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MiR-188-5p inhibits cell proliferation and migration in ovarian cancer via competing for CCND2 with ELAVL1

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ABSTRACT

MicroRNAs (miRNAs) were reportedly demonstrated to participate in ovarian cancer (OC) progression. Here, we inquired into the role of miR-188-5punderneath OC cell proliferation and migration. In this respect, our work examined the miR-188-5p expression and demonstrated its expression level in OC by qRT-PCR analysis. Enforced miR-188-5p expression resulted in a serious downfall of cell growth and mobility, and acceleration apoptosis in OC cells. Furthermore, we identified CCND2 as a target gene of miR-188-5p. RIP assay and luciferase reporter assay verified the interaction of miR-188-5p and CCND2 and exhibited that miR-188-5p greatly hindered the expression of CCND2. Besides, HuR stabilized CCND2 mRNA and counteracted the miR-188-5p suppressive effect on CCND2 mRNA. Functionally, rescue experiments also showed that miR-188-5p-mediated suppression on OC cell proliferation and migration was reverted by CCND2 or HuR overexpression. Our study found miR-188-5p was a tumor suppressor in OC via competing for CCND2 with ELAVL1, contributing to coming up with novel clues for OC therapies.

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Introduction

Ovarian cancer (OC) ranks fifth in common cancer attacking women with low survival rates and a high mortality rate throughout the world (1, 2). In spite of improvements in surgery and chemotherapy, the clinical outcome is way less than satisfactory (3). Therefore, it is imperative to deepen the understanding of pathogenesis and the molecular mechanisms in ovarian cancer for clinical application.

Abundant previous studies elucidated that microRNAs (miRNAs) represent short single-stranded non-coding RNAs, and decreased gene expression through hybridizing to target messenger RNAs (mRNAs) at complementary sites (4). As reported, the abnormally dysregulated miRNAs were strongly correlated with tumorigenesis including ovarian cancer. For example, miR-1287-5p decreased PI3K and then inhibited breast cancer (BRCA) growth (5). MicroRNA34a overexpression inhibits myeloid leukemia cell migration (6). microRNA-4319 over-expression decreased prostate cancer (PRAD) growth via down-regulated Her-2 (7). miR-188-5P could inhibit tumor growth in PRAD, gastric cancer (GC), and hepatocellular carcinoma (HCC) (8-10). No further information about the miR-188-5P function in OV has been detected.

ELAV like RNA binding protein 1 (ELAVL1), as known HuR, is an essential part of RNA-binding proteins and has been elucidated to boost the expression of target genes via stabilising ARE-containing mRNAs (11-13). In addition, the co-regulation of target gene expression by HuR and miRNA in a competitive manner has been introduced in previous research. For example, miR26a/b and HuR induced post-transcriptional regulation of ERBB2 conferring tamoxifen resistance in BRCA cells (14). TNF-α mRNA stability was modulated by miR181s and HuR in sepsis-induced immunoparalysis (15). HuR also regulated miR-873/CDK3 axis to facilitate lung cancer stemness (16). As previously reported, the cytoplasmic HuR expression was associated with unsatisfactory outcomes in serous ovarian carcinoma (17, 18), which may provide convincing evidence for our study. But the correlation between HuR or miR-188-5P and mRNA has not been probed in OV.

Cyclin D2 (CCND2) serves as a putative tumor promoter in multiple cancers, such as glioblastoma (19), lung cancer (20), colorectal cancer (21) and OV (22). The present study carried out was to interrogate the miR-188-5p role and uncover its regulatory mechanism in OV and hopefully might be useful for new emerging clues conducive to OV treatment.

Materials and Methods

Cell lines and culture

Four OV cell lines (HO-8910, SKOV3, OVCAR-3, A-2780) and the counterpart non-cancerous ovarian epithelial cells IOSE80 were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RPMI1640 medium incorporating 10% calf serum was applied to maintain all five cell lines and containing 5% CO_2 at 37 ℃. Cells were collected until they adhered to the walls, and later washed with 0.25% pancreatin, and suspended.

