Abstract. – OBJECTIVE: To explore the role of miR-200a combined with PTEN in the progression of ovarian carcinoma.

PATIENTS AND METHODS: The human ovarian cancer tissues and normal adjacent tissues (n = 57) were obtained from our hospital. The human ovarian cancer cell lines OVCAR3 and A2780, the human ovarian surface epithelial cell line (HOSEpiC), and HEK293T cells were used in this study. Cell migration assay and invasion assay were used to detect the ability of cell migratory. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western blotting were used to detect the expression of miRNA and proteins.

RESULTS: The clinic pathological analysis suggested a significant correlation with lymph node metastasis and tumor-lymph node metastasis (TNM) stage. Moreover, miR-200a was identified as aberrantly up-regulated in ovarian carcinoma tissues and cell lines. Through transwell analysis, the miR-200a overexpression significantly enhanced the cell migratory and invasive abilities. Luciferase assay validated phosphatase and tensin homolog (PTEN) was a miR-200a's direct and functional target gene. The miR-200a overexpression reduced the PTEN expression in OVCAR3 cells while the expression of PTEN was increased via miR-200a inhibitor as confirmed by Western blot. Furthermore, over-expression of PTEN was found reversing the inhibition of cell migration and invasion caused by miR-200a.

CONCLUSIONS: MiR-200a has a carcinogenic effect on ovarian cancer through regulating PTEN.

Key Words
Ovarian carcinoma, miR-200a, Migration, Invasion, PTEN.

Introduction

Ovarian cancer is a malignant tumor among females and one of the leading causes of cancer deaths in the worldwide. Although considerable progress has been made in surgical resection and chemotherapy, the low sensitivity and specificity of ovarian cancer still limit the reform and innovation of treatment methods. What is more, ovarian cancer is often diagnosed in the late stages, when the 5-year survival rates drop below 30%. Therefore, it is essential the discovery and the development of early-stage biomarkers and treatment strategies for ovarian cancer to improve the survival rate.

MicroRNAs (miRNAs) can be bound with the complementary sequences in 3'-untranslated region (UTR) to regulate the expression of target genes. It predominantly led to repress protein translation or cleaving mRNA. The miR-200 family including miR-200a is known for inhibiting the mesenchymal transition (EMT) in many kinds of cancer. Among them, it is reported that miR-200a is a tumor suppressor in various cancers, including nasopharyngeal carcinoma, ovarian carcinoma, etc. However, some studies reported that miR-200a expression in ovarian cancer showed high expression in patients that postoperative survival rate was low. The regulatory role of miR-200a is still controversial in ovarian carcinoma, and the specific regulatory mechanism remains to be further investigated.

Recently, several studies had revealed that miR-200a regulated its target genes including ZEB2, CDK-6, IGF2, EPHA2, TGFB2, and PTEN to participate in organ development and
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tumorigenesis. Among those target genes, PTEN is critical for regulating cell migration and proliferation by activating the PI3K/PTEN/Akt/mTOR pathway\textsuperscript{22}. PTEN has been reported\textsuperscript{23} that lowly expression will weaken the effect on inhibiting the growth of cells in ovarian cancer. Nonetheless, it has not been reported about whether miR-200a targeted PTEN gene expression to regulate the ovarian cancer cell invasion and migration.

We aimed at exploring the role of miR-200a combined with PTEN in the progression of ovarian carcinoma. We speculated that the miR-200a plays a carcinogenic role in the growth of ovarian carcinoma by inhibiting PTEN and the results may provide a novel treatment for ovarian carcinoma.

Patients and Methods

Tissues and Cell Lines

From 2010 to 2015, the human ovarian cancer tissues and normal adjacent tissues (n= 57) were obtained from the Huai’an Hongze District People’s Hospital. All the tissue samples were approved by the Clinical Research Ethics Committee. Human tissues were frozen in liquid nitrogen and then stored in the -80°C refrigerator for further use. This study was approved by the Ethics Committee of Huai’an Hongze District People’s Hospital. Signed written informed consents were obtained from all participants before the study.

The human ovarian cancer cell lines OVCAR3 and A2780, a human ovarian surface epithelial cell line (HOSEpiC) and HEK293T cells were used in this study. All the cell lines came from the Tumor Cell Bank of the Chinese Academy of Medical Science (Beijing, China), supplemented by 10% fetal bovine serum (FBS) and 1% penicillin. These cells were grown in an incubator at 37°C, with 5% CO\textsubscript{2} in the atmosphere (Gibco, Rockville, MD, USA). The medium was replaced every other day according to the culture state.

Cell Transfection

The miR-200a mimic and inhibitor, PTEN siRNA were purchased from RiBoBio (Guangzhou, China) and were transferred into OVCAR3 and A2780 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturers’ protocols.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA, containing miRNA from tissue specimens and cell lines. Quantitative RT-PCR was carried out through the SYBR green assay (Invitrogen, Carlsbad, CA, USA) with ABI 7300 HT Sequence Detection System (Biosystems, Shanghai, China). U6 and GAPDH were used as control of miR-200a and PTEN. The reverse transcription primers and quantitative PCR primers of miR-200a and U6 were purchased from RIBOBIO (Guangzhou, China). The miR-200a and PTEN levels were analyzed using the 2\textsuperscript{−}\Delta\DeltaCT method.

