Original Article

Inhibitory effects of miRNA-133a on the development and metastasis of ovarian cancer cells by targeted regulation of TGF-β1/CTGF signaling pathways

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Abstract: Objective: The present study aimed to explore the inhibitory effects of microRNA-133a on the development and metastasis of ovarian cancer, investigating its mechanisms. Methods: Surgical specimens of 48 ovarian cancer patients were gathered. Ovarian cancer SKOV-3 cells were divided into the experimental group (EG), transfected with microRNA-133a mimic, and the control group (CG), transfected with nonsense microRNA. Expression levels of microRNA-133a were measured using RT-PCR. Moreover, CCK8, Transwell, and flow cytometry were adopted to measure changes in cell proliferation, invasion, and apoptosis. Western blotting was adopted to measure expression levels of TGF-β1 related protein. Results: Relative expression levels of microRNA-133a of the ovarian cancer tissues were lower than those of adjacent tissues (P<0.05). Compared with the control group, overexpression of microRNA-133a remarkably inhibited cell proliferation and invasion in the experimental group (P<0.05). Expression of TGF-β1 of SKOV-3 tissues was remarkably higher than that of adjacent tissues (P<0.05), being negatively correlated with expression of microRNA-133a (r=-0.684, P<0.05). Compared with the EG, invasion and migration of SKOV-3 cells were decreased. Expression levels of mRNA and proteins of TGF-β1 and CTGF were decreased in the EG (P<0.05). Compared with microRNA-133a+CG, invasion and migration of cells in the microRNA-133a mimic+TGF-β1 group were significantly raised (P<0.05). Conclusion: Low expression of microRNA-133a in SKOV-3 tissues may inhibit the development and metastasis of ovarian cancer via regulating the activation of TGF-β1 signaling pathways.

Keywords: miRNA-133a, TGF-β1, CTGF, ovarian cancer

Introduction

Ovarian cancer is one of the most common gynecological malignancies after cervical and uterine cancers. Its mortality rate is the highest among gynecological malignancies, with 5-year survival rates of around 30%. Ovarian cancer is a serious threat to the health of women [1]. Due to the insufficiency of effective early diagnosing methods and sensitive tumor markers, symptoms, such as abdominal distension and abdominal pain, often occur in the middle and late stages [2]. At present, multi-disciplinary combined therapies, including surgery, radiotherapy, chemotherapy, and molecular targeted therapy, are widely used in clinic. However, these are still unable to prevent the recurrence and metastasis of ovarian cancer, leading to high morbidity and mortality rates in China. Extensive metastasis of the abdominal cavity and whole body is an important cause of death. However, the specific mechanisms of metastasis remain unclear, making treatment of advanced ovarian cancer a bottleneck [3]. Therefore, it is of great significance to find targets closely related to incidence and development of the tumors, aiming to clarify potential mechanisms of tumor metastasis. The hope is to promote the research of new methods in ovarian cancer treatment, ultimately improving patient prognosis.

MicroRNAs (microRNAs) are endogenous non-coding small RNAs with 19-25 nucleotides, with no function of coding proteins. However, they can bind to the 3' untranslated region (3'UTR) of the messenger RNA (mRNA). This can reduce the stability of mRNA and negatively regulate
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expression levels of target gene mRNA [4, 5]. The main function of microRNAs is to regulate growth, proliferation, differentiation, apoptosis, aging, death, and other life processes of cells [6, 7]. Some studies have confirmed that the physiological mechanisms of microRNA-133a are similar to those of “tumor suppressor gene”, which can suppress the proliferation and survival of various tumor cells. Moreover, the downregulation of microRNA-133a in tumor cells has been correlated with development and poor prognosis of malignancies [8]. Whether microRNA-133a plays the role of a “tumor suppressor gene” in ovarian cancer, as well as its role and mechanisms in the development and metastasis, are still indistinct. The current study was conducted to explore expression levels of microRNA-133a in ovarian cancer cell lines and its biological function in the development and metastasis of tumor cells. The aim of the current study was to investigate its mechanisms, providing an experimental basis for clinical research on new targets for the treatment of ovarian cancer.

Material and methods

Materials

Specimens: A total of 48 specimens of ovarian cancer and adjacent cancer tissues (more than 4 cm from the tumor margin) were gathered between January 2016 and December 2017. No chemotherapy was performed before the operation. The specimens were reserved in liquid nitrogen at -80°C. Patients provided informed consent and all clinicopathological data was completely preserved. The present study was performed under the approval of the Medical Ethics Committee.

