

Long Noncoding RNA CCAT2 Knockdown Suppresses Tumorous Progression by Sponging miR-424 in Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is the one of most common gynecological malignant tumors with high mortality. A series of long noncoding RNAs (lncRNAs) have been validated to play a vital role in EOC tumorigenesis. Colon cancer-associated transcript 2 (CCAT2) has been verified as an oncogenic lncRNA in multiple tumors; however, the role of CCAT2 in EOC genesis is still unclear. The purpose of the present study was to probe the function of CCAT2 on EOC. Preliminary experiments found that CCAT2 expression was significantly upregulated in EOC tissues and cell lines compared to noncancerous tissue and cells. CCAT2 knockdown induced by interfering oligonucleotides could inhibit proliferation and promote apoptosis and induce cell cycle arrest at the G₀/G₁ phase. Bioinformatics analysis predicted that miR-424 targeted CCAT2, which was confirmed by luciferase reporter assay. Moreover, the miR-424 inhibitor rescued the tumorigenesis inhibition induced by CCAT2 knockdown. In summary, our findings illustrate that CCAT2 acts as competing endogenous RNA (ceRNA) or sponge via negatively targeting miR-424, providing a novel diagnostic marker and therapeutic target for EOC.

Key words: Epithelial ovarian cancer (EOC); Long noncoding RNAs (lncRNAs); CCAT2; miR-424

INTRODUCTION

Epithelial ovarian cancer (EOC) is a primary gynecological malignant tumor with third highest morbidity and highest mortality in women worldwide¹. The 5-year survival rate of EOC patients is near 45%, which is significantly lower than other gynecological tumors, such as breast cancer (about 90%), endometrial cancer (about 80%), and cervical cancer (about 68%)². Because there is insufficient effective early detection, the majority of EOC patients are diagnosed at later stages, and recurrence occurs in most EOC patients within 16 months³. Because of the lack of early symptoms, it is always accompanied with extensive metastases in the pelvic and abdominal cavity as the EOC is diagnosed, leading to lower overall survival rate and poor prognosis⁴. Thus, the in-depth mechanism of pathogenesis for EOC is urgently needed.

Long noncoding RNAs (lncRNAs) are non-protein coding nucleotide sequences with length longer than 200 nt⁵. Many studies have reported that lncRNAs are dysregulated in cancers, suggesting that the aberrant expression of lncRNAs is associated with tumorigenesis, metastasis, and prognosis in tumorigenesis⁶. For instance, lncRNA HOTAIR is overexpressed in EOC patients and predicts

poor prognosis and promotes tumor metastasis⁷. Furthermore, the expression of lncRNA RP11-190D6.2 was markedly decreased in EOC tumor tissues compared with normal tissues, suggesting it is positively correlated with WWOX expression⁸. Colon cancer-associated transcript 2 (CCAT2) was originally identified as an oncogenic lncRNA in colorectal cancer, which has been verified to play an oncogenic molecular role in multiple tumors⁹. However, the role of CCAT2 in EOC genesis is still unclear.

Similar to lncRNAs, microRNAs (miRNAs) have more widely been recognized in oncogenesis due to their comprehensive distribution and vast previous studies^{10,11}. As well as in EOC, miRNAs are widely identified to contribute to the tumorigenesis and progression of multiple tumors, including proliferation, migration, invasion, and apoptosis¹². For instance, miR-127-3p was downregulated and directly regulated the BAG5 gene and acted as a tumor suppressor in EOC¹³. miR-424 has been shown to inhibit tumor cell migration, invasion, and epithelial-mesenchymal transition by downregulating DCLK1 in ovarian clear cell carcinoma¹⁴.

In the present study, we found that CCAT2 was overexpressed in EOC tissue and predicted metastasis and a

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poor prognosis. Thus, this study was aimed to investigate the potential physiological effect of CCAT2 on EOC tumorigenesis and progression and further validate the miRNA sponge role of CCAT2 for miR-424.