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Cell transfection

miR-188-5P mimics and control mimics purchased from Genepharma Company (Shanghai, China) were employed. Besides, CCND2 and ELAVL1 sequences were inserted into pcDNA3.1 vectors (Invitrogen) for generating CCND2 and ELAVL1 stably overexpressing plasmids termed as pcDNA3.1/CCND2 and pcDNA3.1/ ELAVL1, SKOV3 and A-2780 cells prior to transfection were first trypsinised and then seeded onto 6-well plates until they reached approximately 70% confluency. Transfection of miR-188-5P mimics, NC-mimics, pcDNA3.1/CCND2, pcDNA3.1/ELAVL1 and empty vector was conducted and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's guide. SKOV3 and A-2780 cells were further cultured for 48 h before performing subsequent assays.

Real-time PCR (qRT-PCR)

Trizol reagent (Invitrogen, USA) was required for the extraction of the total RNA from SKOV3 and A-2780 cells. To monitor the miR-188-5P expression, the TaqMan MicroRNA Array kit (AppliedBiosystems, USA) was used for cDNA synthesis and TaqMan Universal Master Mix II (Applied Biosystems) was to detect the relative expression ofmiR-188-5P. U6 worked as the internal reference. While, the determination of CCND2 mRNA and ELAVL1 mRNA expression needed PrimeScript RT reagent kit (TaKaRa BIO, Shiga, Japan) for cDNA synthesis and SYBR Premix Ex Taq™ II (TaKaRa BIO) reagent was to measure CCND2 and ELAVL1 mRNA expression level. GAPDH served as the normalization. All qRT-PCR analysis was carried out three times on ABI Prism 7900HT Real-TimePCR System (Termo Fisher Scientifc, Inc.). MiR-188-5p primer was 5′-CATCCCTTGCATGGTGGAGGG-3′. CCND2 forward primer was 5′-TCCAAACTCAAAGAGACCAGC-3′. CCND2 reverse primer was 5′-TTCCACTTCAACTTCCCCAG-3′. ELAVL1 forward primer was 5′-ACTGCA GGGATGACATTGGGAGAA-3′. ELAVL1 reverse primer was 5′-AAGCTTTGCAGATTCAACCTCGCC-3′. GAPDH forward primer was 5′-CTCCTCCACCTTTGACGCTG-3′. GAPDH reverse primer was 5′-TCCTCTTGTGCTCTTGCTGG-3′. U6 forward primer was 5′-CCCTTCGGGGACATCCGATA-3′. U6 reverse primer was 5′-TTTGTGCGTGTCATCCTTGC-3′.

Western blot analysis

RIPA lysis (CWBIO, China) was utilized for the extraction of total protein from the cells and the extracted proteins were quantified using BCA Assay Kit (Bio-Rad, USA). Protein separation was carried out by SDS-PAGE and the proteins were transferred onto a PVDF membrane (Millipore Corporation, USA), which was subsequently blocked by 5% powdered milk for 1 h at room temperature. Afterward, the membrane underwent incubation at 4°C with an anti-GAPDH antibody (internal control) and anti-CCND2 antibody (1:1000, CST, USA) overnight followed by incubation with a secondary antibody, a goat-anti-rabbit conjugated to HRP (1:1000, CST, USA). Enhanced Chemiluminescence (ECL) was applied to develop signals.

Dual-luciferase reporter assay

First, the sequence of CCND2 3′UTR containing the predicted binding sites of miR-188-5p was amplified and named CCND2 (WT), and the amplified mutant CCND2 3′UTR termed as CCND2 (Mut). Later all sequences mentioned before were subcloned into the pGL3 (Promega, USA) plasmids for the construction of luciferase reporters, which were co-transfected with miR-188-5p mimics or NC-mimics into SKOV3 and A-2780 cells. 48 h after transfection, dual luciferase reporter assay was performed on a Synergy 2 Microplate Reader Fluorometer (BioTek) by virtue of a luciferase assay kit (Promega) as per the manufacturer's instruction. The firefly luciferase activity was calculated with renilla luciferase activity as an internal control.

RNA immunoprecipitation (RIP) assay

RIP assay using an RNA-binding protein immunoprecipitation kit (Millipore, USA) was executed. Afterward, SKOV3 and A-2780 cells were lysed with RNA lysis buffer incorporating the protease and RNase inhibitors. Cell lysates were cultured with RIP buffer supplemented with magnetic beads that were coated with either anti-Ago2 antibodies (Millipore, USA) or negative control IgG antibodies (Millipore, USA). Finally, following 2 h of incubation at 4 °C, the co-precipitated RNAs were isolated and underwent reverse transcription and PCR analysis.