Reagents: DMEM Medium (Gibco Company, USA); Lipofectamine 2000 (Shanghai Runcheng Biotechnology Co., Ltd., Shanghai, China); microRNA-133a mimics, nonsense microRNA, and its primers (Bioneer Trading Co., Ltd., Shanghai, China); miRNeasy Mini Kit (Beijing Xiangyue Huanyu Science and Technology Development Co., Ltd., Beijing, China); Transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) antibodies (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China); GAPDH antibodies and second antibodies (Shanghai MultiSciences Biotechnology Co., Ltd., Shanghai, China); Rabbit anti-human TGF-β1 antibody, mouse anti-human GAPDH antibody, and luciferase reporter vector (TGF-β1-wt and TGF-β1-mu) in 3'-UTR of mutant and wild type TGF-β1, microRNA-133a mimics, and over-expression plasmids of and TGF-β1 were packaged and synthesized by Shanghai Genechem Co., Ltd.; Transwell cell and protein lysis reagent (Corning Company, USA); Annexin V-FITC-PI apoptosis detection kit (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, China).

Methods

Culture and treatment of cells

SKOV-3 of ovarian cancer was cultured in an incubator at 37°C with 5% total humidity of carbon dioxide. The DMEM media contained 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Lipofectamine 2000 was used as the transfection reagent. SKOV-3 cells in the experimental group (EG) were transfected with microRNA-133a mimics at a concentration of 5×10^-8 mol/L. SKOV-3 cells in the control group (CG) were transfected with nonsense microRNAs at a concentration of 5×10^-8 mol/L. In the microRNA-133a mimic+TGF-β1 group, all SKOV-3 cells were cultured in an incubator.

RT-PCR was used to detect transfected cells for 48 hours. The cells were collected to extract RNA. The sequence of TGF-β1 gene primers was designed by Premier 5 software. Forward primers were 5'-CCAAGGACGGAATACAGG-3', while the reverse primers were 5'-TGAGGAGCAGGAAGGGGTC-3'. According to miScriptIIRT kit instructions, the RNA was reversely transcribed to cDNA and quantified by fluorescence PCR. With GAPDH as internal reference gene, the 2^ΔΔCt method was applied to calculate relative expression levels of microRNA-133a, where ΔΔCt = ΔCt (sample) - ΔCt (internal reference). The experiment was duplicated three times.

CCK8 assay for cell proliferation

After 24 hours of transfection, the cell suspension was prepared (density: 1×10^5/mL) and inoculated on 96-well plate with 0.1 mL/well. CCK8 reagent was added once every 24 hours. Absorbance (OD) levels of each well at 450 nm were measured after 1 hour of dark culturing. This was repeated three times. A cell proliferation curve was plotted according to the obtained data.
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Transwell assay for cell migration

After transfecting for 24 hours, synchronized cells were cultivated for 12 hours in the serum-free media. FBS-free cell suspension with a density of 1×10⁴/mL was prepared. The routine Transwell chamber was glued and the margin of plate was patted. The suspension was poured to the upper chamber with 0.2 mL/well and cultivated for 24 hours. A chemotactic inducer was decanted into the lower chamber, containing 10% FBS culture media. The slide was rinsed three times, fixed with 4% poly-formaldehyde, rinsed three more times, and dyed with 0.1% crystal violet dye solution. Photos were taken and the numbers of cells were calculated using the microscope.

Flow cytometry for cell apoptosis

After transfecting for 24 hours, the cells were cultured in the serum-free media for 24 hours. They were collected and re-suspended with buffer to form a suspension with a density of 1~5×10⁶/mL. The suspension of 0.1 mL was decanted into the flow tube and stored at 4°C in dark. Apoptosis of the cells was measured via Annexin V/PI double staining. Flow data were processed with Flow Jo software.