Cell Migration Assay and Invasion Assay

Transwell chambers (Corning, NY, USA) were used to evaluate the migratory and invasive ability of ovarian cells. 5 × 10\textsuperscript{4} cells without serum were placed in the upper chamber on the non-coated membrane, and the lower chamber filled with 10% fetal bovine serum (FBS) to induce ovarian cells migrating or invading through the membrane. Also, the cells were placed in the upper chamber with the coated membrane for invasion assay. Then, these cells were incubated for 48 h for the migration assay and 72 h for the invasion assay. The cells were then stained with crystal violet (Beyotime, Shanghai, China).

Luciferase Reporter Assay

The PTEN-wild and PTEN-mut were inserted into the pGL3 promoter vector (Genscript, Nanjing, China) for luciferase reporter experiments. Then, the vector and miR-200a mimic were transfected into HEK-293T cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were cultured in a 24-well plate. About 24 h after transfection, Dual-Luciferase\textsuperscript{®} Reporter Assay Kit (Promega, Madison, WI, USA) was applied to perform luciferase assays.

Western Blot Analysis

The protein samples were obtained using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Proteins were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then incubated with 5% blocking reagent in nitrocellulose membranes at room temperature. Next, we incubated the membranes overnight at 4°C.
with anti-PTEN, anti-GAPDH antibodies (Epitomics, Burlingame, CA, USA). The membranes were washed three times and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein expression levels were measured by Image Lab software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis
Statistical analysis was analyzed with GraphPad Prism 6.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). All the data were presented as mean ± SD. The difference was analyzed by the Student t-test and Chi-square test. Differences were considered significant at \( p < 0.05 \).

Results
The miR-200a Expression is Increased in Human Ovarian Carcinoma
The clinic pathological analysis suggested that miR-200a expression was positively associated with lymph node metastasis (\( p < 0.01 \), Table I). In addition, the qRT-PCR experiment indicated that the miR-200a expression was significantly increased in ovarian cancer tissues (Figure 1A). Consistent with these observations, upregulation of miR-200a was also confirmed in OVCAR3 and A2780 cells compared with normal control (Figure 1B). Taken together, these results suggested that miR-200a might be associated with the pathologic development of ovarian cancer.

### Table I. Clinicopathological characteristics and miR-200a expression in 57 patients with ovarian carcinoma.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (n=57)</th>
<th>miR-200a expression</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \geq 50 )</td>
<td>29</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>(&lt; 50 )</td>
<td>28</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \geq 5 )</td>
<td>27</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>(&lt; 5 )</td>
<td>30</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well+ Moderately</td>
<td>31</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Poor</td>
<td>26</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>20</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>III + IV</td>
<td>37</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>Lymph-node metastasis</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>

Statistical analyses were performed by the \( \chi^2 \) test. TNM, tumor-node-metastasis. *\( p < 0.05 \) was considered significant.

![Figure 1.](image1.png)
Cell Migration and Invasion Are Promoted by miR-200a in Ovarian Carcinoma

Subsequently, we explored the function of miR-200a in ovarian carcinoma. The transfection efficiency verified by qRT-PCR showed that miR-200a mimics enhanced the expression level of miR-200a while miR-200a inhibitor clearly suppressed the miR-200a expression compared with the control (Figure 2A). Additionally, the abilities of cell migration and invasion were significantly enhanced in the cells with miR-200a mimic compared with the control, indicating that miR-200a over-expression could promote cell migration and invasion in OVCAR3 cells (Figure 2B). Moreover, deleting miR-200a by specific miR-200a inhibitor reduced the migrated and invasive cell number in OVCAR3 cell lines (Figure 2C).

MiR-200a Directly Targeted PTEN in Ovarian Carcinoma

Through TargetScan database (http://www.targetscan.org/vert_71/), miR-200a was found to bind with 3′-UTR region of PTEN (Figure 3A). To confirm that PTEN was directly modulated by miR-200a, we performed Luciferase assay. It suggested that luciferase activity of the cells containing miR-200a mimics and the wild-type of PTEN were significantly suppressed, but this inhibition was less changed for 3′ UTR with mutated binding sites (Figure 3B). In addition, endogenous PTEN mRNA or protein levels were markedly decreased in OVCAR3 cells stably expressing miR-200a compared to that of control cells (Figure 3C). On the contrary, miR-200a inhibitor enhanced the expressions of PTEN (Figure 3D). Therefore, these results indicated that miR-200a directly targeted PTEN, and inhibited the PTEN expression in ovarian cancer cells.

