LncRNA myocardial infarction-associated transcript promotes cell proliferation and inhibits cell apoptosis by targeting miR-330-5p in epithelial ovarian cancer cells

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Abstract

Introduction: Long non-coding RNAs (lncRNAs) have been shown to have great importance in cancer development and progression. However, the mechanism of lncRNAs in epithelial ovarian cancer remains unclear. In the present study, we aimed to explore the role of the lncRNA myocardial infarction-associated transcript (MIAT) in epithelial ovarian cancer tumorigenesis.
Material and methods: Quantitative real-time PCR (qRT-PCR) was used to determine MIAT expression in human epithelial ovarian cancer tissues and cell lines, and the effects of MIAT on cell proliferation and cell apoptosis were determined by CCK-8 assay or flow cytometry analysis. Dual-Luciferase Reporter assay and Western blot assay were used to explore the molecular mechanisms of MIAT in epithelial ovarian cancer cells progression.
Results: Our data showed that the expression of lncRNA MIAT was remarkably increased in human epithelial ovarian cancer tissues and cell lines ($p < 0.05$). High MIAT expression was associated with poor overall survival of epithelial ovarian cancer patients ($p < 0.05$). Function assays showed that knockdown of MIAT expression significantly inhibited epithelial ovarian cancer cell proliferation and promoted cell apoptosis *in vitro* ($p < 0.05$). Moreover, we revealed that MIAT might function as an endogenous miR-330-5p sponge to regulate the target gene of miR-330-5p in epithelial ovarian cancer progression.
Conclusions: LncRNA MIAT was found to be a tumor oncogenic lncRNA in epithelial ovarian cancer tumorigenesis. LncRNA MIAT promoted cell proliferation and inhibited cell apoptosis by negative regulation of miR-330-5p in epithelial ovarian cancer cells. Our findings suggested that MIAT might act as a candidate prognostic biomarker and new therapeutic target for treating epithelial ovarian cancer patients.

Key words: long non-coding RNAs, MIAT, miR-330-5p, epithelial ovarian cancer.

Introduction

Epithelial ovarian cancer (EOC), one of the most deadly gynecological malignancies, affected approximately 239 000 people and caused an estimated 151 900 deaths during 2012 according to 2015 global cancer statistics, accounting for about 5% of all cancer and 4.2% of all cancer deaths among females worldwide [1, 2]. Despite the great improvement of early diagnosis and optimal treatment, overall survival of EOC patients

remains unsatisfactory [3]. Therefore, demonstrating the molecular mechanism underlying EOC progression is necessary for development of successful therapeutic strategies to improve EOC patients' survival.

Long non-coding RNAs (lncRNAs) are a new class of non-coding RNAs (> 200 nucleotides) with limited or no protein-coding capacity [4]. With the continuous advances of research approaches, dysregulation of lncRNAs was found to contribute to various malignant tumors, including EOC progression. For example, Zhu *et al.* found that downregulation of lncRNA TUBA4B was associated with poor prognosis for EOC [5]. Jin *et al.* showed that lncRNA MALAT1 promoted proliferation and metastasis in EOC via the PI3K-AKT pathway [6]. Zhang *et al.* suggested that lncRNA HOXD-AS1 promoted epithelial ovarian cancer cell proliferation and invasion by targeting miR-133a-3p and activating the Wnt/β-catenin signaling pathway [7]. However, the roles of lncRNAs in EOC progression are still largely unclear.

Myocardial infarction-associated transcript (MIAT) is one of the noncoding RNAs first identified as an lncRNA in 2006 [8]. Recent studies showed that MIAT plays important roles in microvascular dysfunction [9], myocardial infarction [10], and diabetic retinopathy [11]. Moreover, accumulating evidence has proven that MIAT plays an oncogenic role in tumor progression. However, the roles and underlying mechanism of MIAT in EOC are still unclear.

In the present study, we explored the role of lncRNA MIAT in promoting EOC cell proliferation and apoptosis. In addition, we investigated whether MIAT affected the biological processes of EOC via regulating the miRNA expression. Taken together, our findings suggested that MIAT promoted EOC cell progression through inhibiting miR-330-5p expression.

