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Original Article

TGF beta1 upregulates the expression of MACC1 to promote invasion and metastasis of ovarian cancer

Bingqin Guo[1,2], Nan Yao[1,2], Huaiyong Gan[1,2], Lan Yu[1,2]

[1]*Department of Pathology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233003, P. R. China;* [2]*Pathology Teaching and Research Section, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233003, P. R. China*

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Abstract: Aims: This study is to investigate the effects of transforming growth factor-β1 (TGF-β1) and metastasis associated in colon cancer protein 1 (MACC1) on ovarian cancer. Methods: The ovarian cancer tissues were collected from 60 ovarian cancer patients, and the expressions of TGF-β1 and MACC1 were determined with quantitative PCR. The effects of TGF-β1 on the expression of MACC1 and epithelial-mesenchymal transition (EMT)-related markers in the ovarian cancer cell line A2780 were determined with quantitative PCR and Western blot. Its effect on the proliferation of A2780 was measured by a CCK-8 kit; on the cell invasion and migration was analyzed by Transwell technique; and on the cell cycles was analyzed by flow cytometry. Results: The expression levels of TGF-β1 and MACC1 in the ovarian tissues were obviously upregulated. For the *in vitro* experiment, the EMT related genes changed obviously and EMT occurred in the ovarian cancer cell line A2780 after TGF-β1 treatment, meanwhile the expressions of MACC1 and c-Met genes were obviously upregulated. Results also showed that TGF-β1 could promote the proliferation of the ovarian cancer cells, accelerate the G1/S phase transition and enhance their invasion and metastasis. However, when MACC1 was down regulated by siRNA, the ability of TGF-β1 to promote tumor growth, invasion and metastasis was obviously inhibited. Conclusions: The expressions of TGF-β1 and MACC1 in ovarian cancer were positively correlated and closely related to the occurrence and development of ovarian cancer. TGF-β1 might promote the occurrence and development of ovarian cancer through upregulating the expression of MACC1.

Keywords: Ovarian cancer, TGFβ1, invasion and metastasis

Introduction

Ovarian cancer is one of the most common gynecologic cancers in clinic with a high mortality rate [1]. Because ovarian cancer tissues locate at deep pelvic and the patients have no obvious symptom at the early stage, most of them are at the tumor progression stage when first diagnosed. The recurrence and metastasis of ovarian cancer may lead to death of the patients [2, 3]. It was shown that the 5-year survival rate of early-stage ovarian cancer was up to 90%, while that of medium and late stages were obviously declined [4]. Therefore, in-depth study on the molecular mechanism of the recurrence and metastasis of ovarian cancer has great significance to early diagnosis and treatment.

Currently, researches have confirmed that the invasion and metastasis of tumor are closely related to epithelial-mesenchymal transitions (EMT) [5, 6]. TGF-β1 is an important secretary protein which is abnormally upregulated in many tumor tissues [7]. The TGF-β1-mediated TGF-β/Smad pathway plays important roles in the EMT process [8]. Metastasis-associated in colon cancer 1 (MACC1) gene was first found in colon cancer tissue samples by American scientist Stein using genome-wide expression analysis [9]. Researches indicated that MACC1 is a nuclear transcription factor, which could bind to the promoter of c-Met gene after entering cell nuclear, and thus upregulate the expression of c-Met protein [10, 11]. The c-Met protein is a receptor of hepatocyte growth factor (HGF), and its expression level directly influences the activity of HGF/c-Met signaling pathway [12]. This indicates that MACC1 can activate HGF/c-Met signaling pathway through upregulation of the expression level of c-Met, and thus promote

the invasion and metastasis of cancer cells [13, 14]. Current researches indicate that MACC1 is a new tumor metastasis-related gene, which is obviously increased in multiple tumors, and is positively correlated to tumor stages, lymph node metastasis and differentiation [12, 15, 16]. TGF-β/Smad signaling pathway can activate a large number of downstream oncogenes, which plays important roles in the occurrence and development of ovarian cancer [17]. However, its relation to MACC1 is still unclear.

In this paper, the expressions of TGF-β1and MACC1 in ovarian cancer were studied, and their roles in the ovarian cancer were investigated in order to identify new mechanisms of the invasion and metastasis of ovarian cancer.

