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Circ-PRMT5 stimulates migration in esophageal cancer by binding miR-203

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Abstract. – OBJECTIVE: The study aimed to uncover the role of circ-PRMT5 in triggering the migratory ability of esophageal cancer by regulating microRNA-203 (miR-203) level.

PATIENTS AND METHODS: Circ-PRMT5 levels in 56 matched esophageal cancer tissues and paracancerous ones were detected. The relationship between circ-PRMT5 level and clinical data of esophageal cancer patients was analyzed. Migratory abilities in TE-1 and OE33 cells influenced by circ-PRMT5 were evaluated by transwell and wound healing assay. Regulatory effect of circ-PRMT5 on miR-203 level, and the involvement of miR-203 in the development of esophageal cancer were determined through Dual-Luciferase reporter assay and rescue experiments.

RESULTS: Circ-PRMT5 was upregulated in esophageal cancer tissues and cell lines. The expression level of circ-PRMT5 was positively correlated to the rates of lymphatic metastasis and distant metastasis of esophageal cancer. Knockdown of circ-PRMT5 attenuated migratory abilities in TE-1 and OE33 cells. MiR-203 was verified to be the target gene binding circ-PRMT5, with a negative correlation between each other. Notably, miR-203 was responsible for the regulatory effect of circ-PRMT5 on migratory ability in esophageal cancer.

CONCLUSIONS: Circ-PRMT5 is positively correlated to the rates of lymphatic metastasis and distant metastasis of esophageal cancer. It promotes migratory ability in esophageal cancer by targeting miR-203.

Key Words:
Circ-PRMT5, MiR-203, Esophageal cancer, Migration.

Introduction

Esophageal cancer is a popular digestive system malignancy with relatively high morbidity and mortality, ranking 9th and 6th in global cancer, respectively[1-3]. In developed countries, morbidity and mortality rank 20th and 11th, respectively, which are 8th and 5th in developing countries[4,5]. There are 400,000 people who die of esophageal cancer each year. In China, the incidence of esophageal cancer is very high, and about 150,000 people die of this cancer annually[6,7]. Since symptoms of esophageal cancer in the early phase are atypical and early screening approaches are limited, most people are diagnosed at advanced stage. The 5-year survival of postoperative esophageal cancer ranges from 9.5% to 45%[8,9]. Invasiveness and migration are the major reasons for esophageal cancer death, which are resulted from oncogene activation, tumor-suppressor gene inactivation, and malignant cell phenotypes[10,11]. Clarifying pathological mechanisms of esophageal cancer from the aspects of genetics and epigenetics contributes to improve the therapeutic efficacy[12,13].

Only 1-2% of sequences in human genome are able to encode proteins, and the majority is non-coding RNAs[14,15]. CircRNAs are circular endogenous RNAs produced by special selective splicing, which are widely expressed in eukaryotic cells. Their special structural features result in specific biological functions[16,17]. CircRNAs exert their functions by sponging microRNAs (miRNAs), regulating host gene expressions, interacting RNA-binding proteins, mediating cis-transcription or alternative splicing[17,18]. Some circRNAs are enriched with miRNA binding sites, which serve as miRNA sponges to abolish the inhibitory effects of miRNAs on the corresponding target genes[17,18]. Through bioinformatics analysis, circ-PRMT5 is abundantly expressed in esophageal cancer tissues[19].

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In addition, a specific binding between circ-PRMT5 and miR-203 is predicted. In this paper, we mainly explored the role of circ-PRMT5 and miR-203 in influencing migratory ability of esophageal cancer cells, thus providing a novel idea for clinical treatment of esophageal cancer.

Patients and Methods

Patients and Samples

A total of 56 matched esophageal cancer tissues and paracancerous ones confirmed by H&E staining were collected and stored at -80°C. None of enrolled subjects were preoperatively treated with anti-tumor therapy. Their clinical data were recorded. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of People's Hospital of Shouguang.

Cell Culture

Four human-derived esophageal cancer cell lines (OE19, OE33, TE-1, and EC-109) and one human-derived normal esophageal epithelial cell line (HEEC) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Cells inoculated in 6-well plates with 30-40% confluence were transfected with the corresponding plasmids (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Transwell Migration Assay

200 μL of suspension (2.0×10^5/mL) was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 560 μL of medium containing 10% FBS in the bottom. After 48 h of incubation, the cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40×).

