**Abstract.** – **OBJECTIVE:** Our purpose was to assess the relationship between circ_0005276 and clinical features of epithelial ovarian cancer (EOC), and to illustrate the regulatory effect of circ_0005276 on migratory potential in EOC cells.

**PATIENTS AND METHODS:** EOC tissues and adjacent normal ones were collected from 49 EOC patients. Relative levels of circ_0005276 and ADAM9 in EOC tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between circ_0005276 and clinical features of EOC patients was analyzed. Moreover, migratory potentials of CAOV3 and SKOV3 cells affected by circ_0005276 were examined by transwell and wound healing assay. Regulatory effects of circ_0005276/ADAM9 feedback loop on the development of EOC were finally detected by luciferase assay and rescue experiments.

**RESULTS:** It was found that circ_0005276 was upregulated in EOC tissues and its level was positively linked to rates of lymphatic metastasis and distant metastasis in EOC patients. Survival analysis showed worse OS and DFS in EOC patients expressing a high level of circ_0005276 than those with a low level. Besides, knockdown of circ_0005276 attenuated migratory potentials in EOC cells. ADAM9 was verified to be the target gene binding circ_0005276, and its level was positively regulated by circ_0005276. Notably, circ_0005276 aggravated the development of EOC by targeting ADAM9.

**CONCLUSIONS:** Circ_0005276 is highly expressed in EOC tissues, and its level is positively linked to metastasis. Serving as an unfavorable gene in the process of EOC, circ_0005276 aggravates the development of EOC by targeting ADAM9.

*Key Words:* Circ_0005276, ADAM9, EOC.

**Introduction**

Epithelial ovarian cancer (EOC) is the number one killer among the top three tumors in the female reproductive system. Conventional treatment strategies for EOC include surgical resection, chemotherapy, and immunotherapy, although they have been greatly improved. Nevertheless, the overall survival of patients is still unsatisfactory.

It is reported that in the United States, the 5-year survival of EOC was 46% in 2015, which was 32% and 18% in advanced EOC patients with FIGO III and FIGO IV, respectively. The mortality of EOC in China shows an upward trend. The pathogenesis and etiology of EOC require to be extensively explored.

The role of epigenetics in tumorigenesis has been well concerned. Without changing gene sequences, epigenetics can control gene expression through mediating transcription, histone modification, DNA methylation or chromatin remodeling at various levels. Non-coding RNAs have been highlighted during the malignant development of tumors, which mediate oncogenes and tumor suppressors by degrading target mRNAs. Unlike traditional linear RNAs, circRNAs display a closed loop structure. Due to the specific structure, circRNAs are stably expressed and hardly degraded by RNA exonucleases. The ceRNA hypothesis proposes that circRNAs sponge corresponding miRNAs that share common sequences in the promoter region, and thereafter regulate downstream genes of miRNAs. Differentially expressed circRNAs are involved in the development of many types of tumors. This paper mainly explores the relationship between circ_0005276 and clinical features of EOC, as well as the regulatory effect of circ_0005276 on migratory potential in EOC cells.

**Patients and Methods**

**EOC Patients and Samples**

Baseline characteristics of enrolled 49 EOC patients were listed in Table I. Patients were patho-
logically diagnosed with EOC and older than 18 years old. EOC tissues and adjacent normal ones were collected. Exclusion criteria: (1) secondary ovarian cancer patients, (2) patients with history of other malignancies or anti-tumor treatment, (3) patients with inadequate clinical data. Tumor staging was conducted based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by the Ethics Committee of Binzhou Medical University Hospital and conducted after informed consent was obtained from each subject.

**Cell Culture**

Human ovarian cancer cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and a normal human ovarian surface epithelial cell line (HOSEPiCs) 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% CO2 incubator at 37°C.

**Transfection**

Cells inoculated in 6-well plates were cultured to 30-40% confluence. They were transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, cells were collected for the following use.

**Transwell Migration Assay**

A total of 200 μL of suspension (5.0×10^5/mL) was inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Finally, migratory cells were counted in 5 random fields per sample (magnification 40×).