Materials and methods

Collection of ovarian cancer tissue samples

A total of 60 fresh epithelial ovarian cancer tissues and corresponding peritumoral tissues were collected from the surgery resection during December, 2012 and February, 2014. The tissues were stained with HE, and then diagnosed and classified by two pathologists according to the standard in the World Health Organization (WHO) Classification of Tumors in 2003. According to the International Federation of Gynecology and Obstetrics (FIGO) staging standards and WHO grading standards, there were 21 Stage I, 16 Stage II, 23 Stage III patients. There were 31 patients with high and medium differentiation, and 29 patients with low differentiation. The peritumoral tissues were obtained more than 5 cm from the cancer tissues. The tissues were frozen with liquid nitrogen and stored at -80°C for further analysis. The clinical information and pathological data of the patients were collected. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of Bengbu medical college.

Cell culture

Ovarian cancer cell line A2780 was cultured in RPMI-1640 medium at 37°C in an atmosphere with 5% CO_2, and the medium was changed every two days. The cells were passaged when growth density reaches 80-90% confluent. The ovarian cancer cells at passage 3-6 were used.

Regents

Trizol for the extraction of the total RNA of the tissue cells was purchased from Invitrogen Co. (Carlsbad, CA, USA). Rabbit anti-human Vimentin, rabbit anti-human E-Cadherin polyclonal antibodies and murine anti-human GAPDH monoclonal antibody were purchased from Bioworld Technology Inc. (St Louis Park, MN, USA). Takara Prime Script RT regent Kit and SYBR Prime Script RT-PCR Kit were obtained from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). Cell counting kit-8 (CCK-8) was purchased from Beyotime Biotechnology Co. (Beijing, China).

QRT-PCR analysis

The ovarian cancer tissues and the peritumoral tissues were ground in liquid nitrogen, and then the total RNA was extracted with Trizol. The RNA was transcribed into cDNA with Takara Prime Script RT Regent Kit. A relative quantitative analysis was performed using qRT-PCR on a Step one Plus quantitative PCR instrument. The primers used for the PCR were as follows: F: 5' CCCGGGTTTGGTAGAGTGAC-3'; R: 5' GGAAGTGAGCCCAAGCTTCT-3'.

TGF-β1-induced ovarian cancer EMT model

The ovarian cancer cell A2780 was divided into negative control and TGF-β1 groups. For the TGF-β1 group, 10 ng/ml TGF-β1 was added into the 1640 RPMI. Cells were cultured at 37°C in an atmosphere with 5% CO_2 for 72 h, and then lysed with RIPA lysis solution containing PMSF. The MACC1 and EMT genes and their protein expression levels were detected with quantitative PCR and Western blot after 72 h transfection.

SiRNA interference

The ovarian cell A2780 was divided into negative control and siRNA-MACC1 groups, and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). When the cell confluency reached 70-90%, the macc1 siRNA was added to the cells. After 6 hours, the medium was changed to fresh RPMI-1640 containing 10% FBS. The cells of each group were lysed with RIPA lysis solution containing PMSF after 48 hours routine culture. The MACC1 protein expression level in the cells of each group was

detected by Western blot after 48 h transfection.

Detection of the cell proliferative ability

The cells were divided into negative control (NC), TGF-β1 and TGF-β1+siRNA-Macc1 groups. For each group, cells were seeded on a 96-well plate at a concentration of 1000/well. The experiment was performed in triplicate. The CCK-8 reaction solution was added every 24 hours and incubated for 30 min, and then the absorbance at 490 nm of each well was measured. The experiment was performed for three consecutive days, and a cell proliferation curve was generated.

Transwell experiment

The 24-well Transwell chamber with a diameter of 8 μm (Corning Co., NY, USA) was used in this experiment. In each upper well, 200 μL serum-free 1640 RPMI medium containing 2×10^5 ovarian cancer cells was added, while in each lower well, 500 μL 1640 RPMI medium containing 10% FBS was added. After 24 hours of routine culture, the chamber was taken out, and the cells in the upper wells were removed. The cells in the lower wells were fixed with 4% formaldehyde for 10 min, stained with Giemsa, and observed under a microscope. Under the 200 × objective, 5 views were randomly selected to take photographs. The number of transmembrane cells was counted to evaluate the migration ability of the cells.

