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Effects of CircMGAT5 Downregulation on Invasion and MMP-2 Expression in Ovarian Cancer Cells

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Abstract: *Objective:* To elucidate the regulatory effects and mechanisms of circular RNA MGAT5 (circMGAT5) on the invasive ability of human ovarian cancer (OC) cells. *Methods:* Human OC SKOV-3 cells were transfected with circMGAT5 interference plasmids. The expression level of circMGAT5 was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The proliferation and invasive abilities of cancer cells were determined using soft agar colony formation assay and Transwell assay, respectively. The expression of matrix metalloproteinase-2 (MMP-2) at mRNA and protein levels was detected by qRT-PCR and immunofluorescence, respectively. *Results:* CircMGAT5 level in the circMGAT5-transfected group was significantly downregulated in a concentration- and time-dependent manner ($p < 0.005$). Soft agar colony formation assay exhibited that the number of colonies formed in the 6.25, 12.5, and 25.0 nM siRNA groups was significantly reduced in a concentration-dependent manner ($p < 0.005$). Transwell assay revealed that the number of cells penetrating the filter membrane in the 6.25, 12.5, and 25.0 nM siRNA groups was significantly decreased in a concentration-dependent manner ($p < 0.005$). qRT-PCR and immunofluorescence results demonstrated that the mRNA and protein levels of MMP-2 in OC cells of the siRNA-transfected group were significantly decreased. *Conclusion:* circMGAT5 plays an important role in the invasion of OC cells, and its mechanism may be related to the downregulation of MMP-2 expression.

Keywords: Ovarian neoplasms; Circular RNA; Invasion; Matrix metalloproteinase-2

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1. Introduction

Ovarian cancer (OC) is one of the three most common malignant tumors of the female reproductive system, with the highest mortality rate among gynecological malignancies, seriously endangering women's lives and health. Due to its insidious onset, many cases of OC have already metastasized at the time of diagnosis, which leads to a decrease in patient survival rate [1–3]. Therefore, identifying and investigating biological markers for predicting OC

metastasis at the molecular level is of great significance for formulating effective preventive measures, selecting new therapeutic targets, and establishing reasonable and effective comprehensive treatment regimens.

Circular RNA (circRNA) is one of the current research hotspots. The formation of circRNA depends on the back-splicing of precursor mRNA (pre-mRNA). The spliceosome recognizes specific reverse complementary sequences (such as Alu elements), leading to the covalent linkage of the 3' and 5' ends to form a closed loop [4]. Based on their origin, circRNAs can be divided into three categories, firstly the exonic circRNAs (accounting for 70%), such as circ-0001068. Second, the intronic circRNAs, such as ci-ankrd52, and third, the exon-intron circRNAs (EIciRNAs), such as EIciEIF3J [5,6]. Their structural stability stems from the absence of free ends, which avoids degradation by exonucleases, and their subcellular localization exhibits tissue specificity; the cytoplasmic localization is dominant, while a few are localized in the nucleus [7,8]. CircRNAs have the following core biological functions. First, miRNA sponge effect, where they bind to miRNAs through seed sequences, relieving the inhibitory effect of miRNAs on target mRNAs. For example, circRNA051239 contains 4 binding sites for miR-509-5p, and after adsorption, it releases PRSS3 mRNA to promote translation [4]. Next is the RBP molecular scaffold where they bind to RNA-binding proteins (RBPs) to regulate gene expression; for instance, circFoxp1 binds to AUF1 protein to enhance its own stability [9,10]. (Thirdly is their translation template function where some circRNAs contain open reading frames (ORFs), for example, circ-ZNF609 can encode functional proteins [11]. Besides that, they also comprised of transcriptional regulation where the nuclear circRNAs bind to RNA polymerase II to regulate the transcription of parental genes; for example, circEIF3J promotes the expression of the EIF3J gene [12]. In recent years, researchers have found that some circRNAs are abnormally expressed in OC and are associated with poor prognosis [12–14], but their molecular mechanisms remain unclear.

In this study, circMGAT5 interference plasmids were constructed and transfected into human OC SKOV-3 cells to observe the effect on cancer cell invasion and explore the possible mechanism from the perspective of MMP-2.

