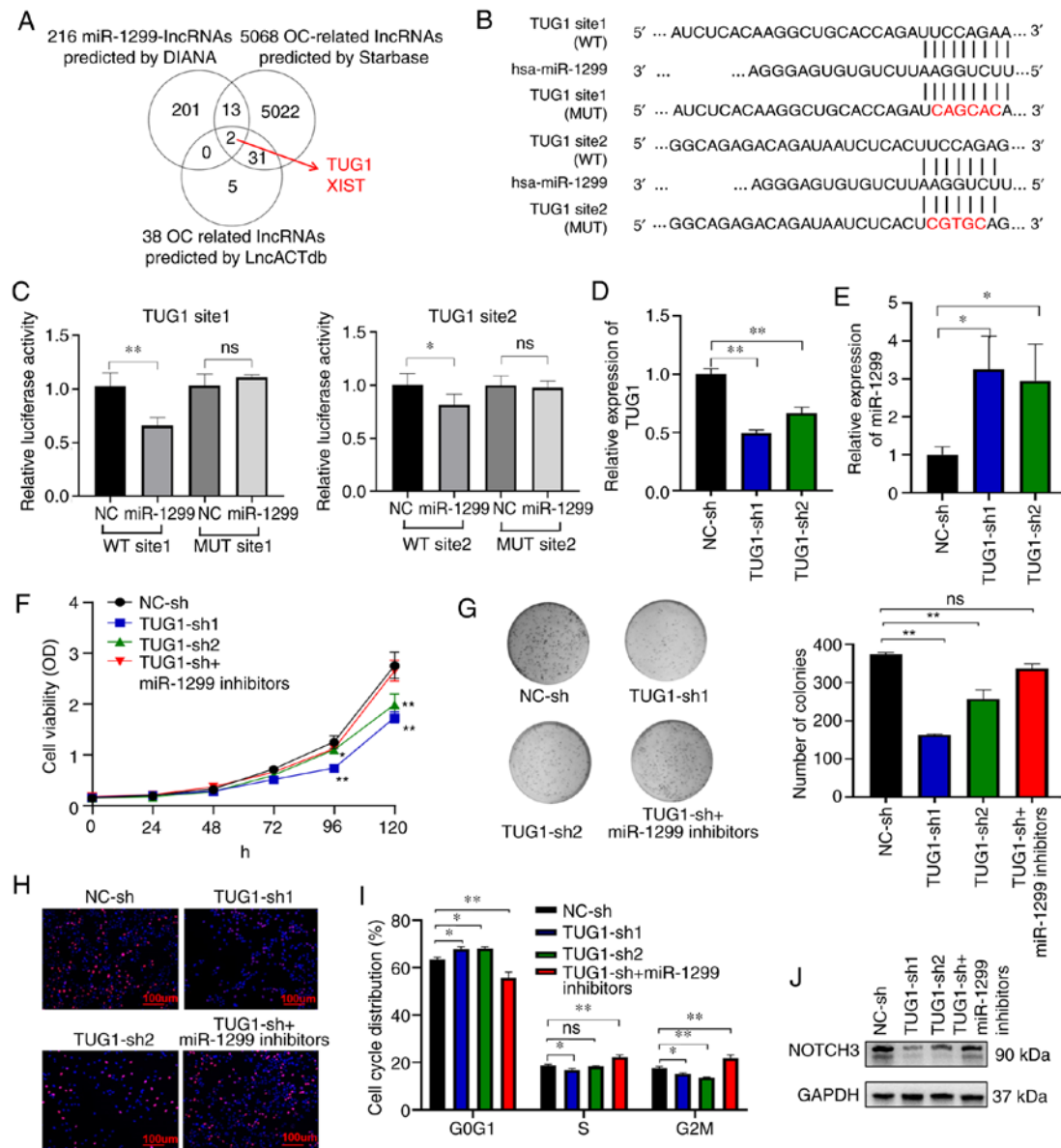
Figure 4. lncRNA TUG1 functions as a sponge of miR-1299 and promotes cell proliferation in OC. (A) Screening methods for regulatory lncRNAs of miR-1299 in OC. (B) The two highest scoring putative miR-1299 binding sites in the TUG1 sequence (WT) and the point mutations in either binding site (MUT). (C) Relative luciferase activity of reporter plasmids carrying wild-type (WT) or mutant (MUT) TUG1 binding sites in A2780 cells co-transfected with NC or miR-1299 mimics. (D) Confirmation of TUG1 knockdown in A2780 cells transfected with TUG1 shRNA by RT-qPCR analysis. (E) RT-qPCR analysis of miR-1299 level in A2780 cells transfected with TUG1 shRNA or scrambled shRNA. Results of the (F) cell proliferation, (G) colony formation, (H) EdU incorporation assays, and (I) cell cycle analysis of A2780 cells transfected with NC-sh, TUG1-sh1, TUG1-sh2, and TUG1-sh+miR-1299 inhibitors. (J) Representative blot image of NOTCH3 protein level in A2780 cells transfected with NC-sh, TUG1-sh1, TUG1-sh2, and TUG1-sh+miR-1299 inhibitors by western blotting. Means ± SD are shown. Statistical analysis was conducted using Student's t-test and one-way ANOVA with Tukey post hoc test. $^*P<0.05$ and $^{**}P<0.01$; ns, not significant; OC, ovarian cancer; NOTCH3, notch receptor 3; TUG1, lncRNA taurine upregulated gene 1.