In order to determine the invasion ability changes of the cells, Matrigel matrix (BD Biosciences, San Jose, CA, USA) was used to simulate the environment of intercellular matrix. The Matrigel matrix was taken out from -20°C one day prior to the experiment and put in 4°C overnight to thaw. The original solution was diluted to 1:2 with serum-free RPMI 1640 medium, and evenly spread 100 μL in the upper wells of the chamber. The chamber was then put into 37°C and incubated for 60 min to let it solidified into a gel. All operation processes were performed on ice; the tips were precooled to 4°C. Other procedures were the same as the cell migration experiment. Results were observed after 72 hours.

Flow cytometry

After TGF-β1 treatment for 72 h and siR-Macc1 transfection for 48 h, 1×10^6 cells were collected from each group and washed with cold PBS twice. The cell cycle assay kit (BD Biosciences) was used for detection and cell cycle analysis was performed according to the manufacture's instruction. Briefly, 200 μL solution A was added and incubated at room temperature for 10 min, and then 150 μL solution B was added and incubated at room temperature for another 10 min, finally 120 μL solution C was added and incubated in dark for 10 min. The cells were detected with a flow cytometry, and the results were analyzed with Modfit software for cell cycle.

Western blot

The cells of each group were washed with cold PBS three times and then added with RIPA protein lysis solution and protease inhibitor PMSF. After lysis, it was centrifuged at 12000 rpm/min under 4°C for 10 min. The supernatant was collected for SDS-PAGE. Protein bands were transferred onto PVDF membrane and then blocked with 50 g/L non-fat milk at room temperature for 1 h. The primary antibody (E-Cadeherin 1:1000, Vimentin 1:1000, MACC1 1:1000, c-Met 1:1000, GAPDH 1:5000) was added and incubated on a shaker at 4°C overnight. On the next day, it was washed with PBST 5 times for 5 min each time, and then HRP-labeled secondary antibody (goat anti-mouse 1:5000, goat anti-rabbit 1:2000) was added and incubated at room temperature for 1 h. The membrane was then washed with PBST 5 times for 5 min each time and developed in ECL luminescent liquid.

Statistical analysis

The statistical software SPSS 17.0 was used for statistical analysis. All metering data were expressed as mean ± standard deviation (SD). The *t* test was used for the comparison between groups. A P value < 0.05 was considered as statistically significant.

Results

Expression of TGF-β1 and MACC1 in ovarian cancer tissues

To determine the expressions of TGF-β1 and MACC1 in the ovarian cancer tissues, quantitative PCR was performed. The results showed that the expressions of TGF-β1 (4.27 ± 0.19) and MACC1 (3.17 ± 0.21) were obviously in-