Wound Healing Assay

5.0×10^4 cells suspended in culture medium containing 1% FBS were inoculated per well and an artificial wound was created. 24 hours later, the percentage of wound closure was calculated.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as the internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. circ-PRMT5: forward: 5'-UCAUCUUCCGGCUCCUCAAGUUCU-3', reverse: 5'-AUCUUCCGGCUCCUCAAGUUC-3'; GAPDH: forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; miR-203: forward: 5'-GTCGTACCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGT-3', reverse: 5'-GCCCGTGAAATGTTTAGGACCAC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Dual-Luciferase Reporter Assay

The cells inoculated in a 24-well plate were co-transfected with pmirGLO-circ-PRMT5-WT/pmirGLO-circ-PRMT5-MUT/pmirGLO and NC/miR-203 mimics, respectively. 48 hours later, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The differences between the two groups were analyzed by the *t*-test. Correlation between expressions of two genes was compared by Pearson correlation test. $p<0.05$ was considered as statistically significant.

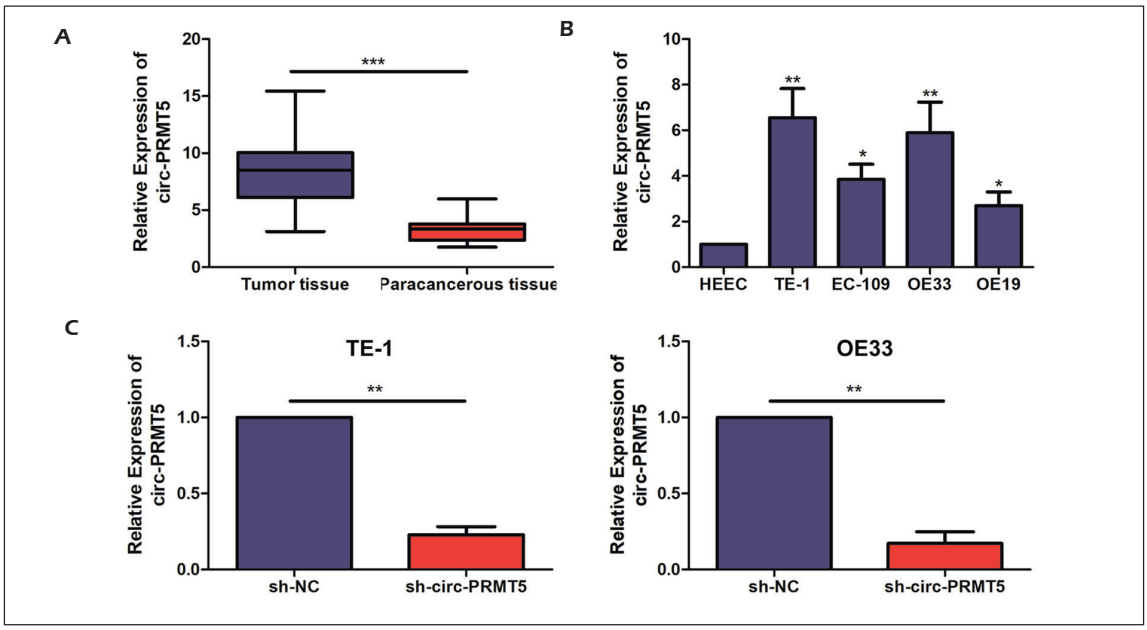

Figure 1. Circ-PRMT5 was upregulated in esophageal cancer. **A,** Circ-PRMT5 levels in esophageal cancer tissues and paracancerous tissues. **B,** Circ-PRMT5 levels in esophageal cancer cell lines. **C,** Transfection efficacy of sh-circ-PRMT5 in TE-1 and OE33 cells. Data were expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

Results

Circ-PRMT5 was Upregulated in Esophageal Cancer

Compared with paracancerous tissues, circ-PRMT5 was upregulated in esophageal cancer tissues (Figure 1A). Consistently, circ-PRMT5 was highly expressed in esophageal cancer cell lines (Figure 1B). Among the four tested esophageal cancer cell lines, TE-1, and OE33 cells showed the most pronounced abundance of circ-PRMT5. Based on circ-PRMT5 level in enrolled 56 esophageal cancer patients, they were assigned into high or low-level group, respectively. It is shown that circ-PRMT5 level was positively correlated to the rates of lymphatic metastasis and distant metastasis of esophageal cancer patients, while it was not correlated to age, sex, and tumor grading (Table I). It is indicated that circ-TRPMT5 may be a biomarker for predicting the malignant progression of esophageal cancer.

