Original Article
MiR-218 suppresses cell progression by targeting APC in cervical cancer

Yifan Mao¹, Liya Zhang¹, Yuan Li³, Minqin Yan², Lianzhi He³

¹Department of Obstetrics and Gynecology, The Second People’s Hospital in Wuhu City, Anhui Province, China; ²Journal of Oncology, ³Department of Geriatrics, The First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui Province, China

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Abstract: Background and aim: MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that play important roles in carcinogenesis and tumor progression. Previous studies have revealed that miR-218 was abnormally expressed in several cancers. In this study, we aimed to analyze the effect on and mechanism of miR-218 in the progression of cervical cancer (CC). Methods: The tissues from patients undergoing CC resection were collected. MicroRNA chip analysis and real-time PCR were used to detect the level of miR-218 in tissues and cells of CC; western blot was used to detect the protein level in tissues and cell lines. Wound healing assay was performed to test the migration ability and transwell assay was used to detect the invasion ability of Hela and C33A cells. Cell proliferation ability was detected by CCK-8 assay and apoptosis level was measured by TUNEL assay. Luciferase assay was used to confirm whether adenomatous polyposis coli (APC)-3'-UTR is the target gene of miR-218. APC over expression plasmid was transfected to Hela cells to detect the relationship between miR-218 and APC. Results: The expression of miR-218 was dramatically down-regulated in CC tissues and cells (P<0.05). Migration and invasion ability of Hela were decreased after transfected with miR-218 mimics. CCK-8 assay and TUNEL assay showed that miR-218 significantly suppressed the proliferation and promoted apoptosis of CC cells. Luciferase reporter assay identified the 3'-UTR of APC mRNA contained a complementary sequence for miR-218, and APC reversed anti-migration role of miR-218. Conclusion: This study discovered that miR-218 may be involved in the development of CC by positively regulating the level of its target gene APC. And our results may contribute to the development of miRNA-directed diagnostic and therapeutic against CC.

Keywords: Cervical cancer, microRNAs, adenomatous polyposis coli, migration

Introduction
Cervical cancer is the second most common cancer and the third leading cause of cancer-related death among females in less developed countries, with an estimated 500,000 new cases and 300,000 deaths per year [1, 2]. The incidence of cervical cancer is on the rise with a younger median age at diagnosis. Although its mortality decreased along with advances in surgery, radiotherapy, and chemotherapy, patients with CC still showed poor prognosis and significantly variable clinical outcomes due to tumor recurrence and metastasis. Therefore, it is more important to explore the molecular mechanism and new biomarkers of cervical cancer for diagnose and therapeutic.

MicroRNAs (miRNAs) are small, non-coding RNA molecules with 18-25 nucleotides. They inhibit mRNA translation and/or negatively regulate its stability and resulting in upregulation or downregulation of the targeted gene by binding to the 3'-untranslated region (3'UTR) of target mRNAs [3, 4]. Studies have shown that miRNA participate in the tumor cell differentiation, proliferation, and apoptosis processes [5-7]. There are some candidate miRNAs for oncogenic or anti-oncogenic factors in cervical cancer [8], and abnormally levels of miRNA can potentially serve as useful biomarkers for cervical cancer diagnosis [9]. For example, miR-7 regulated apoptosis and malignant behaviors of HeLa and C33A cells through targeting XIAP in cervical cancer [10]. Previous studies showed that upregulation of miR-145 were correlated with aggressive progression and poor prognosis of cervical cancer [11]. Therefore, specific miRNA expression and its modulatory behavior must be explored in order to provide a theoreti-
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**Table 1.** Primers sequences used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5’→3’)</th>
<th>Antisense primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6</td>
<td>TGCGGGTGCTCGGTCAGGCG</td>
<td>CCAAGTGCAGGTCCTGGAGGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGGATCAACAGTTTGTCGAT</td>
<td>AGCCCTTCATCCATGGTGGAAGAC</td>
</tr>
<tr>
<td>miR-218</td>
<td>GTGATAATGTAGCGAGATT</td>
<td>AAAATCTGCTACATTACAC</td>
</tr>
<tr>
<td>APC</td>
<td>GTCCAAGGGTACGCAAGAGT</td>
<td>CATCCTTGCTACCCCTGGAC</td>
</tr>
</tbody>
</table>

cal basis for the pathogenesis of cervical cancer.