MATERIALS AND METHODS

Tissue Sample Acquisition

The collection of tumor specimens from 31 cases of EOC patients at Huai'an First People's Hospital, Nanjing Medical University, was used in the present study. Tumor tissues and adjacent nontumor tissues were excised during surgery and immediately stored in -80°C liquid nitrogen for use. No patients experienced radiotherapy and chemotherapy before the operation. The study was approved by the ethics committee and institutional review board of Nanjing Medical University. Informed written consent was signed by all the enrolled patients.

Cell Lines and Culture

EOC cell lines (SKOV3, OMC685, A2780, and HO8910) and a normal ovarian cell line (IOSE386) were purchased from the Shanghai Institute of Cell Biology at the China Academy of Sciences (Shanghai, P.R. China). Cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C with 5% CO_2 atmosphere.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was performed to synthesize cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). PCR was performed using GoTaq qPCR Master Mix with SYBR Green (Promega, Madison, WI, USA) according to the manufacturer's protocol. GAPDH was used as endogenous control. All primers were designed and synthesized by GenePharma (Shanghai, P.R. China) as follows: CCAT2, 5'-CTAGGGTTCGTATAGAGAAAACG-3' (forward) and 5'-AACCATACTCCAATGGTAACAC-3' (reverse); miR-424, 5'-GCTACGCTAACTGGGTAACCTTG-3' (forward) and 5'-GTCGAGGGGCATCGGTT-3' (reverse); and GAPDH, 5'-CGACACTTTATCATGGCTA-3' (forward) and 5'-TTGTTGCCGATCACTGAAT-3' (reverse). The level of gene expression was expressed relative to GAPDH and calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Cell Transfection

Interfering RNAs in the study were obtained from GenePharma Co. Ltd. For the transfection of the miR-424 mimics, inhibitors, and CCAT2 small interfering RNAs (siRNAs) (si-CCAT2), EOC cell lines (2×10^5)

were seeded in six-well plates and transfected with 100 nM siRNAs using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA). The sequences are as follows: miR-424 mimics, 5'-CUUUUUGCGG UCUGGGCUUGC-3'; miR-424 inhibitor, 5'-GCAAGC CCAGACCGCAAAAAG-3'; and si-CCAT2, 5'-AAAC UUUAAAGAACACUACUG-3'.

Cell Proliferation Assay

Cell proliferation assay was performed with the cell counting kit-8 (CCK-8; DOJINDO, Japan). The transfected cells were seeded in 96-well plates with a density of 5×10^3 per well. The cells were then added with 10 μl of CCK-8 solution at 37°C for 90 min, and the absorbance was assessed at 450 nm after incubation.

Colony Formation Assay

EOC cells were plated in six-well culture plates at a density of 100 cells per well. After incubation for 14 days at 37°C , cells were fixed with methanol and stained with 0.2% crystal violet and then washed three times with phosphate-buffered saline (PBS). Finally, the visible colonies consisting of >50 cells were manually counted.

Luciferase Reporter Assays

Luciferase assays were performed using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instructions. Briefly, the full-length 3'-UTR of CCAT2 containing miR-424 binding sites was cloned downstream of the firefly luciferase gene in pGL3 (Invitrogen) to construct pGL3-luc-CCAT2. Cells were seeded into 96-well plates and transfected with pGL3-luc-CCAT2 (50 ng) and *Renilla* luciferase (5 ng), and miR-424 mimics or NC nucleotides (5 pmol) using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions. After 48 h, luciferase activity was detected using Dual-Glo Luciferase Assay System (Promega). For comparison, the activity was normalized with *Renilla* luciferase activity. All experiments were performed in triplicate.

Cell Cycle and Apoptosis Analysis

Cell cycle and apoptosis analysis were performed using the cell cycle analysis kit (Lianke, Shanghai, P.R. China) and Annexin-V/Dead Cell Apoptosis Kit (Invitrogen). Briefly, cells were seeded in six-well plates at a density of 4×10^5 per well. Cells were starved in FBS-free medium for 12 h and then treated with cell cycle and apoptosis agents for 48 h. The adherent cells were washed and trypsinized, and centrifuged at $12,000 \times g$ for 5 min. The collected cells were fixed in 75% cold ethanol at -20°C overnight and resuspended in annexin-binding buffer and 5 μl of 7AAD/annexin V/propidium iodide at room temperature for 30 min, and then incubated at room

temperature for 15 min in the dark. Fluorescence was analyzed by flow cytometry.