2. Materials and methods

2.1. Materials

Human ovarian serous papillary cystadenocarcinoma SKOV-3 cells were cryopreserved in the Tissue and Cell Biobank of our hospital. N-cadherin antibody was purchased from Santa Cruz Biotechnology. TRIzol reagent, RNase inhibitor, and transfection reagent Oligofectamine™ 2000 were purchased from Invitrogen (USA).

2.2. Methods

2.2.1. Cell culture and transfection

OC SKOV-3 cells were continuously cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. One day before transfection, cancer cells were seeded into 24-well culture plates at a density of 1.0×10^5 cells/mL, and cultured overnight. Transfection was performed the next day. After transfection, cells were harvested by trypsinization at different time points for subsequent experiments.

2.2.2. Detection of circMGAT5 in OC cells

Total RNA was extracted from cells using TRIzol reagent. A total of 1 μg of total RNA was used to synthesize the first strand of cDNA with oligo dT as the primer, and 2 μL of this cDNA was used as the template for PCR

amplification. The primers for circMGAT5 were as follows.

(a) Forward primer (5'→3'): TGACAGCTTATGGCAATGGGA

(b) Reverse primer: GCGGCCCAAAAGAAAATGGT

The primers for β-actin (internal reference) were as follows.

(a) Forward primer: CTTCGCGGGCGACGAT

(b) Reverse primer: CCACATAGGAATCCTTCTGACC

For qRT-PCR amplification: Using cDNA as the template, qRT-PCR was performed with the SYBR Green I fluorescent dye method. The $2^{-}\Delta\Delta Ct$ method was used to calculate the relative expression levels of circMGAT5 and MMP-2 mRNA.

2.2.3. Colony formation assay

The experiment was conducted with slight modifications according to the method described in the literature [15]. The colony formation rate was calculated using the formula: Colony formation rate = (Number of colonies / Number of seeded cells) × 100%.

2.2.4. In vitro invasion assay

The assay was performed according to the method described in previous literature [15].

2.2.5. Detection of MMP-2

(1) qRT-PCR: The primer for MMP-2 were: forward primer: CCGCCTTTAACTGGAGCAAA; reverse primer: TTTGGTTCTCCAGCTTCAGG. qRT-PCR was used for detection, following the same method as described in Section 2.2.

(2) Immunofluorescence: Immunofluorescence was used. The assay was performed according to the method described in the literature [16]. The intensity of the fluorescent signal (reflecting protein expression level) and localization (cytoplasm, cell membrane, or secretion into the extracellular matrix and other organelles) were analyzed using software.

3. Statistical methods

Data were expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 26.0 software. A p-value < 0.05 was considered statistically significant, and a p-value < 0.01 was considered highly statistically significant.

4. Results

4.1. CircMGAT5 level in the interference plasmid-transfected group was significantly decreased

To investigate the effects of circMGAT5 on invasion of SKOV-3 cells, circMGAT5 interference plasmids were constructed and transfected into OC SKOV-3 cells, and circMGAT5 expression was detected by qRT-PCR. The results exhibited that compared with the control group, the expression level of circMGAT5 in the interference plasmid-transfected group was significantly decreased ($p < 0.001$) (see **Figure 1**).

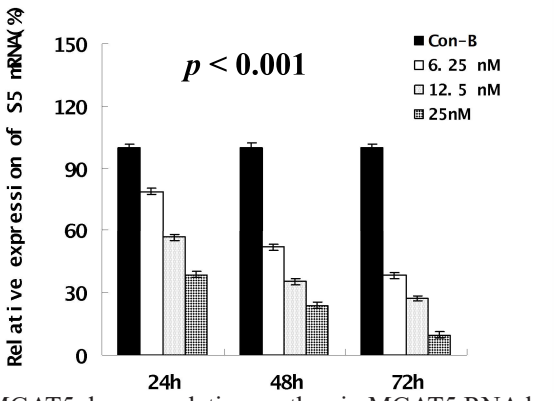

Figure 1. Effect of circMGAT5 downregulation on the circMGAT5 RNA level in OC SKOV-3 cells.