Our microarray result showed that miR-218 was down-regulated in CC cells and real-time PCR also confirmed this result. This study aimed to discover the role of miR-218 in CC and explored its molecular mechanism. The results showed that miR-218 was down-regulated in CC tissues and cell lines and was positive correlated with APC in CC lines. Cell culture indicated that miR-218 acted as a tumor suppressor by affecting CC cell proliferation, migration, and invasion. However, we found that miR-218 as a novel role in CC may exert its biological function by targeting APC.

**Materials and methods**

**Patients and samples**

CC tissue samples (n = 20) and unmatched normal cervical epithelium samples (n = 20) were collected from the Department of Obstetrics and Gynecology, the Second People’s Hospital in Wuhu City from 2013 to 2015. No previous systemic or local treatment had been conducted on these patients before the operation or biopsy. All specimens were confirmed pathologically. Informed consent was obtained from all patients, and the study was approved by the ethics committee of the Second People’s Hospital in Wuhu City. The tissues were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C.

**Cell culture and transfection**

The following human CC cell lines were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA): Hela, SiHa, Caski, C33A. The normal human HaCaT cell line was also employed as normal control. All the cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco, CA, USA), 1% of 100 U/ml penicillin and 1% of 100 mg/ml streptomycin sulfates. The cells were incubated in humidified incubators with 5% CO₂ at 37°C.

The miR-218 inhibitor, miR-218 mimics and the negative control were purchased from Suzhou GenePharma Co., Ltd. (Suzhou, China). Human APC gene was constructed into pcDNA3.1(+) by Life Technologies (Invitrogen, CA, USA), and the empty vector was served as the negative control. cDNAs encoding APC sequences were subcloned into a pcDNA3.1 plasmid. All cloned fragments were verified by sequencing. MiR-218 mimics and pcDNA3.1+ APC or pcDNA3.1(+) empty vector were transfected after the cells were cultured to 70-80% confluence by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions.

**Analysis of miR-218 expression by miRNA microarray and q-PCR**

The microRNA microarray gene expression experiments and data analysis were conducted by Suzhou GenePharma Co., Ltd. (Suzhou, China). Total RNA was extracted from the cell lines and frozen tissue specimens with TRIzol reagent (Thermo Fisher Scientific). Complementary DNA was generated using a miScript Reverse Transcription Kit (Qiagen NV, Venlo, the Netherlands). Primers for miR-218, U6 small nuclear RNA (snRNA) (internal control), GAPDH and APC were purchased from Biotech-solutions. The expression level of miRNA-218 was detected based on the threshold cycle (Ct), and relative expression levels were calculated using the 2^ΔΔCt method, using the expression level of the U6 snRNA as a reference gene. Each polymerase chain reaction (PCR) was performed in triplicate. The primers were presented in Table 1.

**Cell proliferation assay**

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was used for cell proliferation analysis following the manufacturer’s instruction. Hela and C33A cells with established stable expression after transfected with miR-218 mimics, miR-218 inhibitor and/or plasmid were seeded at a density of 5×10⁵ cells per well in 96-well plates and incubated for various periods of time (0 h, 24 h, 48 h, 72 h). The absorbance at 450 nm was measured (Thermo Fisher Scientific, Waltham, MA).
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Table 2. Correlation between clinicopathological variables and miR-218 expression in CC patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>miR-218 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Age ≥55 years</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>≤55 years</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>TNM I-II</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>III-IV</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Lymph node metastasis Yes</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Cell apoptosis analysis

Cells were collected and washed twice with cold phosphate-buffered saline solution (PBS). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL) detection kit (Roche, China) was used to demonstrate cell apoptosis followed the manufacturer’s instructions. The tissue section was examined and photographed with a fluorescence microscope after DAPI counterstain.