Material and methods

Patients and tissue samples

We collected 53 samples of surgical EOC tissues and 19 samples of normal ovarian tissues at the Department of Gynecology of Huaihe Hospital of Henan University between 2011 and 2012. The tissue samples were confirmed by pathological examination and immediately stored in liquid nitrogen after surgery. Written informed consent was obtained from individual patients prior to surgery. The study was approved by the Ethics Committee of Huaihe Hospital of Henan University.

Cell culture and transfection

Human EOC cell lines (SKOV3, OVCAR3, HO8910, and A2780) were purchased from American Type Culture Collection (ATCC, Rockville, USA). The normal cell line human ovarian surface epithelial (HOSE) was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All cells were cultured in RPMI-1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 units/ml penicillin-streptomycin (Invitrogen, USA), and maintained at 37°C in a humidified incubator with 5% CO_2. siRNA against MIAT was designed and synthesized by Shanghai Genechem Co., Ltd. MiR-330-5p mimics and miR-330-5p inhibitors were obtained from Genepharma Co., Ltd. Cell transfection was performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR

TRIzol Reagent (Invitrogen, USA) was used to extract total RNA according to the manufacturer's protocol. The quality and concentration of RNA were determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). QRT-PCR was performed using SYBR Premix ExTaq (TaKaRa, Dalian, China) with the ABI Prism 7900HT thermocycler (Applied Biosystems, USA). GAPDH was employed as the internal control for mRNA quantification. The primers used in the present study were as follows: MIAT forward primer 5′-TTTACTTTAACAGACCAGAA-3′ and reverse primer 5′-CTCCTTTGTTGAATCCAT-3′; GAPDH forward primer 5′-CCACATCGCTCAGACACCAT-3′ and reverse primer 5′-CCAGGCGCCCAATACG-3′. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Cell proliferation assay

Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. Twenty-four h later with transfection, cells were seeded into 96-well plates at a density of 5000 cells per well with 100 µl of medium and continued to incubate at 37°C. At 24 h, 48 h, 72 h, and 96 h, 100 µl of serum-free culture medium and 10 µl of CCK-8 solutions were added to each well, followed by incubation at 37°C for 1 h. The absorbance was measured with a plate reader at 450 nm on an enzyme-linked immunosorbent assay reader. Five independent samples were detected in each experimental group.

Colony formation assay

Cells (1 × 10^3) were seeded into each well of a 6-well plate in quadruplicate. Cells were cultured for 2 weeks in a 37°C incubator. Cells were washed with PBS and fixed with 4% paraformal-

dehyde, stained with 1% crystal violet for 20 min, and then washed with water. Then, the number of colonies was counted manually.

Cell apoptosis assay

The annexin V/PI assay was performed according to the manufacturer's instructions (Invitrogen, USA). Briefly, transfected cells were plated into 6-well plates for 24 h. Afterwards, cells were washed with pre-cold PBS, trypsinized, and re-suspended in 100 μl of binding buffer with 2.5 μl of FITC conjugated annexin-v and 1 μl of PI (100 μg/ml). Cells were then incubated at room temperature for 15 min in darkness. A total of at least 10 000 cells were collected and calculated by flow cytometry (BD Biosciences, USA).

Luciferase assay

We cloned wild-type MIAT with potential miR-330-5p binding sites or mutants of each site into pMIR-REPORT plasmids (Promega, USA). HEK293T cells were placed on a 24-well plate and co-transfected with luciferase plasmids and miR-330-5p mimics or control miRNA. After 48 h of transfection, firefly and Renilla luciferase activities were detected with the Dual-Luciferase Reporter Assay System (Promega, USA).