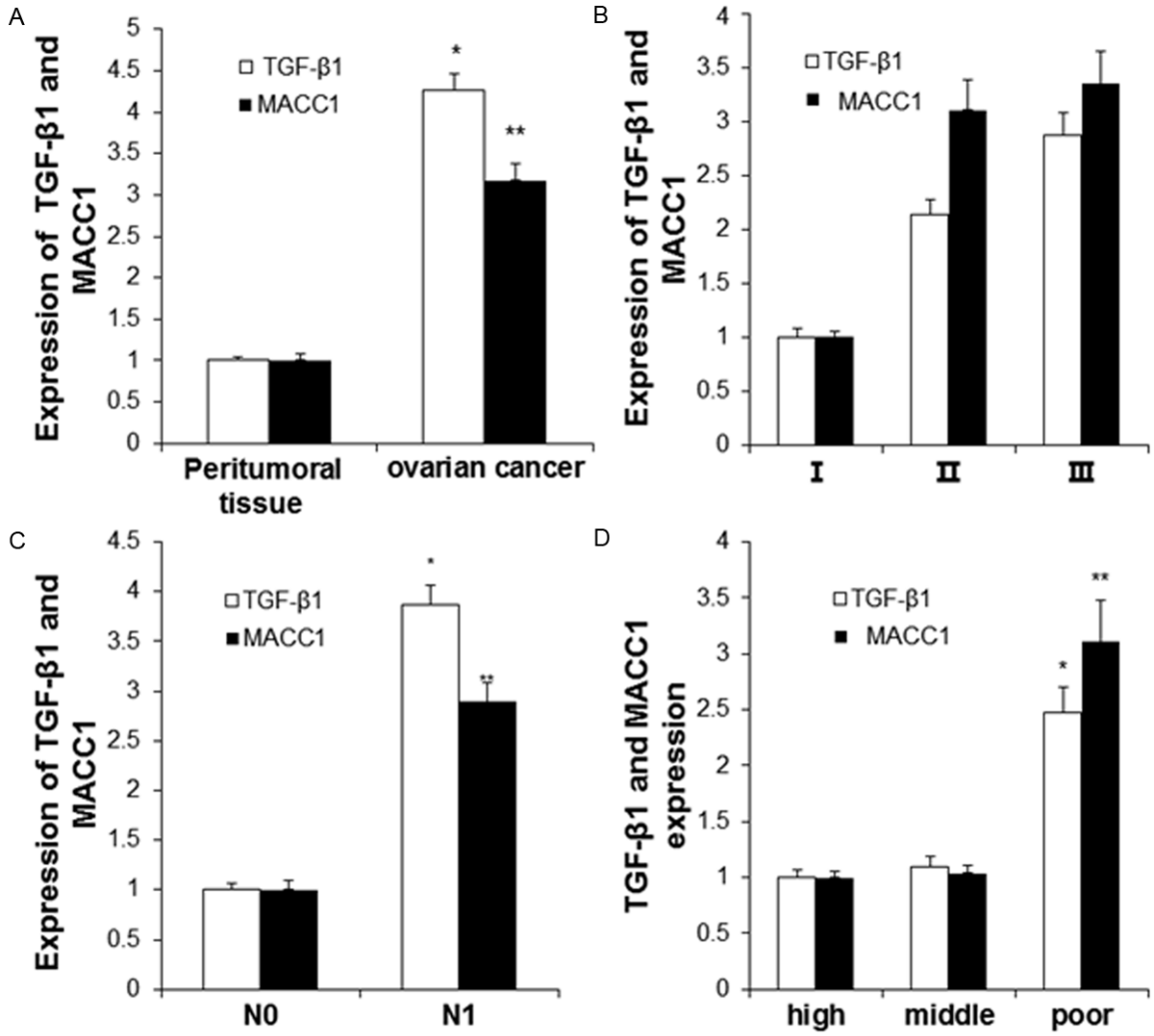

Figure 1. The expressions of TGF-β1 and MCC1 in the ovarian cancer tissues. The expressions of TGF-β1 mRNA and MACC1 mRNA in ovarian tissues was detected by qRT-PCR. A. The expression levels of TGF-β1 and MACC1 in the ovarian cancer tissues and peritumoral tissues. Compared with peritumoral tissues, *P < 0.05, **P < 0.01. B. The expressions of TGF-β1 and MACC1 in the ovarian cancer tissues with different TNM stages. C. The expression levels of TGF-β1 and MACC1 in the lymph node metastasis ovarian cancer tissues. Compared with N0, *P < 0.05, **P < 0.01. D. The correlation of the expression levels of TGF-β1 and MACC1 to the degree of differentiation. Compared with high and middle degree, *P < 0.05, **P < 0.01.

creased in the ovarian cancer tissues, and the difference was statistically significance ($P < 0.05$) (**Figure 1A**). As shown in **Figure 1B**, TGF-β1 and MACC1 increased with the clinical stage ($P < 0.05$). The gene expressions of TGF-β1 (3.87 ± 0.11) and MACC1 (2.89 ± 0.08) in the lymph node metastasis group (N1) were obviously higher than that in the non-metastasis group (N0) ($P < 0.05$) (**Figure 1C**). Their expressions in the high and medium differentiation groups were all lower than that in the poor differentiation group (**Figure 1D**). These results suggested that the expressions of TGF-β1 and MACC1 in the ovarian cancer tissues were all higher than the peritumoral tissues, and they were closely related to the differentiation, invasion and metastasis and clinical stages of the ovarian cancer.