Cell migration and invasion assays

Transwell chambers (8 μm pore size; Millipore) with (invasion assay) or without (migration assay) Matrigel (BD Biosciences, San Jose, CA, USA) matrix was used to detected cell migration and invasion. Firstly, 500 μl complete medium was added to the bottom chamber, transfected cells were suspended in serum-free medium, and 200 μl cell suspension contained 1×10⁵ cells was placed in the upper chamber. 48 hours later, the cells on the top surface of the membrane were mechanically removed with a cotton swab, and the cells on the bottom surface of the membrane were fixed in 95% ethanol and stained with 0.2% crystal violet solution. Five randomly areas of bottom surface of the membrane were selected to count cells under a 200× microscope field. Three independent experiments were performed.

Wound-healing assay

Wound-healing assay was performed using Hela and C33A cells. 8×10⁵ cells were seeded in each well of a 6-well plate, and allowed to grow until confluent. When serum starvation for 24 hours, an artificial homogenous wound (scratch) was created onto the cell monolayer with a sterile 100 μL tip and washed with phosphate buffered saline (PBS) to remove cell debris. After scratching, the cells were washed with serum-free medium, complete media was added, and microscopic images (20× magnification) of the cells were collected at 0 and 48 hours. Image J Plus was used to quantify the wound healing assays. Each experiment was repeated three times.

Western blot analysis

The protein from the cervical tissues and cell extracts were resolved by SDS-PAGE, and analyzed by western blotting. Antibodies used for Western blotting were purchased from Cell Signaling Technology (CST, USA). Following incubation with horseradish peroxidase-coupled secondary anti-mouse/rabbit (ZSGB-BIO, China), protein bands were visualized using ECL Blotting Detection Reagents (Millipore, Germany).

Target prediction and luciferase reporter assay

The target genes of miR-218 were predicted using three Bioinformatics algorithms: Target-Scans, mi-Randa (miRcropedia.org), PicTar (4-way). The APC was chosen to construct dual-Luciferase reporter vectors.

For the luciferase reporter assay, Hela cells were seeded at 1×10⁵ cells per well in a 24-well plate and were transfected after 24 h with 100 ng of pcDNA3.1-APC-3'-UTR (wild type/mutant) or control-luciferase plasmid plus 1 ng of pcDNA3.1 empty vector renilla plasmid (Promega, USA), and 5 nM of miR-218 precursor. Forty-eight hours after transfection, the cells were assayed for both firefly and renilla luciferase using the Dual-luciferase glow assay (Promega, Madison, WI). Three independent experiments were performed and the data was presented as the mean ± standard deviation (SD).

Statistical analyses

All data were repeated for three times and experimental data were analyzed and presented as mean ± SD analyzed by SPSS 17.0 (SPSS, IL, USA). Student’s t test was used for independent groups. miR-218 expression comparisons
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Figure 1. Microarray shows the significant downregulation of miR-218 in HeLa cells compared with HaCaT. Statistical significance is indicated as: P<0.05, Student’s t test.

Figure 2. Expression of miR-218 was downregulated in CC. A. CC tissues showed lower expression of miR-218 compared with normal tissues in 20 cases of unmatched clinical specimens. B. Detection of miR-218 expression in CC cells and normal cell using real-time PCR. The data is shown as the means ± SD. *P<0.05; **P<0.01.

were conducted by chi-squared test. P values that less than 0.05 were considered statistically significant with representation *P<0.05, **P<0.001.

Results

MiR-218 is downregulated in CC cell and tumor tissues

We compared clinicopathological variables between CC patients with positive negative miR-218 expressions, and found that a remarkably negative miR-218 expression was significantly associated with lymph node metastasis (Table 2). Even though previous studies have been reported that a number of miRNAs were implicated in CC progression, another miRNAs might be in this process according to the complexity of CC progression. miRNA expression profile of CC cell lines was detected with HaCaT (normal CC cell) and Hela by miRNA microarray analysis (Figure 1). Among the miRNAs which were identified to be down-regulated in Hela compared with HaCaT, the miR-218 in CC progression remains unknown. Real-time PCR also showed that the level of miR-218 was significantly decreased in CC cell lines selected in the study compared with HaCaT (Figure 2A). Furthermore, we found that miR-218 expression was significantly decreased in CC tissues compared with non-tumor tissues (Figure 2B). These result showed that miR-218 downregulation is correlated with increased CC development, suggesting that miR-218 might inhibit activity of CC cells.