TUG1 is a potential target of NOTCH3 and forms a miR-1299/NOTCH3/TUG1 feedback loop. In the NOTCH signaling pathway, after the cleavage of γ-secretase, the intracellular domain of NOTCH3 protein (NICD3) is translocated to the nucleus and binds the transcription factor complex RBP Jκ (39). When we analyzed the transcriptional start site (TSS, -2 kb to +1 kb) of TUG1 in the JASPAR CORE database (40), we found 5 RBP Jκ binding motifs around its TSS (Fig. 5A), indicating that TUG1 may be directly regulated by NOTCH3. Additionally, there was a significant positive correlation between the expression of TUG1 and NOTCH3 both in our tissues and in the TCGA database (41) (Fig. 5B and C). When we treated A2780 cells with

DAPT, an inhibitor of γ-secretase, that can block the NOTCH signaling pathway without interference in NOTCH3 mRNA activity, we found a subsequent decrease in TUG1 expression in a concentration gradient manner (Fig. 5D). Therefore, we considered TUG1 as a potential downstream target of NOTCH3, and that there may exist a miR-1299/NOTCH3/TUG1 feedback loop in the development of OC (Fig. 5E).

Discussion

Recently, miR-1299 was found to be downregulated in several types of cancer and to act as a tumor suppressor. Zhu *et al* (42)
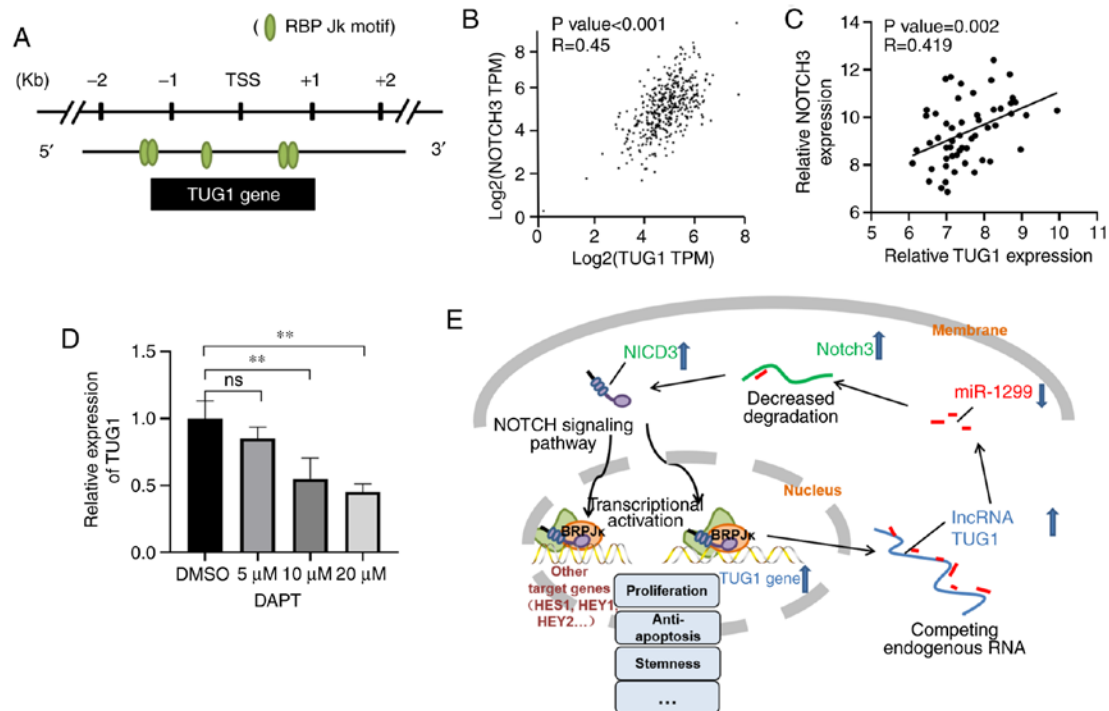
Figure 5. TUG1 is a potential downstream target of NOTCH3. (A) Schematic diagram showing RBP Jκ motifs around the transcriptional start site (TSS) of the TUG1 gene. (B and C) TUG1 expression was inversely correlated with NOTCH3 mRNA in fresh OC tissues (B) and in the TCGA database (C). (D) RT-qPCR analysis of TUG1 level in A2780 cells treated with DAPT. (E) Diagrams showing the miR-1299/NOTCH3/TUG1 feedback loop in the development of OC. Statistical analysis was conducted using one-way ANOVA with Tukey post hoc test and Pearson's correlation analysis. [**]$P<0.01$; ns, not significant; OC, ovarian cancer; NOTCH3, notch receptor 3; TUG1, lncRNA taurine upregulated gene 1; NICD3, NOTCH3 intracellular domain; HES1, hairy and enhancer of split-1; HEY1, Hes related family BHLH transcription factor with YRPW motif 1; HEY 2, Hes related family BHLH transcription factor with YRPW motif 2.