4.2. Transfection with circMGAT5 siRNA inhibited the soft agar colony formation of cancer cells

The results exhibited that OC SKOV-3 cells could spontaneously form colonies in the *in vitro* semi-solid culture system. The soft agar colony formation assay demonstrated a significant, concentration-dependent reduction in the number of colonies formed in the 6.25, 12.5, and 25.0 nM siRNA groups ($p < 0.005$) (see **Figure 2**).

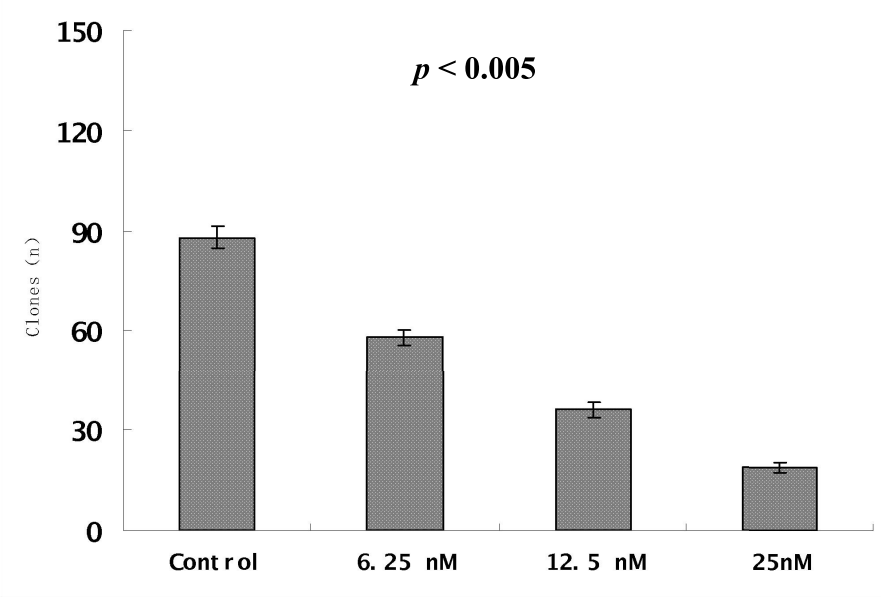

Figure 2. Effect of circMGAT5 siRNA transfection on colony formation of OC SKOV-3 cells.

4.3. Transfection with circMGAT5 siRNA inhibited the invasion of cancer cells

Cells were collected 48 hours after transfection, and the invasion ability of cancer cells was detected by Transwell assay. The results demonstrated a significant, concentration-dependent reduction in the number of invasion cells in the 6.25, 12.5, and 25.0 nM siRNA groups ($p < 0.005$) (see **Figure 3**).

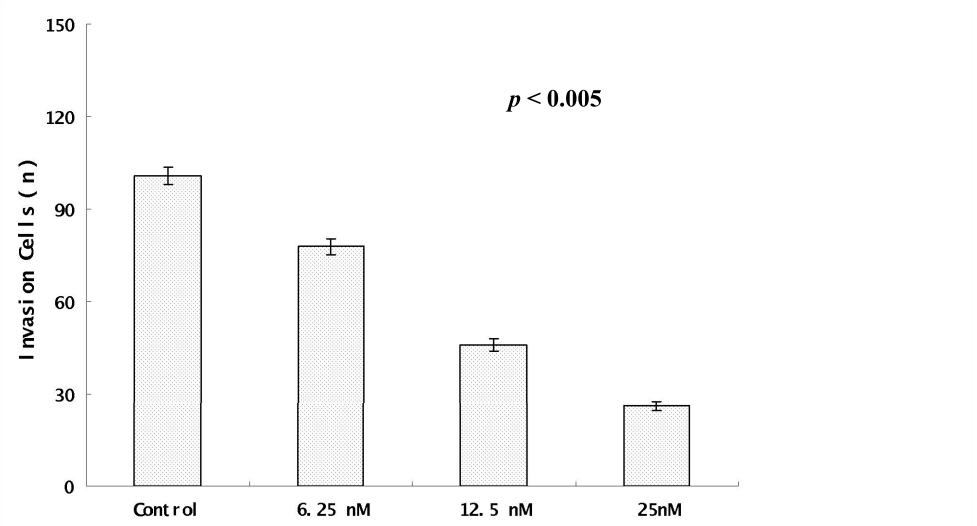

Figure 3. Effect of circMGAT5 siRNA transfection on the invasion of OC SKOV-3 cells.