Western blotting for detection of expression of TGF-β1/CTGF signaling pathway-related proteins

After transfecting for 48 hours, the total cell protein was extracted and transferred to PVDF membranes by SDS-PAGE electrophoresis. The protein was placed in 5% skimmed milk powder at 37°C for 1 hour. The first antibody [TGF-β1, CTGF antibodies (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China); diluted concentration 1:50] was added. The protein was incubated overnight at 4°C and washed for 10 minutes, a total of three times. The second antibody [GAPDH antibodies and second antibodies (Shanghai MultiSciences Biotechnology Co., Ltd., Shanghai, China); diluted concentration 1:1000], labeled with horse-radish peroxidase, was added. The protein was incubated at 37°C for 1 hour. ECL luminescence was adopted for development. Image J software was applied to analyze relative expression levels of target proteins.

Dual luciferase reporter assay

The amplified 3'UTR sequence and mutant 3'UTR sequence of TGF-β1 were cloned into pGL3.0 plasmid to construct wild-type TGF-β1-3'UTR (wt) and mutant-type TGF-β1-3'UTR (mu) reporter vectors. The cells were co-transfected with microRNA-133a mimic and cultured for 48 hours. Fluorescence values were detected.
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using a Dual-Glo dual luciferase detection system.

Statistical analysis

SPSS19.0 statistical software was adopted for statistical analysis of present data. Student’s t-tests were used to analyze measurement data (expressed as mean ± SD), while χ² tests were applied to analyze enumeration data. P<0.05 indicates statistical significance.

Results

Relative expression of microRNA-133a in ovarian cancer tissues and SKOV-3 cell lines

Relative expression levels of microRNA-133a in ovarian cancer tissues (0.725±0.172) were lower than those in adjacent tissues (0.983±0.176) (t=7.264, P=0.000, Figure 1A). Compared with the CG, expression of miRNA-133a in the EG was upregulated after transfecting SKOV-3 with miRNA-133a mimics (0.992±0.188 vs 1.671±0.455; P<0.001, Figure 1B).

Effects of overexpression of microRNA-133a on proliferation of SKOV-3 cells

After 48 and 72 hours of transfection, the proliferation of SKOV-3 cells of the EG was remarkably lower than that of the CG, suggesting that overexpression of microRNA-133a can significantly suppress the proliferation of SKOV-3 cells (P<0.05, Figure 2).

Effects of overexpression of microRNA-133a on invasion of SKOV-3 cells

The Transwell invasion experiment revealed that the number of cell-penetrating cells of the EG (53.543±12.250)
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was remarkably lower than that of the CG (120.544±15.672) (t=23.336, P<0.001, Figure 3), indicating that overexpression of microRNA-133a can significantly inhibit invasion of SKOV-3 cells.

Effects of overexpression of microRNA-133a on migration of SKOV-3 cells

Scratch testing revealed that healing levels of cell scratches of the EG were remarkably lower than those of the CG (P<0.05, Figure 4), implying that overexpression of microRNA-133a can inhibit migration of SKOV-3 cells.

Effects of overexpression of microRNA-133a on apoptosis of SKOV-3 cells

Flow cytometry revealed that the apoptotic rate of the EG (28.542±0.847)% was remarkably higher than that of the CG (7.205±0.226)% (t=168.631, P<0.001, Figure 5), suggesting that overexpression of microRNA-133a can significantly inhibit apoptosis of ovarian cancer SKOV-3 cells.

Dual-luciferase prediction and validation of TGF-β1 as a target gene of microRNA-133a

A target scan network database was applied to predict the target gene of microRNA-133a. It was found that TGF-β1 and microRNA-133a had a similar 3'-UTR binding sequence (Figure 6A), suggesting that TGF-β1 directly targeted microRNA-133a. At the same time, microRNA-133a-mimics and TGF-β1 were co-transfected into ovarian cancer SKOV-3 cells. Luciferase reporter gene assay results showed that microRNA-133a-mimic significantly inhibited luciferase activity (Figure 6B).

Additionally, RT-PCR and Western blotting showed that expression levels of mRNA and proteins of TGF-β1 of the EG were remarkably lower than those of the CG (P<0.05, Figure 6C, 6D). Present results suggest that microRNA-133a directly regulated TGF-β1.