Table I. Association of circ-PRMT5 expression with clinicopathologic characteristics of esophageal cancer.

Parameters	Number of cases	circ-PRMT5 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.592
< 60	23	13	10	
≥ 60	33	21	12	
Gender				0.592
Male	33	21	12	
Female	23	13	10	
T stage				0.592
T1-T2	33	21	12	
T3-T4	23	13	10	
Lymph node metastasis				0.007
No	35	26	9	
Yes	21	8	13	
Distance metastasis				0.004
No	40	29	11	
Yes	16	5	11	

Silence of Circ-PRMT5 Suppressed Migratory Ability of Esophageal Cancer

Transfection efficacy of sh-circ-PRMT5 was tested in TE-1 and OE33 cells (Figure 1C). Transwell assay uncovered that knockdown of circ-PRMT5 in TE-1 and OE33 cells reduced migratory cell number (Figure 2A) and percentage of wound closure (Figure 2B), demonstrating the suppressed migratory ability.

MiR-203 was Downregulated in Esophageal Cancer

Three potential miRNAs binding circ-PRMT5 were analyzed by bioinformatics. In particular, miR-203 level showed the most pronounced change after the knockdown of circ-PRMT5 among the three tested miRNAs (Figure 3A). MiR-203 was lowly expressed in esophageal cancer tissues (Figure 3B) and cell lines (Figure 3D). In addition, its level was negatively correlated to that of circ-PRMT5 in esophageal cancer tissues (Figure 3C). Transfection of miR-203 inhibitor upregulated circ-PRMT5 in TE-1 and OE33 cells, further uncovering their negative relationship (Figure 3E). Decreased Luciferase activity was found after co-transfection of miR-203 mimic and pmirGLO-circ-PRMT5-WT, demonstrating the binding relationship between miR-203 and circ-PRMT5 (Figure 3F).

Circ-PRMT5 Promoted Malignant Progression of Esophageal Cancer by Targeting MiR-203

Downregulated circ-PRMT5 in esophageal cancer cells transfected with sh-circ-PRMT5 was partially upregulated after co-transfection of miR-203 inhibitor (Figure 4A). Moreover, the inhibited migratory ability in esophageal cancer cells with circ-PRMT5 knockdown was partially reversed by silence of miR-203 (Figure 4B).

Discussion

Although therapeutic strategies of esophageal cancer have been greatly improved, most people lose the optimal surgical opportunity because they are developed into advanced stage at the initial diagnosis[19]. The 5-year survival