Changes of the EMT and MACC1 gene expression

After 72 h culture in the RPMI 1640 medium containing 10 ng/ml TGF-β1, the changing levels of the gene and proteins of the EMT markers E-Cadeherin and Vimentin in the A2780 cells were detected by quantitative PCR and

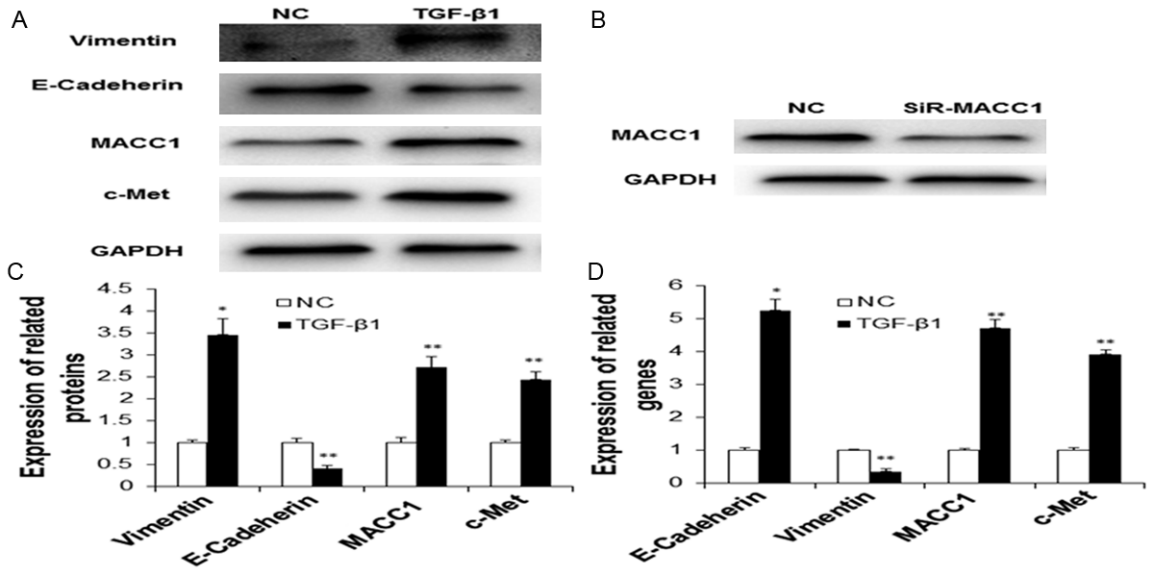

Figure 2. The expression changes of EMT related genes and MACC1 in the ovarian cancer cell line A2780. A. The effects of TGF-β1 on the EMT-related and MACC1 proteins. B. MACC1 protein expression after interfered by SiRNA. C. Quantitative analysis on the TGF-β1 influence on the EMT-related and MACC1 proteins. Compared with NC, *P < 0.05, **P < 0.01. D. TGF-β1 effects on the mRNA expressions of EMT-related and MACC1 genes. Compared with NC, *P < 0.05, **P < 0.01.

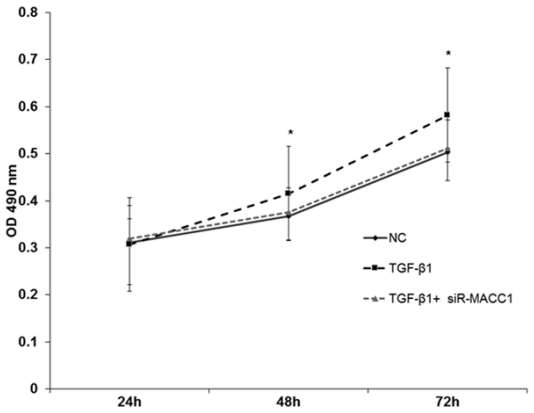

Figure 3. The proliferative ability changes of the ovarian cancer cells determined using CCK-8. It showed that TGF-β1 could promote the proliferation of A2780 cells, but this effect was inhibited when MACC1 was interfered by SiRNA. Compared with NC and TGF-β1+siR-MACC1, *P < 0.05.