Statistical Analyses

The data are presented as the mean \pm SD from at least three independent experiments. Statistical analyses were performed with an analysis of one-way ANOVA or a two-tailed Student's *t*-test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 (Chicago, IL, USA). The significance level was set as $p < 0.05$. Survival was analyzed based on Kaplan-Meier curves with the log-rank test analysis.

RESULTS

CCAT2 Expression Was Upregulated in EOC Tissues and Cell Lines

To test the abundance of CCAT2 in EOC tissue, we performed RT-PCR to assess its expression level in EOC tissue and cell lines. Primarily, the results showed that CCAT2 expression was significantly increased in 31 cases of EOC tissues samples (Fig. 1A). CCAT2 expression was upregulated in 90.32% (28/31) of EOC tissues compared with their adjacent nontumor tissues (Fig. 1B).

Moreover, the expression of CCAT2 of the EOC cell lines (SKOV3, OMC685, A2780, and HO8910) was significantly higher than that of normal ovarian cell line (IOSE386) (Fig. 1C). Overall, the present study revealed that CCAT2 was markedly overexpressed in EOC tissues and cell lines.

CCAT2 Knockdown Suppressed EOC Cell Line Proliferation and Promoted Apoptosis

Because CCAT2 was significantly overexpressed in EOC tissue and cell lines, we hypothesized that CCAT2 acted as an oncogenic lncRNA in EOC oncogenesis and played a vital role. Loss-of-function experiments aimed at CCAT2 were performed to verify the hypothesis. To knock down CCAT2 expression and abundance, small interfering nucleotide (si-CCAT2) and control (si-NC) were synthesized and transfected into EOC cell lines (SKOV3 and OMC685), showing the effective interfering efficiency of si-CCAT2 (Fig. 2A). Cell proliferation and colony formation assays showed that CCAT2 knockdown induced by si-CCAT2 could significantly repress the proliferation and colony formation capacity (Fig. 2B and C). Moreover, the flow cytometry assay showed that CCAT2 knockdown could accelerate the apoptosis of SKOV3 and