Statistical analysis

Statistical analysis were performed using SPSS 17.0. Data were presented as mean ± standard deviation (SD). The Kaplan-Meier method was used to calculate the survival curve, and log-rank test to determine statistical significance. Student's *t*-test or χ^2 test was used to evaluate the statistical significance of differences among different samples. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LncRNA MIAT was upregulated in EOC

To explore the role of lncRNA MIAT in EOC progression, we firstly used qRT-PCR to determine MIAT expression in EOC tissues and cell lines. The results showed that MIAT expression was highly expressed in EOC tissues compared with normal ovarian tissues (NOT) (Figure 1 A; $p < 0.05$). Next, we explored MIAT expression in EOC cell lines. QRT-PCR showed that MIAT expression was significantly higher in EOC cells (SKOV3, OVCAR3, HO8910, and A2780) than normal cell line human ovarian surface epithelial cells (HOSE) (Figure 1 B; $p < 0.05$). In addition, according to the median relative MIAT expression value in EOC, we classi-

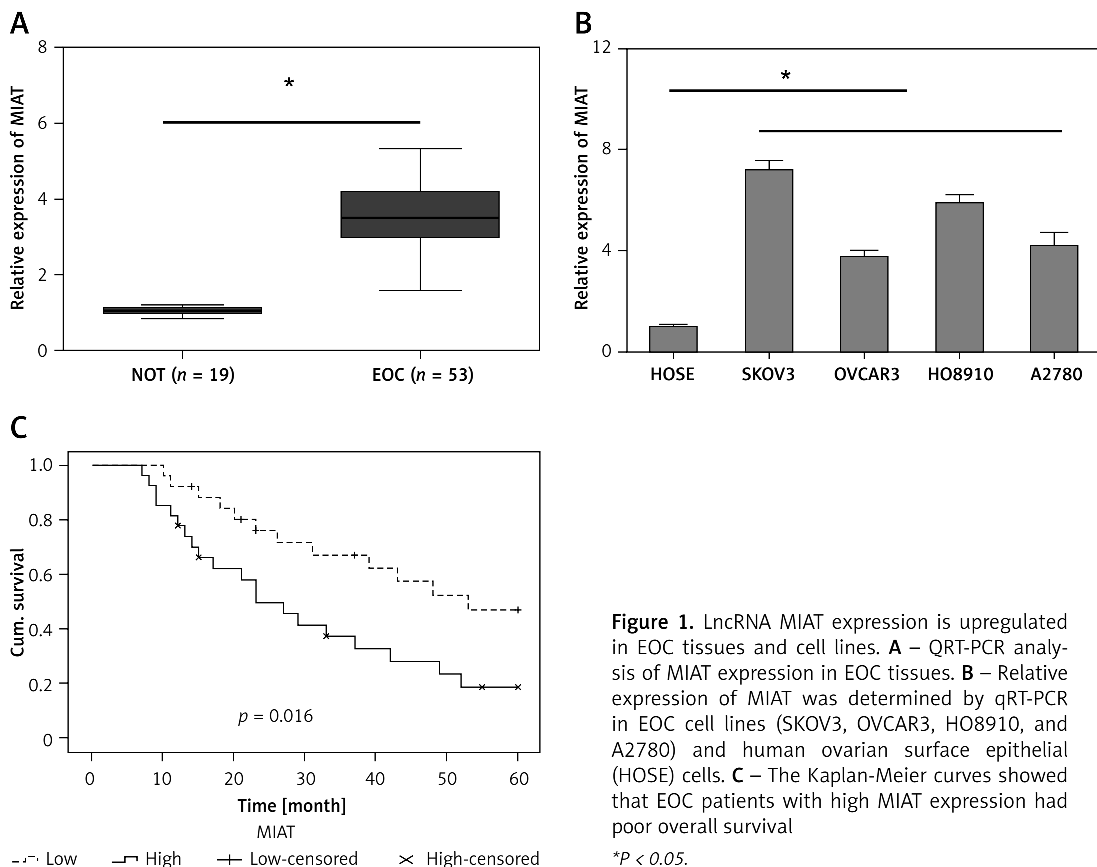

Figure 1. LncRNA MIAT expression is upregulated in EOC tissues and cell lines. **A** – QRT-PCR analysis of MIAT expression in EOC tissues. **B** – Relative expression of MIAT was determined by qRT-PCR in EOC cell lines (SKOV3, OVCAR3, HO8910, and A2780) and human ovarian surface epithelial (HOSE) cells. **C** – The Kaplan-Meier curves showed that EOC patients with high MIAT expression had poor overall survival