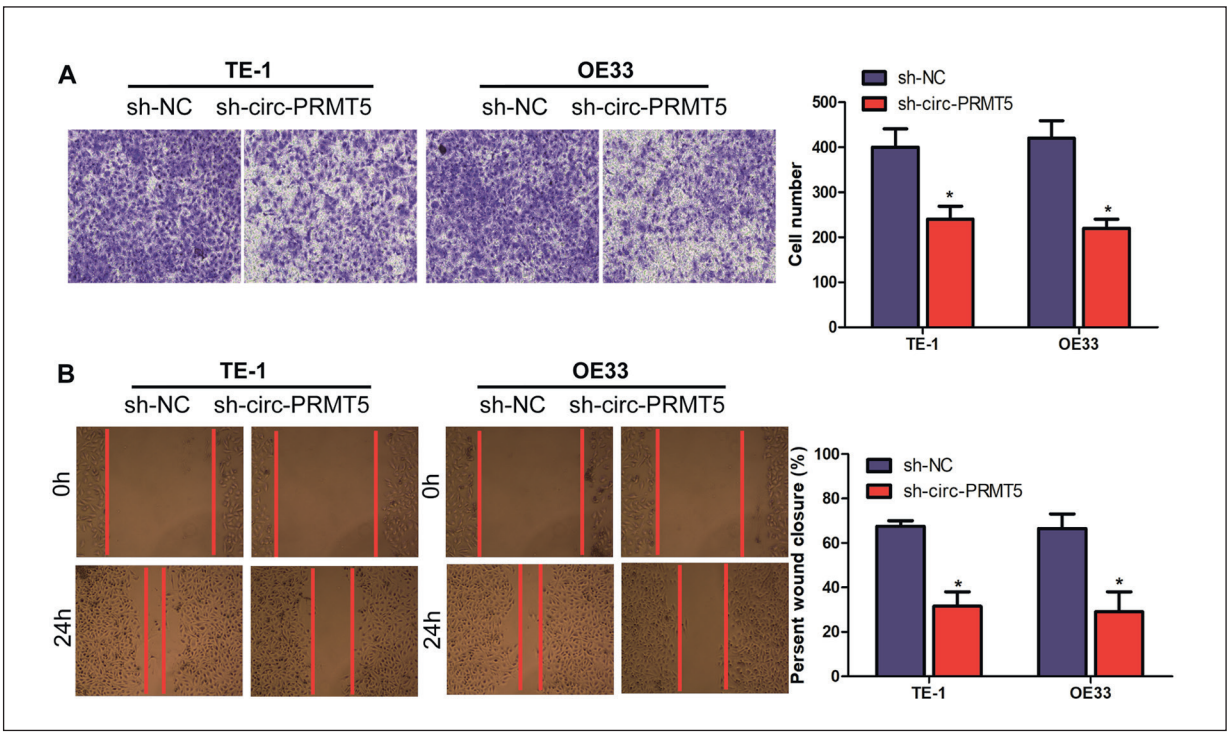

Figure 2. Silence of circ-PRMT5 suppressed migratory ability of esophageal cancer. **A,** Transwell assay showed migratory cells in TE-1 and OE33 cells transfected with sh-NC or sh-circ-PRMT5 (magnification 40×). **B,** Wound healing assay showed wound closure percentage in TE-1 and OE33 cells transfected with sh-NC or sh-circ-PRMT5. Data were expressed as mean ± SD. *$p<0.05$.