Effects of TGF-β1 on inhibition of invasion and migration of SKOV-3 cells mediated by microRNA-133a

Compared with the microRNA-133a mimic+CG group, expression levels of TGF-β1 and CTGF downstream proteins in TGF-β1 mRNA signaling pathways of SKOV-3 cells in the microRNA-133a+TGF-β1 group were remarkably raised (P<0.05, Figure 7A, 7B). The numbers of cell-penetrating cells and scratch healing in the Transwell chambers were significantly increased (P<0.05, Figure 7C, 7D), indicating that microRNA-133a can suppress the invasion and migration of tumor cells via downregulating TGF-β1 and CTGF.
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Figure 6. Dual-luciferase prediction and validation of TGF-β1 as the target gene of microRNA-133a. A: TGF-β1 has similar 3′-UTR binding sequence to miRNA-133a; B: Luciferase activity in cells was shown by Luciferase reporter gene assay; C: TGF-β1 mRNA was detected by RT-PCR; D: TGF-β1 protein expression was detected by Western blotting. Note: Compared with the CG, *P<0.05.

Discussion
Ovarian cancer is one of the most common malignancies in women. It is the most deadly gynecological malignancy with the highest mortality rate. About 70% of patients with ovarian cancer are diagnosed at the advanced stage. Thus, they have missed the best opportunity for treatment [9, 10]. Since the 1970’s, cytoreductive surgery and postoperative chemotherapy, combined with platinum and taxanes, have become the standard treatment for ovarian cancer. Although the scheme has improved the prognosis of patients, the therapeutic effects of ovarian cancer were poor. This was due to the recurrence of the disease, drug resistance, and non-specific molecular targeted treatment, with an overall 5-year survival rate of 30.60% [11-13]. Therefore, investigating the mechanisms of ovarian cancer metastasis is of positive significance, exploring new ideas for treatment of ovarian cancer. Some studies have found that there are about 30 kinds of microRNAs with abnormal expression in ovarian cancer. This factor plays a vital role in the incidence and development of tumors, as well as the invasion and migration of tumor cells [14-16].

Moreover, microRNA-133a has been demonstrated to be downregulated in esophageal squamous cell carcinoma, bladder cancer, colorectal cancer, breast cancer, and other solid tumors. Additionally, microRNA-133a has been shown to have the effects of a tumor suppressor gene and tumor suppressor factor in many tumors. Guo N et al. [17] found that expression of microRNA-133a inhibited the proliferation and invasion of lung cancer cells via targeting AKT/ERK signaling, regulating EGFR. In colorectal cancer, Ruebel K et al. [18] believed that expression of microRNA-133a was remarkably downregulated in the development of tumors. Results implied that microRNA-133a is a tumor suppressor factor and is low-expressed in many solid tumors or cell lines. However, expression of microRNA-133a in ovarian cancer and its mechanisms have not been clearly reported. SKOV-3 is the main tissue type of ovar-
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ian cancer. Results suggest that expression of microRNA-133a in SKOV-3 tissues was remarkably lower than that in adjacent tissues, revealing that low expression of microRNA-133a in SKOV-3 tissues may be related to tumor suppressor factors. In addition, in the present study, microRNA-133a and its control were synthesized and transfected into ovarian cancer SKOV-3 cell line. The Transwell chamber and scratch tests showed that overexpression of microRNA-133a suppressed the invasion and migration and accelerated the apoptosis of SKOV-3 cells, indicating that microRNA-133a provides anti-cancer effects in ovarian cancer tissues. Thus, it suppresses the invasion and migration of tumor cells. A microRNA usually has multiple target genes with different biological functions. Thus, microRNAs play a corresponding biological function by regulating target genes. In the present study, there were binding sites of microRNA-133a in the promoter region of TGF-β1 by bioinformatics prediction. TGF-β1 was identified as the target gene of microRNA-133a via dual-luciferase assay. TGF-β1/CTGF signaling pathways participated in regulating cell differentiation, migration, and growth, playing a vital role in tumorigenesis. TGF-β is a multifunctional peptide growth fac-

Figure 7. Effects of TGF-β1 on the inhibition of SKOV-3 cell invasion and migration mediated by microRNA-133a. A: RT-PCR was used to detect TGF-β1 expression after transfection of SKOV-3 cells with TGF-β1 and microRNA-133a mimic; B: Western blotting was used to measure TGF-β1 protein expression after transfection of SKOV-3 cells with TGF-β1 and microRNA-133a mimic; C: Transmembrane cell number after transfection of SKOV-3 cells with TGF-β1 and microRNA-133a mimic; D: Cell scratch healing after transfection of SKOV-3 cells with TGF-β1 and microRNA-133a mimic. Note: Compared with the CG, *P<0.05.
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Disclosure of conflict of interest

None.

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