**P < 0.05.*

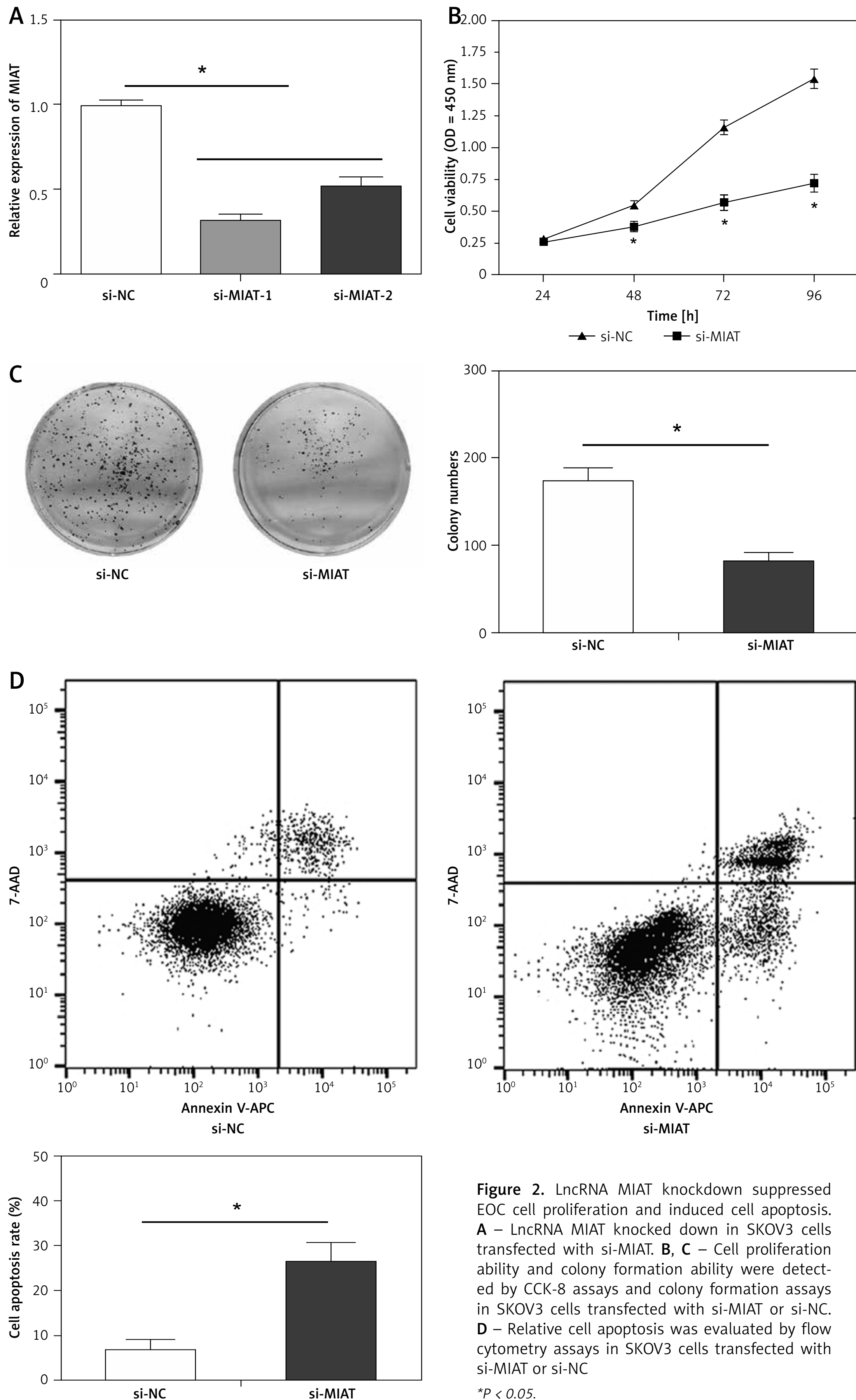

Figure 2. LncRNA MIAT knockdown suppressed EOC cell proliferation and induced cell apoptosis. **A** – LncRNA MIAT knocked down in SKOV3 cells transfected with si-MIAT. **B**, **C** – Cell proliferation ability and colony formation ability were detected by CCK-8 assays and colony formation assays in SKOV3 cells transfected with si-MIAT or si-NC. **D** – Relative cell apoptosis was evaluated by flow cytometry assays in SKOV3 cells transfected with si-MIAT or si-NC