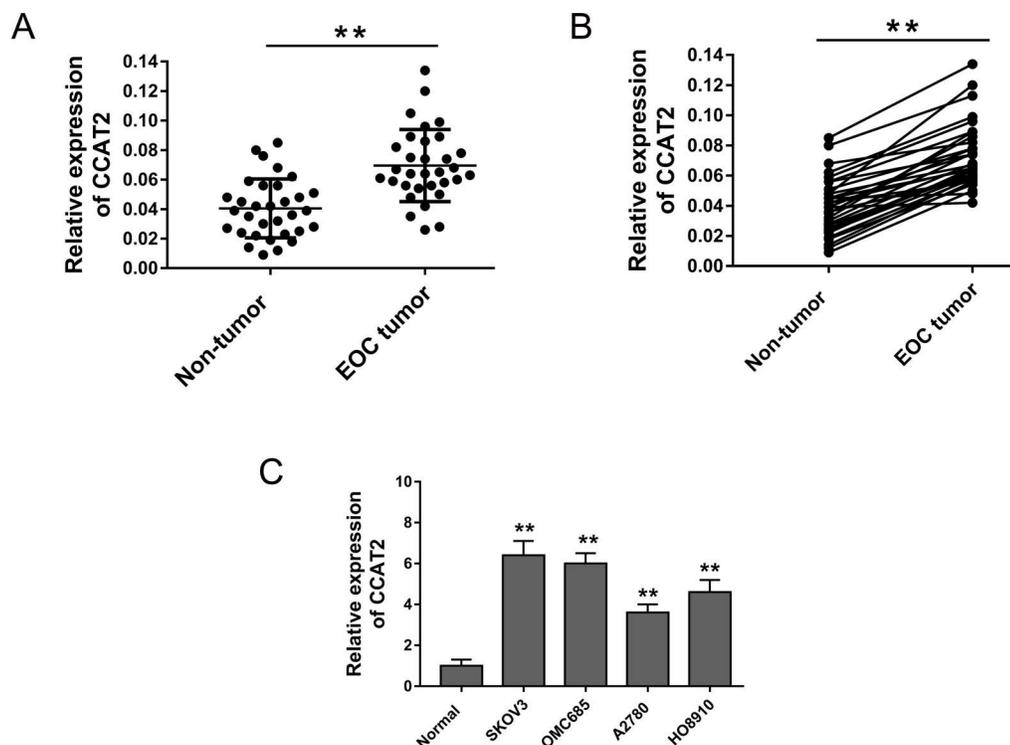


Figure 1. Colon cancer-associated transcript 2 (CCAT2) expression was upregulated in epithelial ovarian cancer (EOC) tissue samples and cell lines. (A) RT-PCR showed the expression level of CCAT2 in EOC tumor tissues and adjacent nontumor tissues. (B) CCAT2 expression was upregulated in 90.32% (28/31) of EOC tissues compared with pair-matched adjacent noncancerous tissues. (C) Expression of CCAT2 was detected in EOC cell lines (SKOV3, OMC685, A2780, and HO8910) and a normal ovarian cell line (IOSE386). Data are presented as mean \pm SD. ** $p < 0.01$ compared with the corresponding control group.