MiR-218 inhibits cell proliferation and promotes cell apoptosis

MiR-218 mimics, inhibitor and negative control were synthesized and transfected into the Hela and C33A cells, respectively. The results indicated that the expression of miR-218 in Hela and C33A cells transfected with miR-218 mimics was increased compared with cells transfected with the negative control, and the expression of miR-218 transfected with miR-218 inhibitor was decreased compared with cells transfected with the negative control (P<0.05, Figure 3A). Then the effect of miR-218 on cell proliferation was performed in cells by CCK-8 assay. The OD = 450 nm value in the CCK-8 assay revealed that proliferation of Hela and C33A was markedly decreased by the transfection of miR-218 mimics compared to the negative control (P<0.05). However, miR-218 inhibitor significantly promoted the proliferation of Hela and C33A cells (P<0.05, Figure 3B). TUNEL assay showed that cell apoptotic following miR-218 mimics was drastically increased compared to that in negative control group both showed in Hela and C33A cells (P<0.05, Figure 3C).
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**Effect of miR-218 on migration and invasion of CC**

Cell migration and invasion is a significant aspect of cancer progression. To test the role of miR-218 on the ability of CC cell migration and invasion, we transfected miR-218 mimics and inhibitor into Hela and C33A cells, respectively. And wound healing assay was used to detected cell migration, transwell assay was used to show cell invasion ability. The migration ability of Hela and C33A cells was decreased in the miR-218 mimics group compared with the negative control showed in Figure 4A. Transwell results showed that miR-218 mimics significantly reduce the invasiveness of Hela and C33A cells compared with negative control whereas miR-218 inhibitor promoted the cell invasion ability (Figure 4B). Western blot were applied to verify protein level of APC, E-cadherin and β-catenin in Hela and C33A cells, so as to further explore the relationship between miR-218 and EMT. The results indicated that miR-218 induced APC and E-cadherin protein expression and suppressed the protein level of β-catenin in CC cells (Figure 4C). These results suggested that miR-218 had a negative effect on cell migration and invasion in CC.

**APC is a direct target of miR-218**

Bioinformatics analysis was used to predict potential target genes of miR-218. We found that the 3'-UTR of APC mRNA contained a target site for miR-218 (Figure 5A). Furthermore, luciferase reporter assay was performed to confirm APC as a direct target of miR-218. Our results showed that miR-218 significantly suppressed the luciferase activity of the wild type (wt) but not the mutant (mut) 3'-UTR of APC.
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Figure 4. MiR-218 inhibited cell migration and invasion in CC. A. Wound healing assay showed that miR-218 mimics significantly inhibited cell migration and miR-218 inhibitor promoted cell migration in HeLa and C33A. B. Transwell assay demonstrated that miR-218 suppressed cell invasion in HeLa and C33A. C. To detect the expression of APC, E-cadherin, and β-catenin in HeLa and C33A transfected with miR-218 mimics or inhibitor. The data is shown as the means ± SD. *P<0.05; **P<0.01.
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APS 3'-UTR 5' ...CAGGUACUGUUUGUCCUGUUGG...

miR-218 -3p 3' CAAAGGUAGCAAGGUGGG

APC 3'-UTR mutant ...CAGGUACUUGAGCGUGUGG...

Figure 5. APC is a direct target of miR-218. A. Bioinformatics prediction of the 3'-UTR of APC mRNA contained a target site for miR-218. B. Luciferase activity assay revealed that miR-218 suppressed wild APC 3'-UTR luciferase activity, while it had no effect on mutant APC 3'-UTR luciferase activity compared to control in HeLa cells. C. Expression of APC mRNA affected by miR-218. The data is shown as the means ± SD. *P<0.05; **P<0.01.