**P < 0.05.*

fied EOC patients into a high MIAT group (n = 27) and a low MIAT group (n = 26). Kaplan-Meier survival analysis showed that patients with higher MIAT expression had significantly reduced overall survival compared with patients with lower MIAT expression (Figure 1 C; $p < 0.05$). Those data indicated that MIAT plays important roles in EOC progression.

LncRNA MIAT knockdown inhibited EOC cell proliferation

To clarify the role of MIAT in EOC cell proliferation and apoptosis, si-MIAT was transfected into SKOV3 cells, and the transfection efficiency was determined by qRT-PCR (Figure 2 A; $p < 0.05$). CCK-8 assays showed that MIAT inhibition significantly decreased SKOV3 cell proliferation (Figure 2 B; $p < 0.05$). Colony formation assays showed that the clonogenic survival was inhibited in si-MIAT transfected SKOV3 cells (Figure 2 C; $p < 0.05$). Furthermore, flow cytometric analysis revealed that MIAT inhibition markedly promoted cell apoptosis in SKOV3 cells (Figure 2 D; $p < 0.05$). Thus, our data indicated that MIAT suppression might inhibit EOC cell proliferation by inducing EOC cell apoptosis.

MIAT directly interacted with miR-330-5p

Increasing evidence has demonstrated that lncRNA might act as a molecular sponge in regulating the biological functions of miRNA [12]. In the present study, bioinformatics prediction (DIANA) results showed that the MIAT sequence harbors a putative miR-330-5p binding region (Figure 3 A).