Western blot to evaluate the EMT changes of the ovarian cancer cells. Additionally, the effect of TGF-β1 on the gene and protein expressions of c-Met and MACC1 was also tested. The results showed that TGF-β1 could induce down-regulation of the epithelial marker E-Cadeherin in the ovarian cancer cell A2780 ($P < 0.05$) and upregulation of the expression of the mesenchymal marker Vimentin ($P < 0.05$), suggesting the degree of EMT of the ovarian cancer cells was enhanced (**Figure 2A**, **2C** and **2D**). The expressions of MACC1 and c-Met were also obviously upregulated after treatment with TGF-β1 ($P < 0.05$). The Western blot results of the siRNA interfering MACC1 expression showed that the expression of MACC1 was obviously decreased at 48 h after siRNA transfection, indicating the interference was effective (**Figure 2B**). These results indicated that MACC1 might be related to the TGF-β1-induced EMT.

Changes of the proliferative ability of the ovarian cancer cell A2780

To further investigate the proliferative effects of TGF-β1 and MACC1 on the ovarian cancer cell A2780, the cell proliferation was measured by CCK-8. The results showed that the proliferative ability of the ovarian cancer cell A2780 was obviously increased compared to the control group after treatment with TGF-β1, indicating that TGF-β1 could promote A2780 cell proliferation (**Figure 3**). In the TGF-β1+siR-Macc1 group, the cell proliferative ability had no obvious difference from the control group ($P > 0.05$), indicating that TGF-β1 might influence the proliferation of A2780 through promoting the expression of MACC1. Its high expression in