4.4. Effect of circMGAT5 interference plasmid transfection on the MMP-2 in OC cells

The qRT-PCR and immunofluorescence, respectively, showed that the mRNA level of MMP-2 in the circMGAT5 interference plasmid-transfected group was significantly inhibited (all $p < 0.05$) (see **Figure 4**).

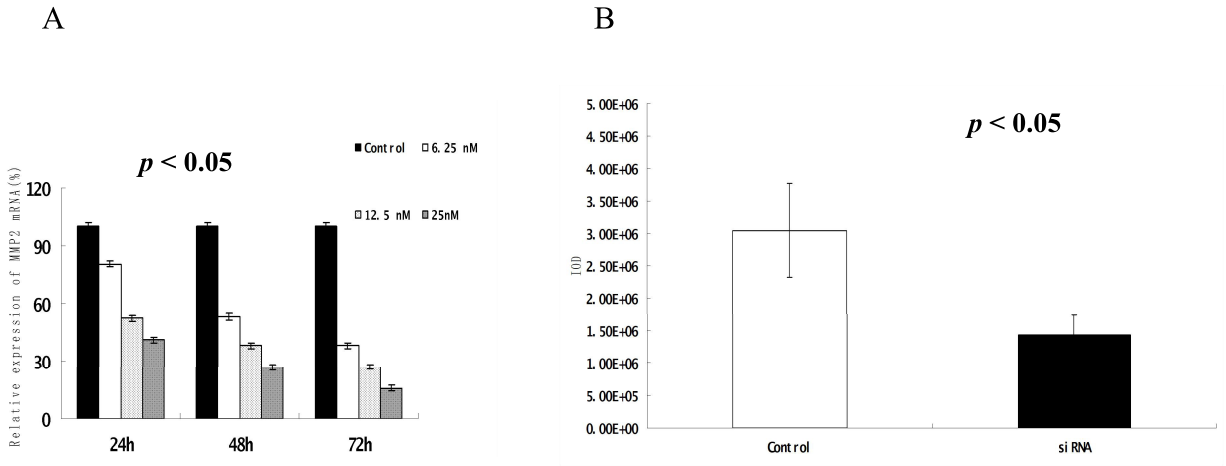

Figure 4. Effect of circMGAT5 siRNA transfection on MMP-2 in OC SKOV-3 cells (A: Detection by qRT-PCR; B: Detection by immunofluorescence).

5. Discussion and limitations

5.1. Discussion

OC accounts for 3.4% of female malignant tumors but contributes to more than 50% of gynecological cancer-related deaths [1]. Its clinical characteristics exhibit "three highs and three lows": high mortality rate where 5-year survival rate of 44%, high recurrence rate , with 70% of patients relapse within 2 years, high rate of late-stage diagnosis, where 70% of cases are diagnosed at stage III/IV; insidious early symptoms including only 20% of patients are diagnosed at stage I/II, limited screening methods, specificity of CA125 is less than 70%, and insufficient targeted drugs where only PARP inhibitors are approved for maintenance therapy [2]. The lack of

early diagnosis results in most patients missing the opportunity for radical surgery, and chemotherapy resistance where platinum resistance rate reaches 50% further exacerbates the treatment dilemma [3]. Therefore, identifying and investigating biological markers for predicting OC metastasis at the molecular level is of great significance for formulating effective preventive measures, selecting new therapeutic targets, and establishing reasonable and effective comprehensive treatment regimens.

To understand the effects and mechanisms of circMGAT5 in the invasion of OC cells, the circMGAT5 interference plasmids were transfected into human OC SKOV-3 cells. The circMGAT5 expression was detected by qRT-PCR, and the results showed that the expression level of circMGAT5 in the transfected group was significantly decreased, indicating successful knockdown and suggesting that this interference plasmid can be used as a powerful tool for studying the function of circMGAT5. Subsequently, we observed the effect of circMGAT5 interference plasmid transfection on the invasion of OC cells. In vitro experiments exhibited that the anchorage-independent proliferation and invasive abilities of cancer cells were significantly reduced, suggesting that downregulation of circMGAT5 can inhibit the invasion of OC cells.