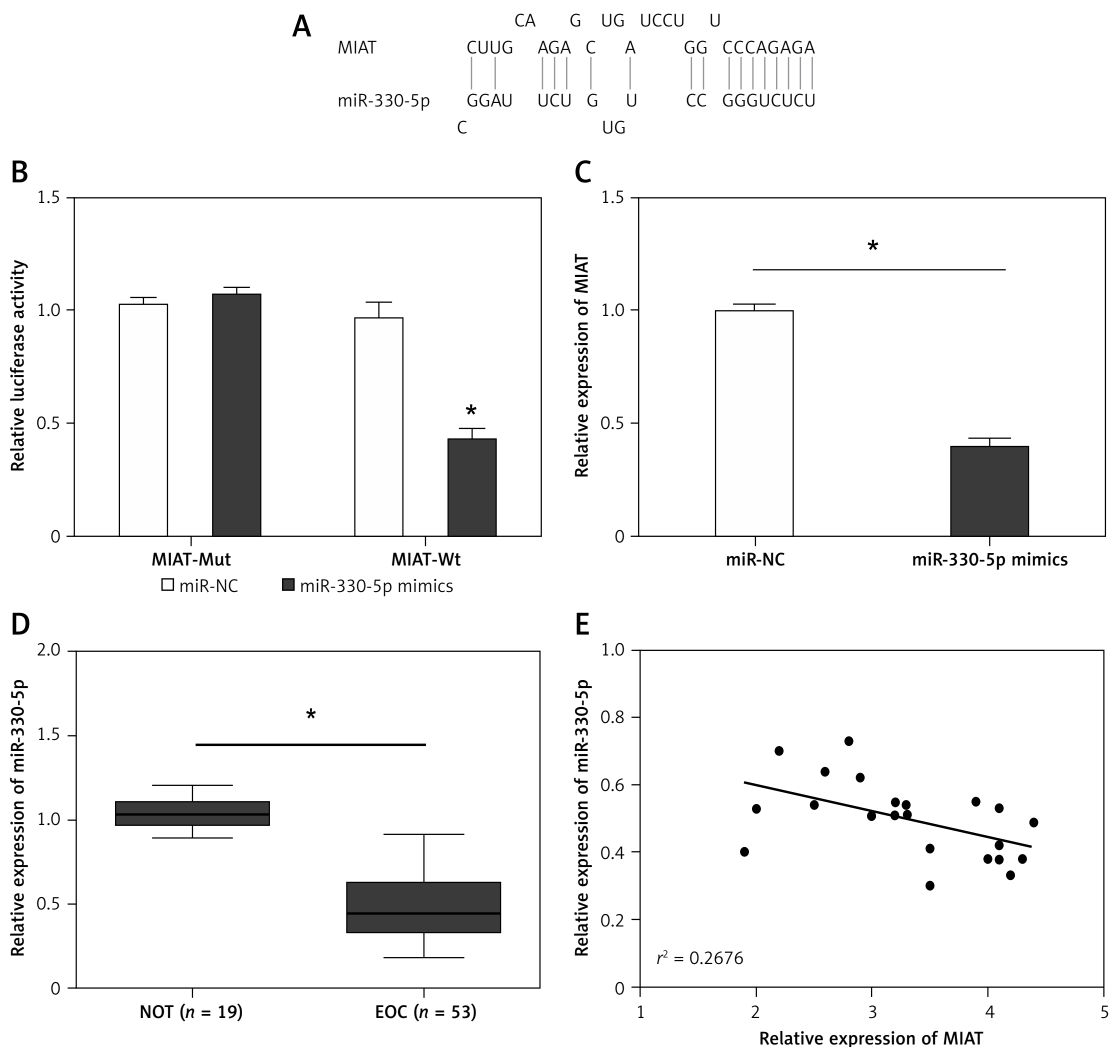

Figure 3. LncRNA MIAT act as a direct target of miR-330-5p. **A** – The sequence of the predicted miR-330-5p targeting site within MIAT 3'-UTR. **B** – MiR-330-5p obviously repressed the luciferase activity of the pMIR-MIAT-Wt but not of pMIR-MIAT-Mut in HEK293T cells. **C** – QRT-PCR analysis of MIAT expression in EOC cells transfected with miR-330-5p mimics. **D** – QRT-PCR analysis determined miR-330-5p expression in EOC tissues. **E** – The correlation analysis showed that miR-330-5p expression was negatively associated with MIAT expression in EOC tissues