CCK-8 assay

CCK-8 solution (Dojindo, Japan) was utilized for monitoring the cell proliferation potential. SKOV3 and A-2780 cells transfected with different plasmids were seeded into 96-well plates with 2×10^3 cells distributed in each well, which was later treated with cell counting kit-8 solution. Cellular viability was observed at 24 h, 48 h and 72 h and detected by measuring the absorbance at 450 nm of each well.

Caspase-3 activity assay

A Caspase-3 Fluorometric assay kit (Enzo Life Sciences, USA) was used to check Caspase-3 activity in transfected cells. Briefly, cells were incubated in lysis buffer and then centrifuged for 10 min at 4°C. Subsequently, the supernatants were harvested and cryopreserved at −70°C. The BCA Assay kit (Thermo Fisher Scientific, Inc.) was conducted for determining protein concentration. 50 μg protein samples were later incubated with Ac-DEVD-pNA overnight at 37°C. Caspase-3 relative activity at 405 nm was estimated via a microplate ELISA reader.

Transwell assay

In order to evaluate cell migration capacity, a transwell chamber (8um pore size, Corning) was included in the assay.48 h post-transfection, SKOV3 and A-2780 cells were seeded into the upper chamber (serum-free media) without being coated with 10ug/ml Matrigel. In the meantime, the lower chamber incorporated media with the addition of 10% FBS. After 48h of incubation, we wiped the cells that remained in the upper membrane, in contrast, cells that migrated to the lower chamber were immobilized by methanol and stained with 0.1% crystal violet. A microscope was to count migrated cells. At least three independent experiments were conducted.

Statistical analysis

All experiment data are presented as the means ± standard error of at least three independently performed expe-

riments and statistical analysis was executed by means of the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Two group comparisons and more than two group comparisons were drawn respectively using a Student t-test and ANOVA. There is statistical significance when a $P< 0.05$.

Results

miR-188-5p hinders cell proliferation, migration, and boosts apoptosis in OV cells

To monitor the biological function of miR-188-5P in OC cells, we examined the miR-188-5P expression status in OC and ovarian epithelial cells. It turned out that as shown in qRT-PCR analysis, declined miR-188-5P was observed in OC cells (IOSE80) (Figure. 1A). SKOV3 and A-2780 were chosen for the subsequent gain-of-function assay. OC cell proliferation was assessed with a miR-188-5p overexpression efficiency test performed before (Figure. 1B) and it demonstrated that cell proliferation was remarkably impaired in miR-188-5p up-regulated cells (Figure. 1C). Caspase-3 activity in the presence of miR-188-5p overexpression was notably elevated (Figure. 1D). Above results showed miR-188-5P impeded cell proliferation, migration, and advanced cell apoptosis in ovarian cancer.

CCND2 is a direct target of miR-188-5p in OV cells

To clarify the mechanism of miR-188-5p, a bioinformatics prediction program was browsed for identifying miR-188-5p potential target genes. CCND2 was suggested to be a potential target of miR-188-5p and the substantially upregulated expression of CCND2 at mRNA and protein levels was also presented in OC cells, not in normal cells (Figure. 2A and 2B). qRT-PCR and WB assay suggested that miR-188-5p reduced CCND2 mRNA and protein (Figure. 2C and 2D). RIP assay indicated that CCND2