**Wound Healing Assay**

A total of 5.0×10^4 cells suspended in culture medium containing 1% FBS were inoculated per well of 6-well plates, and an artificial wound was created. 24 h later, the percentage of wound closure was calculated.

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

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primerscript 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) was the internal reference. Each sample was performed...
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in triplicate, and relative level was calculated by 2-\Delta\DeltaCt. Circ_0005276: forward: 5'-GCTAATGGTATCCAGGTGTC-3' and reverse: 5'-CCCTCTCTCAGATGAAAGC-3', ADAM9: forward: 5'-GCTAGTTGGACTGAGATTTGG-3' and reverse: 5'-TTATTACACAGGAGGGAC-3', GAPDH: forward: 5'-GCTTTCTTTCCTTTCGCGCT-3' and reverse: 5'-TTTGCGGTGGAAATGTCCTT-3'.

Western Blotting
Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay
EOC cells were inoculated in 24-well plates, and co-transfected with circ_0005276-WT/circ_0005276-MUT and NC/pCDNA-ADAM9, respectively, using Lipofectamine 2000 cells were lysed for determining relative Luciferase activity 48 h later.

Statistical Analysis
Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the t-test. Kaplan-Meier curves were plotted for survival analysis, followed by the log-rank test. Pearson correlation test was applied for evaluating the relationship between expression levels of circ_0005276 and ADAM9 in EOC tissues.

Results
Circ_0005276 Was Highly Expressed In EOC
Circ_0005276 was highly expressed in EOC tissues than that in normal ones (Figure 1A). Identically, in vitro level of circ_0005276 was upregulated in EOC cell lines (Figure 1B).

Circ_0005276 Expression Was Correlated With EOC Metastases
Included EOC patients were assigned into two groups based on the median level of circ_0005276. By analyzing their clinical data, it was found that circ_0005276 level was positively correlated with rates of lymphatic metastasis and distant metastasis in EOC patients (Table I). Furthermore, Kaplan-Meier curves illustrated worse overall survival (Figure 1C) and disease-free survival (Figure 1D) in EOC patients expressing a high level of circ_0005276. Results indicate that circ_0005276 may be a predictive marker for the prognosis of EOC.

Knockdown of Circ_0005276 Suppressed Migratory Potential of EOC
Transfection efficacy of sh-circ_0005276 was first tested in CAOV3 and SKOV3 cells (Figure 1E). Transwell assay unveiled that migratory cell number was decreased after knockdown of circ_0005276 (Figure 2A). Similarly, wound closure percentage was markedly decreased in EOC cells transfected with sh-circ_0005276 (Figure 2B). It is concluded that circ_0005276 promotes migratory potential of EOC.

Circ_0005276 Bound to ADAM9
Through online prediction, binding sites in the promoter regions of circ_0005276 and ADAM9 were discovered (Figure 3A). Overexpression of ADAM9 markedly decreased Luciferase activity in wild-type circ_0005276 vector, verifying the binding relationship between circ_0005276 and ADAM9 (Figure 3B). Western blotting and qRT-PCR showed that the protein and mRNA levels of ADAM9 were downregulated in EOC cells transfected with sh-circ_0005276, compared with those in EOC cells transfected with sh-NC (Figure 3C, 3D). Similar to circ_0005276, ADAM9 was upregulated in EOC as well (Figure 3E, 3F). Moreover, a positive relationship was identified between expression levels of circ_0005276 and ADAM9 in EOC tissues (Figure 3G).

Overexpression of ADAM9 Reversed Regulatory Effect of Circ_0005276 on Migratory Potential of EOC
To further uncover the involvement of ADAM9 in EOC development, pCDNA3.1-ADAM9 was constructed. It was found that co-transfection of pCDNA3.1-ADAM9 upregulated the decreased level of ADAM9 in EOC cells with circ_0005276 knockdown (Figure 4A). Notably,
overexpression of ADAM9 increased migratory cell number (Figure 4B) and wound closure percentage (Figure 4C) in EOC cells transfected with sh-circ_0005276. It is demonstrated that circ_0005276 aggravates the development of EOC by upregulating ADAM9.