**P < 0.05.*

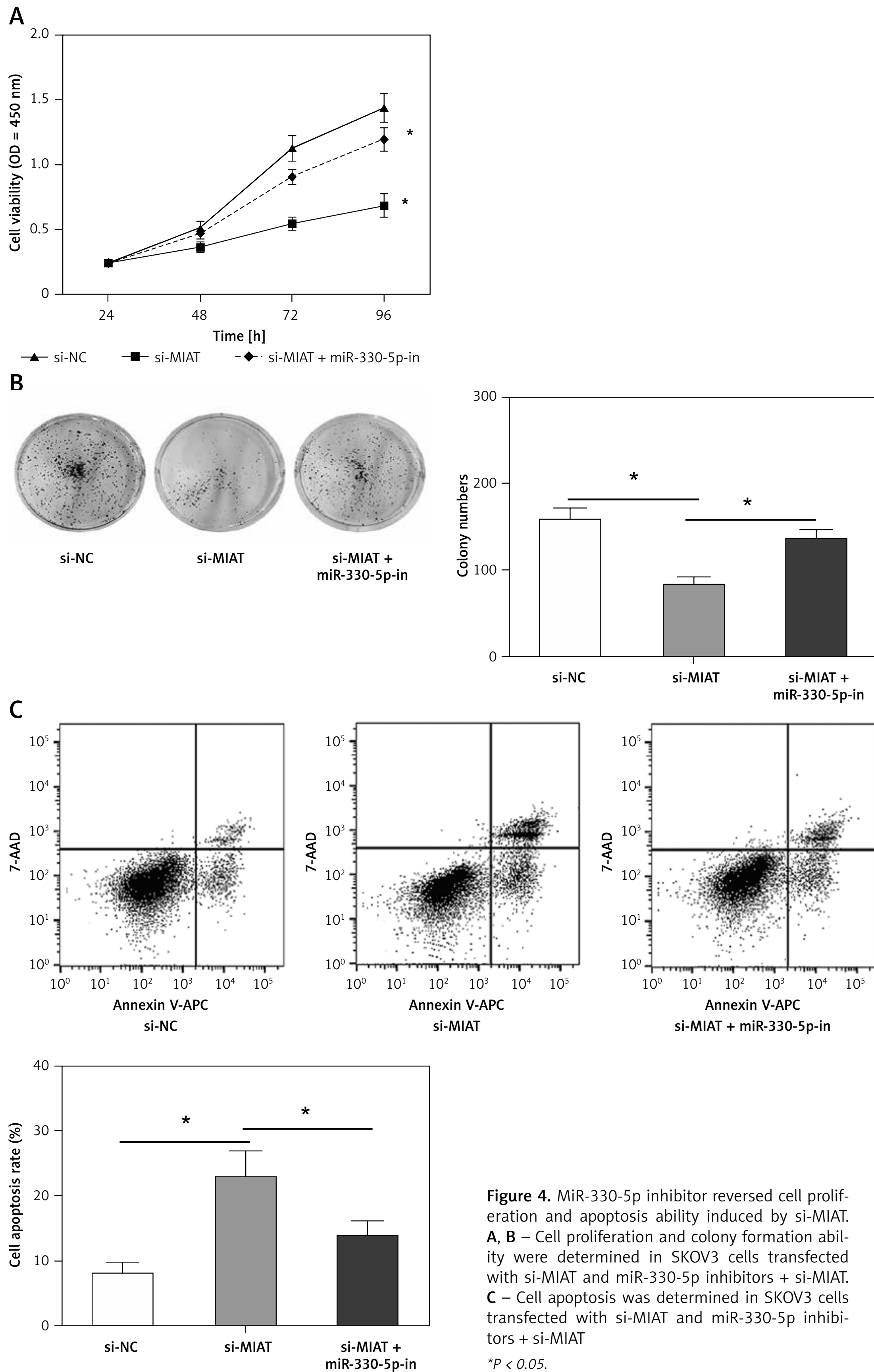

Figure 4. MiR-330-5p inhibitor reversed cell proliferation and apoptosis ability induced by si-MIAT. **A**, **B** – Cell proliferation and colony formation ability were determined in SKOV3 cells transfected with si-MIAT and miR-330-5p inhibitors + si-MIAT. **C** – Cell apoptosis was determined in SKOV3 cells transfected with si-MIAT and miR-330-5p inhibitors + si-MIAT

**P < 0.05.*

To identify whether MIAT is target of miR-330-5p, the wild type sequence of MIAT (Wt-MIAT) or the mutant (Mut-MIAT) with the miR-330-5p recognition sequence was subcloned into the pMIR luciferase reporter, and then transfected into HEK293T cells together with miR-330-5p mimics and miR-NC. The results showed that miR-330-5p mimics decreased the luciferase activity of Wt-MIAT, but not the luciferase activity of Mut-MIAT (Figure 3 B; $p < 0.05$). Furthermore, we explored the correlation between MIAT expression and miR-330-5p. The results showed that MIAT expression was downregulated by transfection with miR-330-5p mimics (Figure 3 C; $p < 0.05$). In addition, the results showed that miR-330-5p was downregulated in EOC tissues and was inversely correlated with the expression of MIAT (Figures 3 D, E; $p < 0.05$). These results indicated that miR-330-5p could directly bind to MIAT and negatively regulate MIAT expression in EOC.

MIAT affected EOC cell proliferation and apoptosis by regulating miR-330-5p

In order to explore whether the effect of MIAT on EOC tumorigenicity is mediated by miR-330-5p, si-MIAT and miR-330-5p inhibitors + si-MIAT were transfected into SKOV3 cells. CCK-8 assays and colony formation assays showed that miR-330-5p inhibitor abrogated the effect of si-MIAT in reducing cell viability (Figures 4 A, B; $p < 0.05$). Meanwhile, the alterations in EOC cell apoptosis caused by silencing MIAT were also rescued by miR-330-5p inhibitors (Figure 4 C; $p < 0.05$). Therefore, our study indicated that MIAT could act as an endogenous sponge by binding to miR-330-5p in EOC cells.