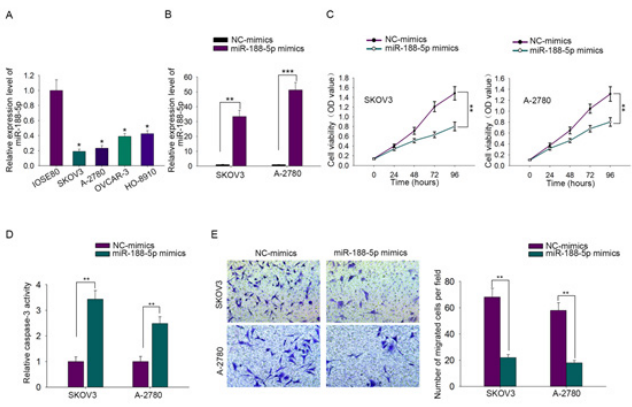

Figure 1. The upregulation of miR-188-5P hinders cell proliferation, migration and boosts apoptosis in ovarian cancer cells. (A) qRT-PCR analysis demonstrated that miR-188-5P is markedly decreased in ovarian cancer cells (HO-8910, SKOV3, OVCAR-3, A-2780), in contrast to normal cell line (IOSE80). (B) The stable expression of miR-188-5p in SKOV3 and A-2780 cells was confirmed by qRT-PCR analysis post-transfection with miR-188-5p mimics. (C) CCK-8 assay showed that overexpression of miR-188-5p in SKOV3 and A-2780 cells remarkably inhibited cell proliferation. (D) Caspase-3 activity assay displayed enhanced caspase-3 activity in the presence of miR-188-5p mimics, suggesting promoted cell apoptosis caused by miR-188-5p overexpression.(E) Transwell assay revealed the impeded migration potential of SKOV3 and A-2780 cells after transfection with miR-188-5p mimics. $^{*}P < 0.05$, $^{**}P < 0.01$.

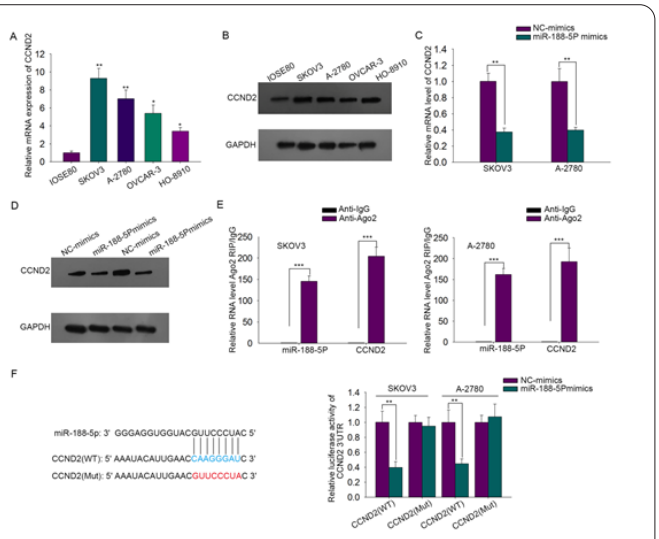

Figure 2. CCND2 is a direct target of miR-188-5p in ovarian cancer cells. (A) qRT-PCR analysis exhibited the upregulated mRNA expression level of CCND2 in OC cells not in IOSE80 cells. (B) Western blot analysis showed the elevated protein content of CCND2 in OC cells not in IOSE80 cells. (C) The decreased CCND2 mRNA level was observed in stably overexpressing miR-188-5p cells by qRT-PCR analysis. (D) The declined protein content of CCND2 was found in stably overexpressing miR-188-5p cells by western blot analysis. (E) RIP assay followed by qRT-PCR analysis suggested that miR-188-5p and CCND2 mRNA were enriched in the anti-Ago2 group compared with the anti-IgG group in SKOV3 and A-2780 cells. (F) Dual luciferase reporter assay disclosed the significantly inhibited 3' UTR of CCND2 mRNA luciferase activity by miR-188-5p augmentation. $^{*}P < 0.05$, $^{**}P < 0.01$.

mRNA had a higher level in the anti-Ago2 group than in the negative control group (Figure. 2E). Besides, a further luciferase reporter assay indicated that miR-188-5p targeted CCND2 in OV cells.

ELAVL1 and miR-188-5p regulate CCND2 expression in a competitive manner

ELAVL1, as an indispensable part of the Elav family of RNA-binding proteins, was observed implicating in enhancing mRNA stability via binding to adenine and ARE of the mRNA in the 3' untranslated regions (3'-UTRs) (11, 23). In addition, the participation and upregulation of ELAVL1 in several cancers were also reported including ovarian cancer (24-28). Therefore, ELAVL1 protein level was respectively examined by and western blot assay and unsurprisingly remarkable upregulation of ELAVL1 protein presented among OC cells, not in normal cell line IOSE80 (Figure. 3A). Based on this premise, we speculated whether ELAVL1 regulated downstream gene expression through the above mechanism. With the aid of starBase V3.0, it predicted the binding of ELAVL1 to CCND2 mRNA. A test of ELAVL1 overexpression efficiency was performed before further validation (Figure. 3B). qRT-PCR and WB assay showed that ELAVL1 augmentation notably advanced the CCND2 mRNA and protein in OC cells (Figure. 3C and 3D).