**Discussion**

Malignant biological behaviors, pathogenesis, and progression mechanisms of EOC are research focuses. Atypical symptoms in the early stage of EOC result in the poor prognosis since most of EOC patients are initially diagnosed in middle or advanced stage. Seeking abnormally expressed genes in EOC and analyzing their potential functions contribute to improve survival of affected patients. Epigenetics is a hot topic in cancer researches, i.e., gene expressions are heritably changed while nuclear DNA sequences remain unchangeable. Genetics provides information on various proteins that synthesize epigenetic modified proteins, and epigenetic information regulates a set of expressed genes and the degree of expressions. Precise expressions of genes are not only controlled by DNA sequences, but also subject to epigenetics. Posttranscriptional and transcriptional regulations are two major components of epigenetics. By regulating protein
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Epigenetics is responsible for cell phenotype changes and is closely involved in tumor development.

CircRNAs contain exon sequences and are spliced at classical splice sites. Circ_0005276 has been identified to participate in cell apoptosis, cell cycle, and other phenotypes. It is upregulated in many types of tumor cells and related to tumor development. However, the mechanism of circ_0005276 in EOC is not clear. Therefore, the objective of this study was firstly to elucidate the

Figure 2. Knockdown of circ_0005276 suppresses migratory potential of EOC. A, Migration in CAOV3 and SKOV3 cells transfected with sh-circ_0005276 or sh-NC (magnification: 40×). B, Wound closure percentage in CAOV3 and SKOV3 cells transfected with sh-circ_0005276 or sh-NC (magnification: 40×). Data are expressed as mean ± SD. **p<0.01.
Oncogenic role of circ_0005276 in the progression of EOC, as well as the specific mechanism of circ_0005276 regulating ADAM9. Circ_0005276 was upregulated in EOC tissues, and its level was positively linked to rates of lymphatic metastasis and distant metastasis. Survival analysis showed worse OS and DFS in EOC patients expressing a high level of circ_0005276. Besides, the
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Knockdown of circ_0005276 attenuated migratory potentials in EOC cells. The above findings demonstrate that circ_0005276 aggravates the development of EOC.

CircRNA abolishes the inhibitory effect of miRNAs on their downstream genes, thus upregulating their levels, that is, the ceRNA theory. CircRNAs are able to directly bind to proteins, thus inhibiting their activities or components of protein complexes. They also guide protein synthesis as translation templates. Through bioinformatics prediction and Luciferase assay verification, ADAM9 was confirmed to be the downstream gene binding circ_0005276. ADMA9 is a transcription factor involved in malignant development of tumors. ADAM9 participates in angiogenesis, tumor growth, and metastasis. Here, ADAM9 level was positively regulated by circ_0005276. Of note, ADAM9 was found to be responsible for migratory potential of EOC.

**Figure 4.** Overexpression of ADAM9 reverses regulatory effect of circ_0005276 on migratory potential of EOC. A, ADAM9 levels in CAOV3 and SKOV3 cells co-transfected with sh-circ_0005276 and pcDNA3.1-NC/pcDNA3.1-ADAM9. B, Migration in CAOV3 and SKOV3 cells co-transfected with sh-circ_0005276 and pcDNA3.1-NC/pcDNA3.1-ADAM9 (magnification: 40×). Wound percentage in CAOV3 and SKOV3 cells co-transfected with sh-circ_0005276 and pcDNA3.1-NC/pcDNA3.1-ADAM9 (magnification: 40×). Data are expressed as mean ± SD. **p<0.01.
ulated by circ_0005276. This research provides novel targets for clinical treatment and prognosis of EOC.

Conclusions

These results showed that circ_0005276 is highly expressed in EOC tissues, and its level is positively linked to metastasis. Serving as an unfavorable gene in the prognosis of EOC, circ_0005276 aggravates the development of EOC by upregulating ADAM9.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References