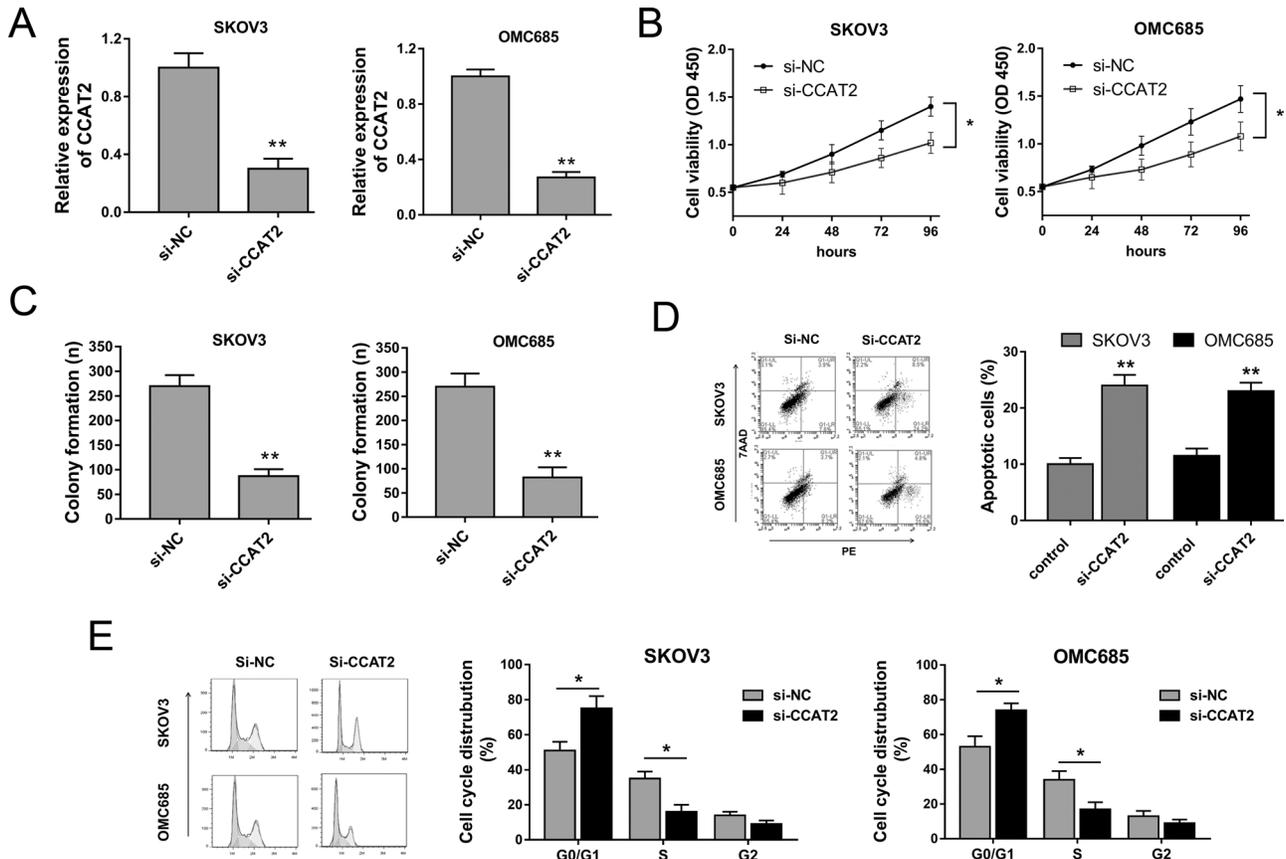


Figure 2. CCAT2 knockdown suppressed EOC cell lines' proliferation and promoted apoptosis in vitro. (A) Expression level of CCAT2 in EOC cell lines (SKOV3 and OMC685) transfected with small interfering nucleotide (si-CCAT2) and control (si-NC), showing effective interfering efficiency. (B) Cell proliferation ability was detected by the cell counting kit-8 (CCK-8) assay. (C) Cell proliferation ability was detected by colony formation assay. (D) Apoptosis of SKOV3 and OMC685 cells was detected by flow cytometry assay. (E) Cell cycle analysis of SKOV3 and OMC685 detected by flow cytometry revealed the cell cycle arrest at the G₀/G₁ phase. Data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ compared with the corresponding control group.

OMC685 cells and induce cell cycle arrest at the G₀/G₁ phase (Fig. 2D and E). In summary, the results strongly revealed that CCAT2 knockdown could suppress EOC cell lines' proliferation and promoted apoptosis, suggesting the oncogenic role of CCAT2 in EOC oncogenesis.

Bioinformatics Analysis Predicted That CCAT2 Was a Target of miR-424

The present study confirmed that CCAT2 was upregulated in EOC tissue, indicating that CCAT2 might function as oncogenic lncRNA in EOC oncogenesis. Admittedly, the major approach that lncRNA exerted physiological function is to act as miRNA "sponge" to recede the target miRNAs' abundance to indirectly modulate functional miRNAs. Thus, we probed the possible target miRNAs of CCAT2 using bioinformatics analysis. Bioinformatics prediction programs (starBase, <http://starbase.sysu.edu.cn>) revealed that miR-424 shared complementary binding sites with CCAT2 (Fig. 3A). The luciferase reporter assay

verified that miR-424 mimics bound with CCAT2 wild type and decreased luciferase activity (Fig. 3B). RT-PCR was performed to test the miR-424 expression in EOC tissue, and the results showed the lower-expressed miR-424 in EOC tissues compared to the adjacent nontumor tissues (Fig. 3C). miR-424 expression was downregulated in 87.09% (27/31) of EOC tissues compared with their adjacent nontumor tissues (Fig. 3D). Moreover, in EOC cell lines, miR-424 expression levels were significantly suppressed (Fig. 3E). All the results showed that miR-424 was negatively correlated with CCAT2 and could act as target for CCAT2.

miR-424 Targeted CCAT2 to Modulate Tumorous Progression of EOC Cells

In EOC tumor tissue and cell lines, miR-424 expression was negatively correlated with CCAT2; thus, we hypothesized that miR-424 and CCAT2 exerted inversely regulating roles in EOC cell tumor progression.