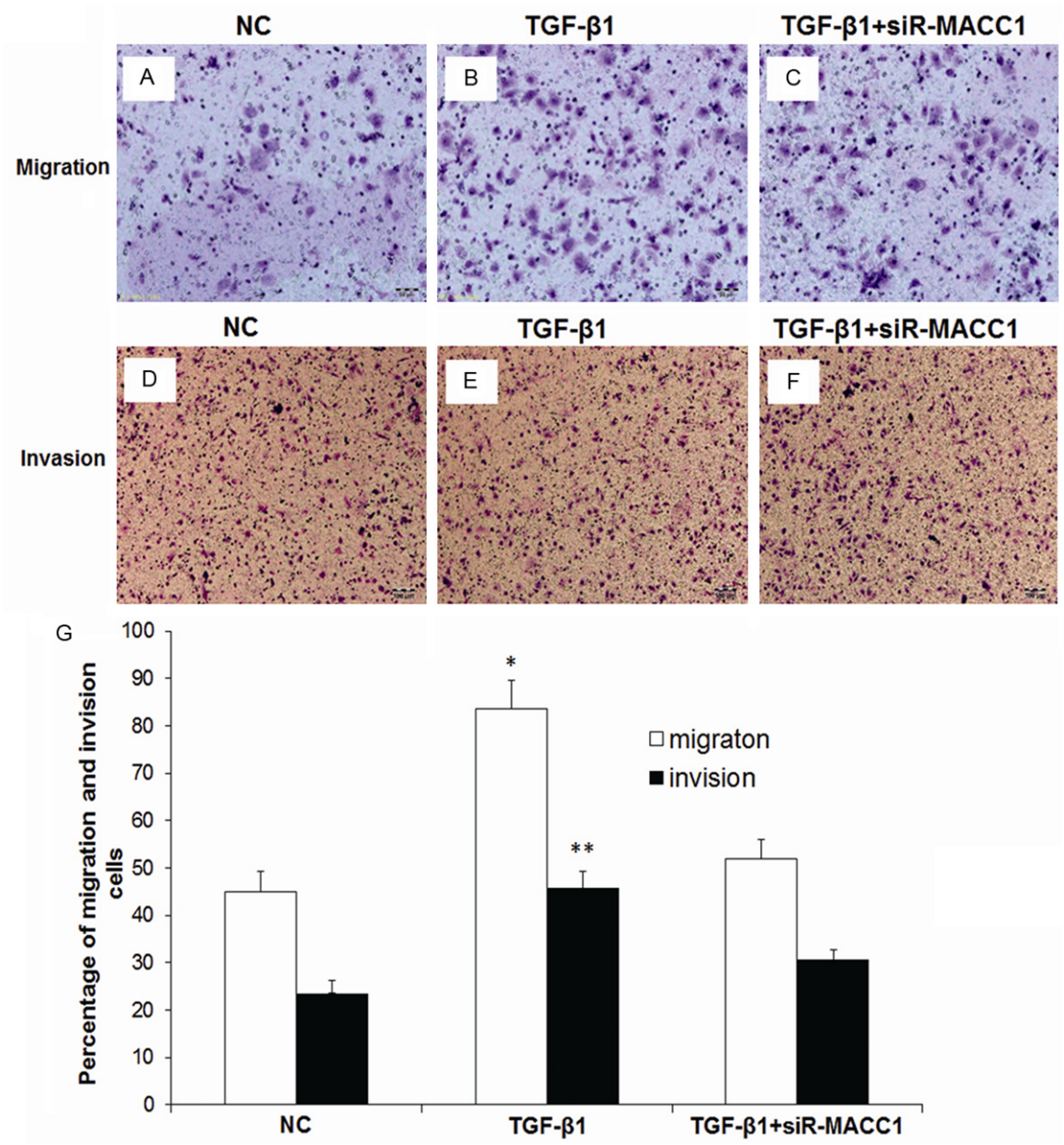

Figure 4. The effects of TGF-β1 on the invasion and metastasis abilities of the ovarian cancer cell line A2780 determined using Transwell analysis. A-C. Cell migration; C-F. Cell invasion. A, D. NC groups; B, E. TGF-β1 treated groups; C, F. TGF-β1+siR-MACC1 groups. G. Migration and invasion cell numbers. Compared with NC and TGF-β1+siR-MACC1, *P < 0.05, **P < 0.01.

the ovarian cancer tissues could be one of the reasons to promote the occurrence and development of ovarian cancer.

Invasion and migration ability of the ovarian cancer cell A2780

To determine whether TGF-β1 influences the invasion and metastasis of the cancer cell A2780 through regulating the expression of macc1, the Transwell experiment was carried out. As shown in **Figure 4**, the migration ability of A2780 cells increased obviously after treatment with TGF-β1 in comparison with the control group. The number of cells passed through the well membrane was more than that in the NC group (83.5 ± 6.20 *vs.* 44.9 ± 4.5), and the difference had statistical significance ($P < 0.05$). The invasion experiment result showed that the number of cells penetrated the well membrane of the Transwell in the TGF-β1-

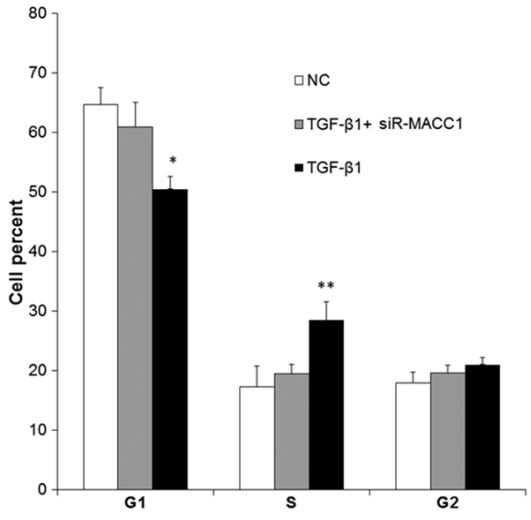

Figure 5. Cell cycle changes determined by flow cytometry. It showed that TGF-β1 could promote G1/S phase transition of A2780 cells, and this effect was inhibited when MACC1 was interfered by SiRNA. Compared with NC and TGF-β1+siR-MACC1, *P < 0.05, **P < 0.01.

treated group increased obviously (45.8 ± 8.1 *vs.* 23.5 ± 5.50), which indicated that TGF-β1 could promote the invasion and metastasis of the ovarian cancer cells. However, the invasion and metastasis ability of the ovarian cancer cells in the TGF-β1+siR-Macc1 group had no obvious difference compared to the control group, demonstrating that MACC1 might play important roles in the TGF-β1-induced invasion and metastasis.

TGF-β1 regulated MACC1 to influence the cell cycle of the ovarian cancer cells

The effects of TGF-β1 and MACC1 on the cell cycle of the ovarian cancer cells were detected by flow cytometry. The results showed that the G1/S phase transition was accelerated for the TGF-β1-treated ovarian cancer cells in comparison with the NC group (**Figure 5**). However, in the siR-Macc1 group, the percentage of cells at each phase had no obvious difference from the control group. These indicated that TGF-β1 could regulate G1/S phase transition through promoting MACC1 expression, and thus promote the proliferation of the ovarian cancer cell A2780.