showed that in hepatocellular cancer, miR-1299 overexpression inhibited cell proliferation and arrested the cell cycle in the G0/G1 phase, while miR-1299 knockdown promoted cell proliferation and accelerated G1/S transition. In breast cancer, miR-1299 was found to inhibit CDK6 expression and to bind to its 3'UTR region, suppressing cell proliferation and migration ability (43). A similar tumor suppressive role of miR-1299 was found in esophageal squamous cell carcinoma (44), prostate cancer (45), triple-negative breast cancer (46), and cholangiocarcinoma (47). In this study, we demonstrated that miR-1299 was significantly decreased in OC tissues and was associated with clinical features, and overexpression of miR-1299 inhibited tumor growth both *in vitro* and *in vivo*, suggesting that miR-1299 also acted as a tumor suppressor in ovarian cancer (OC). In addition, we first demonstrated that miR-1299 was a negative regulator of NOTCH3, a vital oncogene in OC. Transcriptome analysis showed that suppression of NOTCH signaling in ovarian and breast cancer cells led to downregulation of genes in pathways involved in cell-cycle regulation (48). Both NOTCH3 siRNA and pathway inhibitors caused cell cycle arrest by reducing cyclin D1 and cyclin D3 levels while elevating p21 and p27 levels. In this study, we observed a similar cellular phenotype and protein change (data not shown) after transfection of miR-1299. However, there is also a limitation to our study as we used miR-1299 mimics to transfect two cell lines, and did not use inhibitors in the functional experiments considering the low expression of miR-1299 in OC.