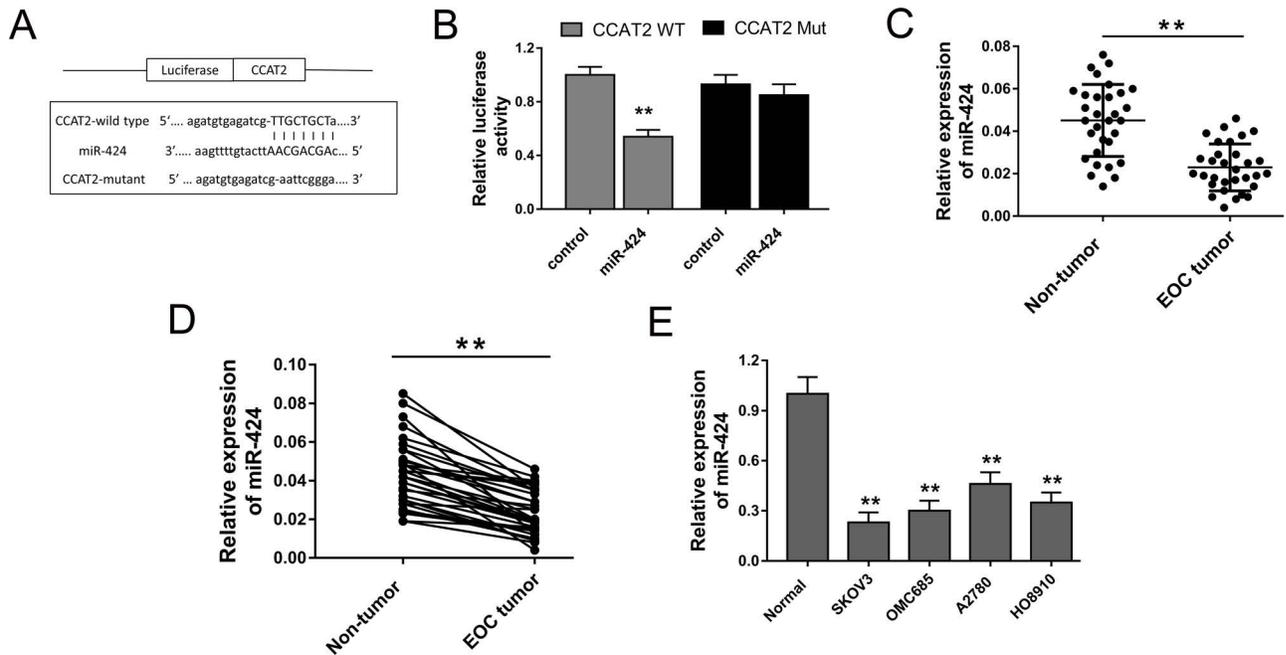


Figure 3. CCAT2 was directly targeted with miR-424. (A) The predicted complementary binding sites of miR-424 and CCAT2. (B) Luciferase reporter assay showed that luciferase activity was decreased in miR-424 mimics binding to CCAT2 wild type. (C) RT-PCR showed lower miR-424 expression in EOC tissues compared to adjacent nontumor tissues. (D) miR-424 expression was downregulated in 87.09% (27/31) of EOC tissues compared with pair-matched adjacent noncancerous tissues. (E) Expression of miR-424 in EOC cell lines. Data are presented as mean \pm SD. $**p < 0.01$ compared with the corresponding control group.

The existing literature has reported that miR-424 acted as a tumor suppressor in multiple tumorigenesis^{15,16}. Previous experiments had shown that CCAT2 knockdown could suppress EOC cell tumorigenesis; hence, we performed miR-424 knockdown and rescue experiments to verify the competing endogenous role of CCAT2 in SKOV3 cell lines. CCAT2 knockdown induced by si-CCAT2 could increase the miR-424 expression level, which was reversed by the miR-424 inhibitor (Fig. 4A). The CCK-8 assay and colony formation assay showed that the miR-424 inhibitor rescued the inhibition of CCAT2 knockdown on cell proliferation ability in SKOV3 cells (Fig. 4B and C). Furthermore, flow cytometry demonstrated that CCAT2 knockdown accelerated apoptosis and induced cell cycle arrest at the G_0/G_1 phase, whereas it was reversed by the miR-424 inhibitor (Fig. 4D and E). In summary, a series of rescue experiments revealed that the miR-424 inhibitor could reverse the tumor suppressor role of CCAT2 knockdown, suggesting the targeting binding within CCAT2 and miR-424.