Discussion

Researchers have confirmed that there are many cytokines in the microenvironment of tumor, such as TGF-β, HGF, IL-1, and TNF, and they play very important roles in the occurrence and development of tumor [18, 19]. TGF-β is the most important start factor of EMT. After its binding to cell membrane TGF-βR I and II receptors, Smad protein starts to enter cell nucleus, bind to the promoters of multiple genes, and greatly activate their expressions to play biological functions [20, 21]. Researchers have confirmed that TGF-β1 is detected to be expressed in many human tumors, and plays different biological functions in different tumors, including tumor promotion and suppression roles. For example, TGF-β1 is highly expressed in kidney, pancreas and colon cancer and promotes the invasion and metastasis ability of tumor [22-24]. On the contrary, the expression level of TGF-β1 is obviously declined or absent in some cancer cell lines such as lung cancer and breast cancer cells [25, 26]. In addition, if exogenous TGF-β1 is transferred to recover its expression, the invasion and metastasis of the tumor will be inhibited [27]. These fully indicate that TGF-β1 could serve as a suppressor gene to play its role in suppressing tumor occurrence and development [28].

MACC1 is a newly found oncogene in recent years, which can significantly promote tumor proliferation, invasion and metastasis [29], but the mechanisms of regulation and expression are still unclear. Researches show that abnormal expression of MACC1 is usually related to tumor microenvironment, metabolic stress and other factors [30, 31]. MACC1 gene is expressed at a high level in ovarian cancer, colon cancer and liver cancer tissues [32-34]. As an important cytokine in the tumor microenvironment, TGF-β1 is also expressed in ovarian cancer at an abnormally increased level [35]. The TGF-β1/Smad signaling pathway can activate a lot of tumor-related genes; however, there is no literature about whether TGF-β1/Smad signaling pathway participates in the transcription of Macc1 gene.

In this study, it was found that the expressions of TGF-β1 and MACC1 in the ovarian cancer tissues were all significantly increased and closely related to the occurrence and development of ovarian cancer. For example, the higher the stage of the ovarian cancer, the higher the expressions of TGF-β1 and MACC1; the lower the degree of differentiation of the ovarian cancer, the lower the expressions of TGF-β1 and

MACC1. In addition, the expression levels of TGF-β1 and MACC1 in the lymph node metastasis group were obviously higher than that in the non-metastasis group. These also indicated that the expressions of TGF-β1 and MACC1 were positively correlated.

For the *in vitro* experiment, the proliferation of the ovarian cancer cell line A2780 was accelerated after stimulated by TGF-β1, and the G1/S phase transition was also obviously increased. Meanwhile, the invasion and metastasis ability of the cancer cells were enhanced. These demonstrated that TGF-β1 could promote the occurrence and development of ovarian cancer. In this process, the expression of EMT related protein E-Cadeherin declined and the expression of Vimentin increased, which indicated that the epithelial cell characteristics were lost gradually and mesenchymal cell features started to appear. At the same time, the expressions of MACC1 gene and its downstream gene c-Met were obviously increased. These results indicated that in the TGF-β1-mediated EMT process, the expressions of MACC1 and c-Met were increased. When the expression of Macc1 was interfered by siRNA, the functions of TGF-β1 to promote tumor proliferation, invasion and metastasis were all obviously inhibited, indicating that MACC1 could be one of the important downstream genes of TGF-β1 and may participate in the TGF-β1-mediated tumor proliferation, invasion and metastasis.

Therefore, TGF-β1 and MACC1 are closely related to the clinical stage, lymph node metastasis and differentiation degree of the ovarian cancer. TGF-β1 can promote invasion and metastasis of the ovarian cancer by stimulating the expression of MACC1 gene, which is a potential target for biological treatment and has significant clinical values.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bingqin Guo, Department of Pathology, The First Affiliated Hospital of Bengbu Medical College, 801 Zhihuai Road, Bengbu 233003, P. R. China. Tel: 86-552-3064469; Fax: 86-552-3064469; E-mail: Gbq333@126.com

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