The invasion and metastasis of OC is a multi-step process. It refers to the process by which tumor cells detach from the primary tumor and invade surrounding and/or distant tissues, involving the penetration of tumor cells through the extracellular matrix (ECM), vascular basement membrane, and penetrate the vascular wall into the patient's microenvironment. A large number of experiments have confirmed that the invasive and metastatic abilities of tumor cells are closely related to their ability to induce the production of proteases that degrade the ECM and basement membrane. These proteases include matrix metalloproteinases (MMPs), the most important group that play a pivotal role in tumor invasion and metastasis, acting as "molecular scissors" in this process. MMP-2, also known as gelatinase A, is a key enzyme that degrades the ECM and basement membrane. In OC, its overactivation is a core link in the acquisition of invasive, migratory, and metastatic abilities by tumor cells. MMP-2 can degrade the ECM and basement membrane, promote vasculogenic mimicry (VM), and open up channels for cancer cell invasion and metastasis [17,18]. MMP-2 is precisely regulated by TIMP-2, with dual effects, miRNAs such as miR-34a, transcription factors such as SOX15, and signaling pathways such as Substance P [17–20]. MMP-2 is associated with poor prognosis (stage, metastasis, drug resistance, survival rate) and has been used as a biomarker for diagnosis/differential diagnosis (in combination with CA125/HE4) [19,20].

With the aim of exploring the molecular mechanism through which circMGAT5 modulates OC cell invasion, this study centered on measuring the expression level of MMP-2. The results of qRT-PCR and immunofluorescence showed that downregulation of circMGAT5 could significantly reduce the mRNA and protein expression levels of MMP-2 in SKOV-3 cells, suggesting that circMGAT5 may affect the invasive ability of OC cells by regulating the expression of MMP-2. Combined with existing studies, this study speculates that circMGAT5 may regulate the expression of MMP-2 through the following pathways:

(1) Acting as a miRNA sponge to adsorb miRNAs that target MMP-2, thereby relieving the inhibitory effect of miRNAs on MMP-2 mRNA and further promoting the expression of MMP-2;
(2) Interacting with transcription factors or RNA-binding proteins that regulate MMP-2 expression, thereby affecting the transcription or translation process of MMP-2;
(3) Indirectly influencing the expression of MMP-2 by regulating upstream and downstream signaling pathways (such as PI3K/Akt, MAPK, etc.).

However, the specific molecular mechanism by which circMGAT5 regulates MMP-2 expression still needs to be further verified through techniques.

5.2. Limitations and future perspective

This study has certain limitations. Firstly, this study only explored the function of circMGAT5 in the SKOV-3 cell line, lacking verification in other OC cell lines and clinical samples, so the universality of its conclusions needs to be further confirmed. Secondly, this study only initially confirmed that circMGAT5 can reduce the invasive ability of OC cells by downregulating the expression of MMP-2, and the specific molecular mechanism by which circMGAT5 regulates MMP-2 has not been clarified. Finally, this study lacks animal experiments to verify the effect of circMGAT5 on OC metastasis in vivo.

Future research will focus on the following directions, first, expanding the scope of cell lines and clinical samples to verify the expression and clinical significance of circMGAT5 in OC; secondly, clarifying the specific molecular mechanism by which circMGAT5 regulates MMP-2 through molecular biology experiments; thirdly, constructing animal xenograft models to verify the effect of circMGAT5 on OC metastasis in vivo, so as to provide more sufficient evidence for its clinical transformation and application.

6. Conclusion

In summary, this study is the first to verify that circMGAT5 serves a key regulatory function in the invasive process of OC cells; specifically, downregulating circMGAT5 can markedly diminish the invasive capacity of these cells by suppressing MMP-2 expression. The findings not only offer a novel perspective for deeply comprehending the molecular mechanisms underlying OC invasion and metastasis but also provide a potential molecular target for the targeted treatment of this disease.

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Disclosure statement

The authors declare no conflict of interest.

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