Discussion

Despite the great therapeutic advances made in EOC, including surgical resection and adjuvant therapy, the long-term prognosis of patients with distant metastases remains unfavorable. Increasing numbers of studies have suggested that lncRNAs could serve as effective therapeutic targets for cancer treatment [13]. For example, Feng *et al.* found that RMRP might act as an oncogene and could be used as a therapeutic target for the treatment of glioma [14]. Zhang *et al.* showed that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [15]. Recently, studies showed that lncRNA MIAT has an oncogenic effect in many cancers. For example, Lai *et al.* indicated that MIAT promoted non-small cell lung cancer proliferation and metastasis through MMP9 activation [16]. Luan *et al.* showed that MIAT promoted breast cancer progression and functions as a ceRNA to regulate DUSP7 expression by sponging miR-155-5p [17]. Li *et al.* showed that lncRNA MIAT regulated cell biological behaviors in gastric cancer through a mechanism involving the miR-29a-3p/HDAC4 axis [18]. However, the role of MIAT in EOC is still unclear.

In the present study, we found that MIAT expression was highly expressed in EOC tissues and cell lines. Kaplan-Meier survival analysis showed that patients with high MIAT expression had significantly reduced overall survival compared with patients with low MIAT expression. Moreover, we explored the role of MIAT in EOC cells. Our data showed that MIAT knockdown inhibited EOC cell proliferation and colony formation and induced cell apoptosis. Thus, those results indicated that MIAT was involved in EOC progression.

MiR-330-5p was down-regulated in various tumors, and regulated the development of tumor cells [19]. For example, Su *et al.* showed that miR-330-5p suppressed cell proliferation and invasion in cutaneous malignant melanoma [20]. Kong *et al.* found that miR-330-5p overexpression repressed cell growth of non-small cell lung cancer by inhibiting NOB1 expression [21]. Bibby *et al.* indicated that miR-330-5p acted as a putative modulator of neoadjuvant chemoradiotherapy sensitivity in oesophageal adenocarcinoma [22]. In the present study, our data showed that miR-330-5p was downregulated in EOC tissues, and downregulated expression of miR-330-5p was negatively corrected with MIAT expression in EOC tissues.

Recently, studies have reported that there is a novel regulatory mechanism between lncRNAs and miRNAs. lncRNA might function as a ceRNA or a molecular sponge in modulating miRNA [23]. For example, Cui *et al.* showed that upregulated lncRNA SNHG1 contributed to progression of non-small cell lung cancer through inhibition of miR-101-3p and activation of the Wnt/β-catenin signaling pathway [24]. Wang *et al.* indicated that lncRNA XIST exerted oncogenic functions in human glioma by targeting miR-137 [25]. Xie *et al.* suggested that lncRNA ZFAS1 sponged miR-484 to promote cell proliferation and invasion in colorectal cancer [26]. So we hypothesized that MIAT also has similar effects on EOC progression. Bioinformatics analysis found that the MIAT sequence harbors a putative miR-330-5p binding region. Luciferase activity assays confirmed the direct binding of MIAT to miR-330-5p. QRT-PCR showed that miR-330-5p mimics significantly decreased MIAT expression in EOC cells. Furthermore, we explored whether the effect of MIAT on EOC tumorigenicity is mediated by miR-330-5p. CCK-8 assays and colony formation assays showed that miR-330-5p inhibitor abrogated the effect of si-MIAT in reducing EOC cell proliferation ability. Meanwhile, the alterations in EOC cell apoptosis caused by silencing MIAT were also rescued by miR-330-5p inhibitors.

In conclusion, in this study, we demonstrated that lncRNA MIAT acted as an oncogene in EOC progression by suppressing the expression of miR-330-5p. These data suggested that the MIAT/miR-330-5p axis might act as a potential therapeutic target for the treatment of EOC patients.

Conflict of interest

The authors declare no conflict of interest.

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