PTEN is Involved in Cell Migration and Invasion

Furthermore, the mRNA expressions level of PTEN in OVCAR3, A2780 cell lines, and normal cell line were detected. These two cell lines had significantly low mRNA expression of PTEN (Figure 4A). Then, we transfected si-PTEN into OVCAR3 cells to explore its role in ovarian carcinoma. The decreased expression of PTEN was found in transfected cells detected by qRT-PCR (Figure 4B). Transwell analysis suggested that knockout of PTEN promoted the migration and invasion in OVCAR3 cells (Figure 4C, 4D). Furthermore, the down-regulation of PTEN mediated by miR-200a mimics could be partially reversed by PTEN plasmid (Figure 5A, 5B). The results of transwell assay showed that overexpression of PTEN partially attenuated the acceleration of miR-200 for cell migration and invasion (Figure 5C). Taken together, miR-200a might promote cell invasion and migration of ovarian carcinoma by targeting PTEN.

Discussion

Ovarian carcinoma is one of the most lethal gynecologic malignancies which are often diagnosed in the late stages. Although the treatment of ovarian cancer has improved because of more effective surgery and optimized combinational chemotherapy, the complete cure rate is only 30%\(^2\). Moreover, the mechanisms of its tumorigenesis and progression are unclear, thus ovarian cancers are urgent to confirm the molecular mechanisms involved in their development.

MiRNA as a critical regulator in the cancer-related processes has been recognized by more and more scholars. Moreover, in a variety of biological pathways such as cell proliferation, differentiation, metabolism, and apoptosis has been reported to be influenced by miRNA\(^25\). It has been reported\(^26-28\) that miR-200a belonging to the miR-200 family is down or up-regulated in all kinds of cancers, such as esophageal cancer, liver cancer, nasopharyngeal carcinoma, and other malignant tumor cells. In addition, the significant role of miR-200a was found during the inhibition of epithelial-to-mesenchymal transition (EMT) and metastasis\(^29\). However, in ovarian carcinoma, miR-200a function is rarely reported and controversial. In recurrent ovarian cancer, miR-200a has been found to have a potentially important function as a biomarker\(^9\). Moreover, miR-200a has been reported inhibiting ovarian cancer migration and invasion with the target of E-cadherin repressor ZEB2\(^31\). The same conclusions are also confirmed in many tumors including nasopharyngeal carcinoma, ovarian cancer, thyroid cancer, and lung cancer\(^30,32\). However, Zhu et al\(^25\) found that the increase in miR-200a had greatly promoted cell proliferation, and it also contributed to the invasion of ovarian cancer cells. Notably, this study proved that miR-200a expression was also up-regulated and promoted cell migration and invasion in ovarian cancer. Therefore, this research about the detailed regulation mechanism of miR-200a in ovarian carcinoma deserves further research.
Figure 2. miR-200a overexpression could promote ovarian carcinoma migration and invasion in vitro. A, The miR-200a expressions in OVCAR3 and A2780 cells containing miR-200a mimic and inhibitor were measured via qRT-PCR. B, miR-200a overexpression increased the migratory and invasive abilities in OVCAR3 cells. C, The miR-200a inhibitor decreased migratory and invasive abilities in OVCAR3 cells. *p<0.05, **p<0.01.
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Figure 3. MiR-200a directly targeted PTEN in ovarian carcinoma. A, The binding site of miR-200a with the wild PTEN 3'-UTR. B, Luciferase activity. C-D, The mRNA and protein expressions of PTEN were analyzed in cells transfected with miR-200a mimic or inhibitor. GAPDH was used as internal control. *p<0.05, **p<0.01.
Furthermore, miR-200a directly targeted PTEN. Moreover, the current work found that the reduction of PTEN could promote cell migration and invasion of ovarian carcinoma. Although a miRNA can regulate hundreds of target genes in a tumor, the role of miR-200a/PTEN axis has not been reported in ovarian cancer. In this study, miR-200a overexpression inhibited PTEN expression through miR-200a targeted PTEN. The PTEN as an inhibitor has been studied in numerous cancers. Li et al. proposed that the PTEN was involved in cell migration and proliferation in Hirschsprung’s disease. Especially, miR-200a participated in the proliferation and apoptosis of endometrial adenocarcinoma cells and the target gene was PTEN. In addition, it has been reported that the function of miR-214/PTEN promoted ovarian carcinoma cell survival and cisplatin resistance. Our conclusion about the inhibited function of PTEN is consistent with all those previous investigations. Collectively, miR-200a promotes cell invasion and migration through inhibiting PTEN in ovarian carcinoma.

Conclusions

Our results suggested that miR-200a had a carcinogenic effect on ovarian cancer through regulating PTEN. This finding may provide an opportunity to develop efficient biomarkers for ovarian carcinoma in the future.

Conflict of Interest:
The authors declared no conflict of interest.
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Figure 5. PTEN is involved in cell migration and invasion. A, PTEN expression was confirmed via qRT-PCR after transfection of PTEN overexpression plasmid and miR-200a mimics. B, PTEN overexpression was confirmed by Western-blots. C, Transwell assay in OVCAR3 cells that co-transfected with PTEN overexpression plasmid and miR-200a mimics. *p<0.05, **p<0.01.
References


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