DISCUSSION

EOC is the most aggressive subtype of ovarian cancer with a significantly higher recurrence and mortality rate. Recently, lncRNAs have become a research hotspot involving cardiovascular system disease, endocrine system disease, and tumors¹⁷. For ovarian cancer, lncRNAs

are performed to elucidate the complex mechanisms of tumorigenesis.

CCAT was originally identified as an oncogenic lncRNA in colorectal cancer, which similarly has been identified as oncogenic molecular in other tumors¹⁸. In the present study, CCAT2 was significantly upregulated in EOC tissue and cell lines. CCAT2 knockdown suppressed EOC cell lines' proliferation and promoted apoptosis, suggesting the oncogenic role of CCAT2 in EOC oncogenesis. In addition to CCAT2, several lncRNAs have been identified to modulate carcinogenesis. The lncRNA HOTAIR was increased in EOC tissues compared with corresponding normal tissues, acting as a useful biomarker for the predisposition to EOC and for the early diagnosis of the disease¹⁹. Moreover, urothelial cancer-associated 1 (UCA1) was aberrantly upregulated in EOC tissues and cells, acting as a prognostic factor for EOC patients' overall survival and endogenous sponge by directly binding to miR-485-5p²⁰. Hence, lncRNAs had been recognized for their vital pathological roles in EOC tumorigenesis.

Admittedly, the major approach for lncRNAs to exert physiological function is to act as an miRNA "sponge" to recede target miRNAs' abundance to indirectly modulate functional miRNAs. Thus, we investigated the possible target miRNAs of CCAT2 using bioinformatics analysis. The results showed that miR-424 shares complementary