RIP assay showed that CCND2 mRNA was immunoprecipitated by an Anti-ELAVL1 antibody, not an anti-IgG antibody (Figure. 3E), verifying the interaction between CCND2 mRNA and ELAVL1.qRT-PCR analysis was performed in SKOV3 and A-2780 cells after treated with Actinomycin D to measure the stability of CCND2 mRNA. The half-life of CCND2 mRNA was prolonged in the pre-

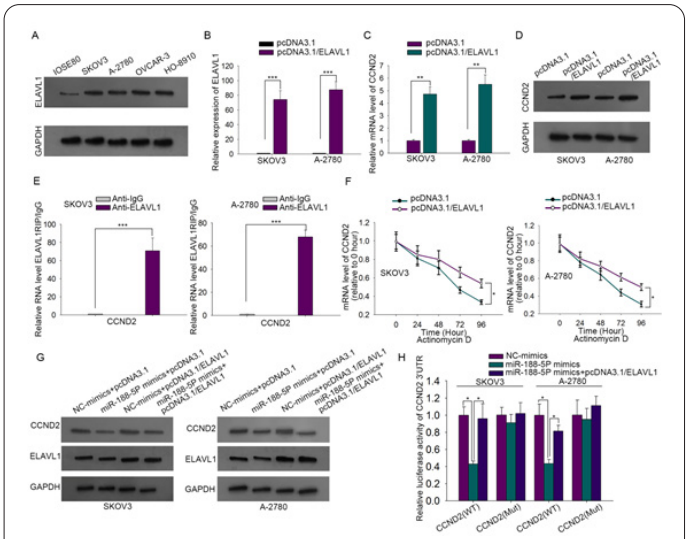

Figure 3. ELAVL1 and miR-188-5p competitively regulate CCND2 expression. The expression profile of ELAVL1 was determined by western blot analysis. (B) Stable expression of ELAVL1 was ensured in SKOV3 and A-2780 cells. (C) The protein content of CCND2 was boosted in the presence of pcDNA3.1/ELAVL1 by western blot analysis. (D) The enrichment of CCND2 mRNA in SKOV3 and A-2780 cells was observed in the anti-ELAVL1 group not in the Anti-IgG group by RIP assay. (F) The stability of CCND2 mRNA in SKOV3 and A-2780 cells after Actinomycin D treatment was determined by qRT-PCR analysis. (G) Western blot analysis suggested that ELAVL1 overexpression abolished the inhibitory effect of miR-188-5p on CCND2 expression. (H) Dual luciferase reporter assay indicated that the inhibited 3' UTR of CCND2 mRNA luciferase activity in the presence of miR-188-5p mimics was restored by ELAVL1 overexpression. $^{*}P < 0.05$, $^{**}P < 0.01$.

sence of pcDNA3.1/ELAVL1 (Figure. 3F). So far, it suggested that ELAVL1 was able to stabilize CCND2 mRNA and accelerate CCND2 mRNA translation. Increased ELAVL1 abrogated miR-188-5p-mediated suppressive effect on CCND2 expression (Figure. 3G). Besides, overexpression of ELAVL1 revisedmiR-188-5p-induced the decline of luciferase activity in SKOV3 and A-2780 cells (Figure. 3H). Collectively, ELAVL1 reversed the inhibitory impact of miR-188-5p on CCND2 expression.

ELAVL1 and CCND2 rescue miR-188-5p-mediated repression of proliferation and migration in OC cells

To figure out how ELAVL1 and miR-188-5p regulated CCND2 to affect OC cell viability, mobility and apoptosis, rescue experiments were implemented after confirming the overexpression efficacy of CCND2 (Figure. 4A). CCK-8 assay presented that the addition of CCND2 or ELAVL1reversed the impaired SKOV3 growth and the promoted SKOV3 apoptosis caused by miR-188-5p (Figure. 4B and 4C). Introduction of CCND2 or ELAVL1 reverted miR-188-5p inhibited cell migration in SKOV3 cells (Fig. 4D). Taken together, ELAVL1 and CCND2 reversed the suppressive impact of miR-188-5p on OC cell proliferation and migration, and promotive OC cell apoptosis.