Figure 6. MiR-218 regulated cell invasion through affecting the expression of APC. A. Change of invasion ability of HeLa detected by transwell assay. B. The protein level of APC and β-catenin in HeLa cells transfected with miR-218 inhibitor and/or pcDNA3.1-APC. The data are shown as the means ± SD. *P<0.05; **P<0.01.

(P<0.05, Figure 5B). Moreover, the results of real-time PCR and western blot showed that over-expression of miR-218 significantly promoted the expression of APC in Hela and C33A cells (P<0.05, Figure 5C). These results discovered that APC was one of the direct targets gene of miR-218.

Effect of APC on cell migration and invasion

To investigate whether the tumor migration and invasion suppressor function of miR-218 is mediated by APC, HeLa cells were co-transfect-
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progression of cancers [12] and function as tumor promoters or suppressors [13]. Therefore, we hypothesized that miR-218 may act as a tumor-suppressor in cervical cancer. Previous studies demonstrated that miR-218 was down-regulated in colorectal cancer, lung squamous cell carcinoma, breast cancer, gastric cancer and played a tumor-suppressor role in these malignant carcinomas [14-17]. MiR-218 sensitized HCT-116/L-OHP cells to L-OHP-induced cell apoptosis via inhibition of cytoprotective autophagy by targeting YEATS4 expression in colorectal cancer [14]. In addition, epigenetic silencing of miR-218 by the IncRNA CCAT1, acting via BMI1, promotes an altered cell cycle transition in the malignant transformation of HBE cells induced by cigarette smoke extract [18]. miR-218 suppressed metastasis of colorectal cancer by post-transcriptionally inhibiting the MACC1 expression [19]. However, the novel functions and its mechanisms of miR-218 in CC still need in-depth study. In this study, we discovered that miR-218 was down-regulated in CC, and suppressed proliferation, migration and invasion of CC cell lines by directed targeting APC.

Adenomatous polyposis coli, also known as APC, GS, DP2 and BTPS2. APC located at chromosomal band 5q21-q22 and encodes a tumor suppressor protein that related to the Wnt signaling pathway [20]. Furthermore, it is also involved in other cancer processes including metastasis, transcriptional activation, and apoptosis [21-23]. Defects in APC can lead to familial adenomatous polyposis (FAP), an autosomal dominant pre-malignant disease may result in malignancy [24].

Previous study demonstrated that the inactivation of APC gene leads to dysfunction of β-catenin degradation, and then activates Tcf/Lef and causes abnormal transcription of oncogenes, such as c-myc, c-jun and cyclin D1, finally leads to carcinogenesis [25]. On the other way, APC plays an integral role in the wnt-signaling pathway and in intercellular adhesion. The decreased expression of APC activated Wnt/β-catenin pathway then regulated cell proliferation, migration and a wide range of malignancies [26]. Nicola S et al [25] showed that mutation in the APC gene is the basis of inherited predisposition to colorectal cancer and is also the primary event in initiation of sporadic colorectal tumors. However, mutant APC may also break intercellular adhesion and stability of the cytoskeleton, both of which play a important part in tumor progression. However, APC remains a controversial gene in function, for example, Masaru Shinozaki [27] thought APC methylation was related to breast cancer (P<0.05), but So Yeon Park [28] and Susan R. Sturgeon [29] thought APC methylation had no relationship with breast cancer (P>0.05). But a meta-analysis showed that APC was indeed associated with breast cancer.

In this study, we first found that miR-218 was down-regulated in CC cells and tissues compared with normal cervical cells and tissues, respectively. And these results demonstrated that miR-218 might act as CC suppressor. However, the subsequent inhibition assays, luciferase reporter assay, and rescue assays further demonstrated that miR-218 exerts its tumor suppressor role in CC cells by targeting APC. In conclusion, this study explored a novel miR-218 targeted APC axis which showed potential therapeutic value for CC treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Lianzhi He, Department of Geriatrics, The First Affiliated Hospital of Wannan Medical College, Wuhu 241000, Anhui Province, China. E-mail: helianzhiahwh@163.com

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