The lncRNA taurine upregulated gene 1 (TUG1), a 7.1-kb lncRNA, was first discovered in a genomic screen for genes upregulated by taurine treatment in mouse retinal cells (49).

Recently, TUG1 was characterized as a new oncogene and found to be upregulated in multiple human cancers (50-56). TUG1 was upregulated in OC tissues and cells and was positively correlated with advanced disease and poor prognosis. Knockdown of TUG1 significantly inhibited cell proliferation and epithelial-mesenchymal transition (EMT) and induced cell apoptosis in OC (56-58). The biological functions of lncRNAs largely rely on their distinct subcellular localization. Cytoplasmic lncRNAs can regulate mRNA stability or translation by acting as sponges for miRNAs (59). TUG1 localizes both in the nucleus and the cytoplasm, indicating that it could have a multi-oncogenic role. Using bioinformatics analyses and luciferase reporter assays, our study provided the first evidence that TUG1 directly bound to and inhibited miR-1299 expression. Knockdown of TUG1 was found to inhibit cell proliferation, colony formation, and cell cycle progression, which could be partially rescued by inhibition of miR-1299. Our observations were consistent with previous studies and confirmed the involvement of TUG1 in the ceRNA mechanism. Notably, there have been several other miRNAs negatively regulated by TUG1 in cancers (60-62), among which quite a few miRNAs have been reported to have a tumor suppressive role in OC (63-65). TUG1 could sponge more than one miRNA in OC and affect the development of OC through different signaling pathways.

Interestingly, we found that TUG1 possessed five RBP Jk motifs around its promoter region, which can be bound by the NOTCH intracellular domain and trigger transcriptional activation by classical NOTCH signaling pathway. Additionally, treatment with DAPT, an inhibitor of γ-secretase, in OC cells decreased the TUG1 level in a concentration gradient-depen-

dent manner. Therefore, we hypothesized that there could be a miR-1299/NOTCH3/TUG1 feedback loop in OC development. Downregulation of miR-1299 in OC upregulated NOTCH3 level, and overexpression of NOTCH3 activated the expression of various oncogenes by the NOTCH signaling pathway, including the downstream molecule TUG1. In addition, overexpression of TUG1 was a sponge for miR-1299, triggering a positive feedback in tumorigenesis. A recent study in glioma showed that TUG1 was a NOTCH-regulated lncRNA (66), which confirmed our hypothesis. However, we have provided preliminary evidence on this feedback loop hypothesis. The direct regulation of TUG1 by NOTCH3 at the molecular level requires further experimental evidence such as RIP or RNA pulldown results. Therefore, further confirmation of the miR-1299/NOTCH3/TUG1 feedback loop, as well as its role in other important issues, including stemness and drug-resistance in OC, will be the main aim of our future work.

To date, the possible regulators and mechanisms underlying NOTCH3-mediated regulatory behaviors in OC have not been completely elucidated. Our study showed that miR-1299 is a novel negative regulator of NOTCH3 and is downregulated in OC. Overexpression of miR-1299 was found to play a tumor suppressor role both *in vitro* and *in vivo* by partially inhibiting cell proliferation. lncRNA TUG1 acted as a sponge for miR-1299 and promoted cell proliferation by upregulating NOTCH3. TUG1 was also a potential target of NOTCH3, forming a miR-1299/NOTCH3/TUG1 feedback loop in OC cells. Our findings improve the understanding of OC pathogenesis and facilitate the development of miRNA- and lncRNA-targeted diagnostics and therapeutics against this disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP, DZ and WC designed the research and analyzed the data. YP, KL and XL performed the cell function experiments. YP and XD performed the animal experiments. YW, WW and NL collected the OC and normal ovary tissues and reviewed the clinical features. YP prepared the images and drafted the original manuscript. WC and DZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing [grant no: NCC2017G-115], and informed consent was obtained from all participating patients. Animal experiments were conducted with the approval of the Animal Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing (ACC2019A060) and in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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