Discussion

Ovarian cancer was widely recognized as a leading female malignancy throughout the world with an alarming mortality rate of which poor survival rate was unanimously ascribed to its strong invasion (29-31). Even though past decades had witnessed developing therapies, the prognosis of ovarian cancer remains discouraging, indicating that novel targets are in urgent need for ovarian treatment (32, 33).

Surging reports demonstrated that the dysregulated expression of miRNAs has been frequently associated with various pathological progresses and multiple cancer progressions (5-7, 34). Among this, miR-188-5p served as a tumor suppressor in PRAD, GC and HCC, whose function in ovarian cancer was undecided. Consistently the anti-tumor effect of miR-188-5p was further confirmed in OV. We confirmed that miR-188-5p was unveiled to inhibit OV cell growth, migration and advance apoptosis.

In addition, previously the mechanism of miRNA action is implicated in binding to the mRNAs 3' UTR region through sequence complementarities, responsible for mRNA destabilization and translation inhibition. For example, miRNA-194 regulates the Wnt/β-catenin signaling pathway in GC via targeting SUFU (35). And the novel mechanism that miRNA competed for target gene mRNA with ELAVL1 (HuR) identified as an ARE-containing mRNAs stabilizer also unmasked before (14, 36, 37). Furthermore, the ELAVL1 expression in the cytoplasm in ovarian carcinoma was correlated with unsatisfactory outcomes (38-40). Consistently, the ELAVL1 was up-regulated in OV cells in our study. However, our study exhibited the competition between miR-188-5p and ELAVL1 for target mRNA for the first time. Cyclin D2 (CCND2) functioned as a putative carcinogenic factor in glioblastoma (19), lung cancer (20), and colorectal cancer (21) including OV (22). But the present study first revealed miR-188-5p- mediated repression of CCND2 via binding CCND2 3′-UTR and ELAVL1-dependent upregulation of CCND2 through binding CCND2 3′-UTR. Moreover, it was verified in the study that ELAVL1 antagonized the miR-188-5p-regulated inhibition of CCND2 in the OV cells. Likewise, res-

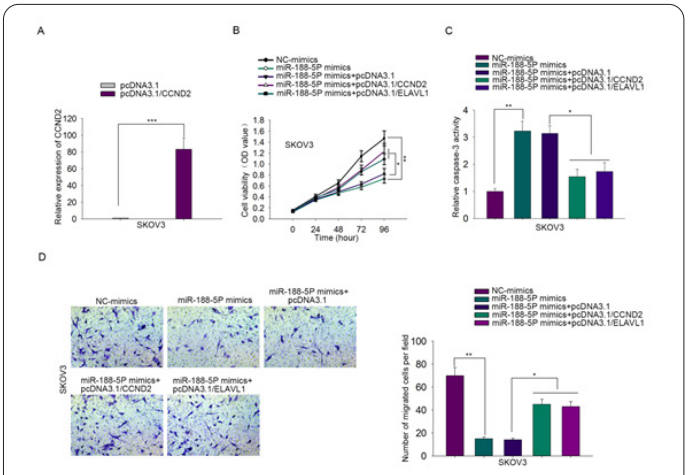

Figure 3. ELAVL1 and CCND2 rescue miR-188-5p-mediated repression of proliferation and migration in OC cells. The transfection efficiency of pcDNA3.1/CCND2 in SKOV3 cells was determined by qRT-PCR analysis. (B) Elevated expression of ELAVL1 or CCND2 mitigated the suppressed SKOV3 cell viability induced by miR-188-5p augmentation. (C) Elevated expression of ELAVL1 or CCND2 attenuated the promoted SKOV3 cell apoptosis regulated by miR-188-5p augmentation. (D) Upregulated expression of ELAVL1 or CCND2 reverted the impaired SKOV3 cell migration mediated by miR-188-5p overexpression. $^{*}P < 0.05$, $^{**}P < 0.01$.

cue experiments demonstrated that ELAVL1 abrogated the miR-188-5p-regulated suppression of malignant phenotypes in OV.

In conclusion, the current study further confirmed a novel mechanism in ovarian cancer whereby ELAVL1 antagonized the inhibition of proliferation, migration, and acceleration on cell apoptosis imposed by miR-188-5p, carrying important implications for generating creative options for ovarian cancer therapies.

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Conflicts
None.

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