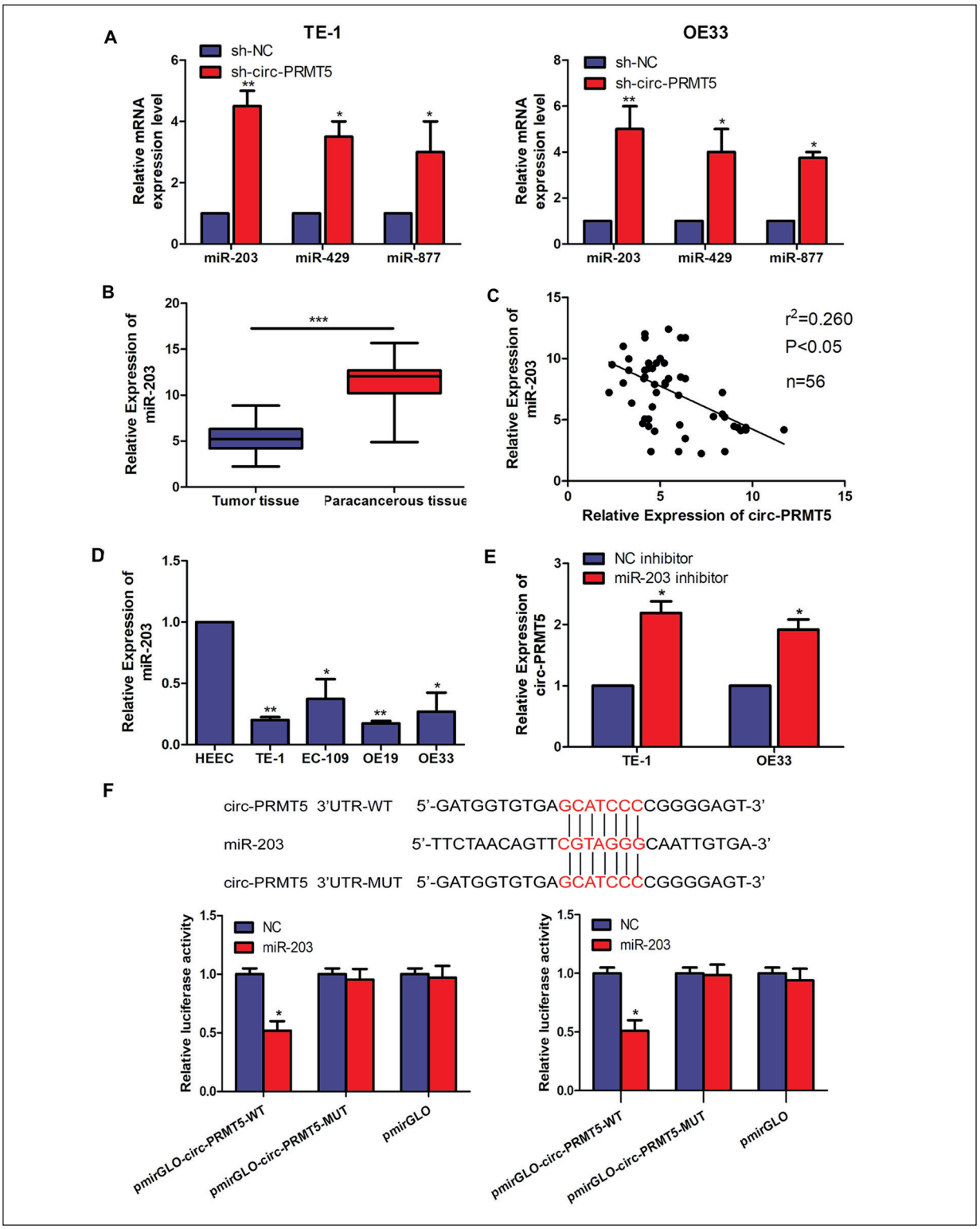

Figure 3. MiR-203 was downregulated in esophageal cancer. **A,** Expression levels of three potential miRNAs binding circ-PRMT5 in TE-1 and OE33 cells transfected with sh-NC or sh-circ-PRMT5. **B,** MiR-203 levels in esophageal cancer tissues and paracancerous tissues. **C,** A negative correlation between expression levels of miR-203 and circ-PRMT5 in esophageal cancer tissues. **D,** MiR-203 levels in esophageal cancer cell lines. **E,** Circ-PRMT5 level in TE-1 and OE33 cells transfected with NC or miR-203 inhibitor. **F,** Luciferase activity in TE-1 and OE33 cells co-transfected with pmirGLO-circ-PRMT5-WT/pmirGLO-circ-PRMT5-MUT/pmirGLO and NC/miR-203 mimics. Data were expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