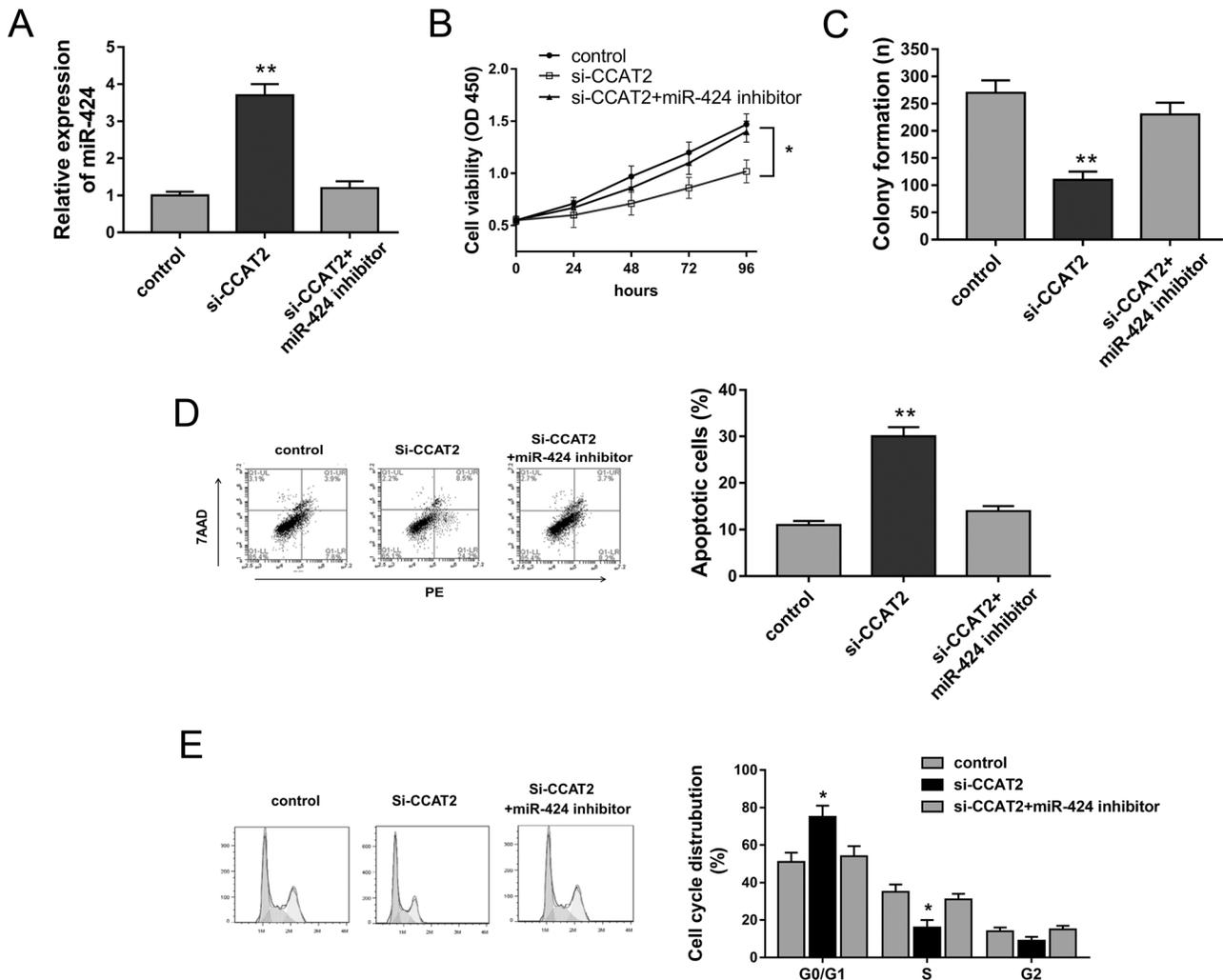


Figure 4. miR-424 targeted CCAT2 to modulate EOC cells' proliferation and apoptosis. (A) miR-424 expression in SKOV3 cells transfected with si-CCAT2 and/or miR-424 inhibitor. (B) Cell proliferation of SKOV3 cells was detected by CCK-8. (C) Colony formation assay. (D) Apoptosis of EOC cells was assessed by flow cytometry. (E) Cell cycle analysis showed the cycle distribution. Data are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ compared with the corresponding control group.

binding sites with CCAT2 at the 3'-UTR, indicating potential targeting miRNA for CCAT2. It has been validated that miR-424 functions as a tumor suppressor in the carcinogenesis of breast cancer, non-small cell lung cancer, and cervical cancer²¹⁻²³. In the present study, miR-424 was downregulated in EOC tissues and cell lines, being inversely correlated with that of CCAT2. The luciferase reporter assay revealed internal matching function. Generally, CCAT2 acts as an miR-424 sponge, and the miR-424 inhibitor rescued the tumorigenesis inhibition induced by CCAT2 knockdown. The regulation of miR-424 and CCAT2 is antagonistic, suggesting paired binding. The role that lncRNAs acts as an miRNA sponge is a widespread acting mechanism in multiple types of tumorigenesis²⁴. In hepatocellular carcinoma, lncRNA MALAT1 downregulated miR-146b-5p, which inhibited

the phosphorylation of Akt mediated by TRAF6, to promote cancer growth and metastasis²⁵. Thus, miRNA sponge is a vital regulatory mechanism in oncogenesis, integrating miRNA and targeting gene mRNAs and composing a complete regulating loop.

Aberrant expression of lncRNAs might act as diagnostic and therapeutic targets for multiple tumors, as well as EOC. Because lncRNAs exist in the cytoplasm or nuclei, their expression levels could be detected in the early phase of solid tumors. Huang et al. reported that CCAT2 was upregulated in the EOC tissue and was associated with shorter overall survival and disease-free survival, predicting poor prognosis²⁶. lncRNA-mRNA coexpression network analyses are conducted to predict lncRNA expression trends and the potential target genes of lncRNAs²⁷.

In summary, the present study demonstrates the over-expression of lncRNA CCAT2 and indicates the potential correlation with miR-424 in tumorigenesis, revealing the ceRNA mechanism or the miRNA sponge and providing a novel diagnostic marker and therapeutic target for EOC.

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