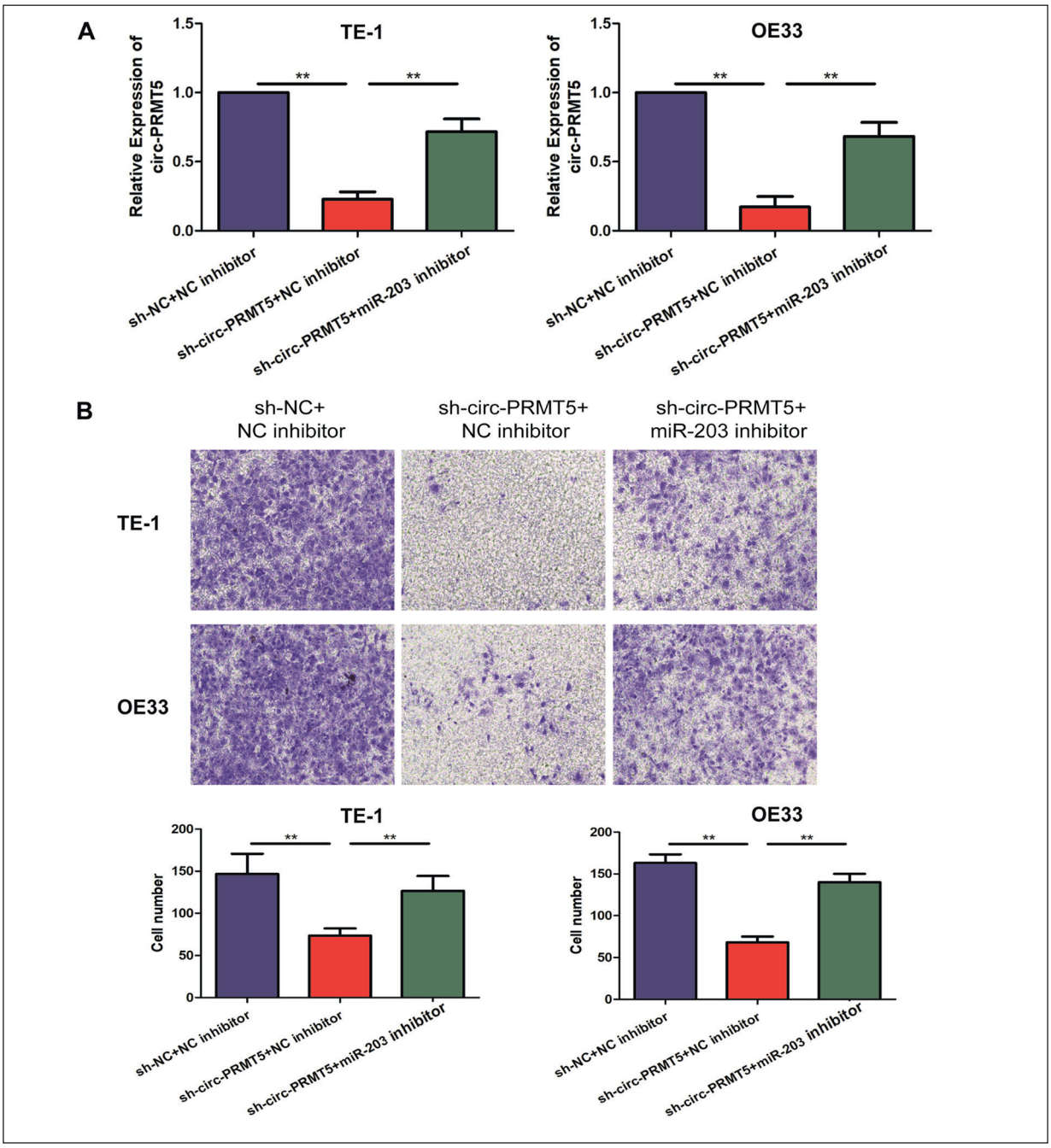

Figure 4. Circ-PRMT5 promoted malignant progression of esophageal cancer by targeting miR-203. **A,** Circ-PRMT5 level in TE-1 and OE33 cells transfected with sh-NC+NC inhibitor, sh-circ-PRMT5+NC inhibitor or sh-circ-PRMT5+miR-203 inhibitor. **B,** Transwell assay showed migratory cells in TE-1 and OE33 cells transfected with sh-NC+NC inhibitor, sh-circ-PRMT5+NC inhibitor or sh-circ-PRMT5+miR-203 inhibitor (magnification 40×). Data were expressed as mean ± SD. $^{**}p<0.01$.

of early-stage esophageal cancer is satisfactory. Therefore, effective screening and intervention of esophageal cancer as early as possible contribute to enhance detective rate[11-13]. Currently, clinical reports[12,13] have shown the promising aspect of tumor biomarkers. Seeking for effective and specific biomarkers of esophageal cancer is of great significance in improving early detective rate.

CircRNAs are extensively distributed in eukaryotic transcriptome, which are featured by the closed loop structure[16,17]. They are not sensitive to nuclease and present expression specificities in dif-

ferent phases, which make them potentials as clinical biomarkers[18]. In this paper, circ-PRMT5 was upregulated in esophageal cancer tissues. High level of circ-PRMT5 was linked to high metastatic rate of esophageal cancer. Subsequently, our *in vitro* experiments verified the promotive effect of circ-PRMT5 on migratory ability of esophageal cancer cells. It is suggested that circ-PRMT5 may aggravate the development of esophageal cancer, which was consistent to a previous finding[19].

Some studies[17,18] have shown the potential of circRNAs as tumor targets. With the discovery of miRNA management by circRNAs, the latter may be a second-generation miRNA inhibitor[20]. It is believed that synthetic circRNA inhibitors can be utilized as novel cancer treatments[21,22]. Through bioinformatics prediction and Dual-Luciferase reporter assay, we indicated that miR-203 was the direct target of circ-PRMT5. In esophageal cancer tissues, miR-203 was downregulated. Moreover, miR-203 level was negatively correlated to that of circ-PRMT5 in esophageal cancer tissues. Notably, miR-203 was responsible for the regulatory effect of circ-PRMT5 on migratory ability in esophageal cancer. As a result, circ-PRMT5/miR-203 axis was important in regulating the development of esophageal cancer.

Conclusions

Our results showed that circ-PRMT5 is positively correlated to the rates of lymphatic metastasis and distant metastasis of esophageal cancer. It promotes migratory ability in esophageal cancer by targeting miR-203.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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