Original Article

MiR-489-3p inhibits cervical cancer cell migration and invasion through regulating Wnt/β-catenin signaling pathways

Yongjie Pan¹, Jinhua Dai¹, Yufeng Liao¹, Jun Xu²

Departments of ¹Clinical Laboratory, ²Nursing, Ningbo No. 2 Hospital, Ningbo 315010, Zhejiang, China

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Abstract: MicroRNAs (miRNAs) play crucial roles in the development and progression of human cancers, including cervical cancer. The discovery of miRNAs may provide a new and powerful tool for studying the mechanism, diagnosis, and treatment of cervical cancer. In this study, we aimed to investigate the role and mechanism of miR-489-3p in the development and progression of cervical cancer. Quantitative real-time PCR (qRT-PCR) was used to measure the expression level of miR-489-3p in cervical cancer tissues and cell lines and found that miR-489-3p was significantly down-regulated. In addition, over-expression of miR-489-3p inhibited cervical cancer cell migration and invasion. Subsequent dual-luciferase reporter assay identified one of the proto-oncogene RAB1 as direct target of miR-489-3p, and RAB1 can save metastasis inhibition caused by miR-489-3p. We also confirmed that the metastasis inhibition caused by miR-489-3p is associated with Wnt/β-catenin pathway activation. Taken together, our results indicate that miR-489-3p could serve as a potential diagnostic biomarker and therapeutic option for cervical cancer in the near future.

Keywords: Cervical cancer, miR-489-3p, RAB1, migration and invasion, Wnt/β-catenin pathways

Introduction

Cervical cancer has been recognised as one of the most frequent malignancies, which is one of the most serious threat to women’s health with the highest incidence and mortality of women’s tumors in the world [1-3]. Current studies suggest that cervical cancer is a complicated multi-factor and multi stage process, involving a large number of tumor related gene structure change and abnormal expression, among them, transcription regulation level control, and the mRNA degradation regulation are under the influence of miRNA [3-6]. Researches about cervical cancer have achieved great progress, while effective therapies for treating cervical cancer are lacking.

MicroRNAs (miRNAs) are short non-coding RNAs that act as important regulators of gene expression, which play a critical role in regulating varieties of the biological and pathologic processes, including various cancers [7-9]. MiRNA affects the expression of oncogenes or tumor suppressor genes in cervical cancer from multi-angle, multi-level, therefore, and has become the hot topic in the cervical cancer study [10, 11]. For instance, Deng Y, et al, has found that miR-376c was significantly downregulated in cervical cancer cell lines and clinical tissues and the upregulation of miR-376c inhibits cell proliferation [12]. Wang N, et al, has found that down-regulation of miR-31 impaired cell proliferation, cell migration and invasion in vitro [13].

Studies has found that miR-489 is a kind of miRNA closely correlated with tumor progress and development [14, 15]. Patel Y, et al, has indicated that miR-489 high expressed in the mammary epithelial cells than adjacent tumor cells, which proved the direct evidence of role of miR-489 in the development of cancer [14]. Study of Xie Z, et al, has confirmed that miR-489-3p low expressed in non-small cell lung cancer cell line, and overexpression of miR-489-3p can inhibit invasion of non-small cell
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lung cancer cells [1]. However, role of miR-489-3p in cervical cancer has not been reported.

In the current study, we aimed to explore the expression and the underlying mechanism of miR-489-3p in cervical cancer. We used quantitative real-time PCR (qRT-PCR) to measure the expression level of miR-489-3p in cervical cancer tissues and cell lines. We found that miR-489-3p was significantly down-regulated. In addition, over-expression of miR-489-3p inhibited cervical cancer cell migration and invasion. Subsequent dual-luciferase reporter assay identified RAB1 as direct target of miR-489-3p, and RAB1 can save metastasis inhibition caused by miR-489-3p. We also confirmed that the metastasis inhibition caused by miR-489-3p is associated with Wnt/β-catenin pathway activation.

All of our efforts will provide theoretical basis and new insights into the treatment of cervical cancer.

Material and methods

Clinical samples

The cervical cancer and non-cancerous cervix tissues were collected in Obstetrics and Gynecology Hospital of Fudan University, China. The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Obstetrics and Gynecology Hospital of Fudan University [2].

Cell culture and transfection

Human cervical cancer cell lines HeLa, SiHa, C33A and CaSki were obtained from Chinese Academy of Sciences (Shanghai, China). The normal human cervical cancer cell lines were restored in our own laboratory. All cell lines were seeded in DMEM medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% CO₂.

The miRNA mimic and ASO-miR were synthesized by RiboBio (Guangzhou, China), and the pcDNA-3.1-RAB1 plasmid was designed by GenePharma (Shanghai, China). Cells were seeded at 50% confluence, and 16 h later, cells were transfected with the miRNA mimic/inhibitor or pcDNA-3.1-RAB1 plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Scrambled miRNA or the pcDNA-3.1 plasmid were used as negative controls. After 48 h, cells were harvested for subsequent analysis [2].

RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated according to the standard procedure using TRizol reagent (Invitrogen, Carlsbad, CA, USA). The miRNA expression was measured using miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed on ABI 7500 Real-Time PCR System (Applied Biosystems, USA) with the following conditions: 95°C, 10 min for 1 cycle, then 95°C, 15 sec, 60°C, 1 min for 40 cycles [2].

Luciferase activity assay

Luciferase reporters were generated based on the firefly luciferase expressing vector pMIRREPORT (Ambion, Foster City, CA, USA). Cells were seeded in 24-well plates at the density of 5×10⁴ cells per well one day advance the transfection. Luciferase reporter (500 ng), 50 pmol (miRNA mimic, inhibitor or negative control) and 40 ng of pRL-TK were added in each well. Cells were analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after transfection.

Transwell migration assay and matrigel invasion assay

The cell migration and invasion assays were performed by Transwell chamber with a pore size of 8 μm (Costar, New York, NY, USA). The inserts were coated with 50 μl of Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) for the Matrigel invasion assay. Cells were plated into the upper chamber containing 100 μl of serum-free medium, and the lower chamber was filled with medium containing 20% FBS as a chemotactrant. The cells in the upper chambers were removed after incubation for 24 h at 37°C under 5% CO₂ in a humidified incubator. And the cells that migrated to the lower surface of the filter were fixed in 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min [16].
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Figure 1. The expression level of miR-489-3p. A: The expression level of miR-489-3p analyzed by RT-PCR in cervical cancer tissues. B: The expression levels of miR-489-3p in cervical cancer cells and normal cells. Error bars indicate means ± SD and * indicates significant difference compared with blank group (P<0.05).

Western blotting

The cell were washed twice with PBS, and lysed in RIPA lysis buffer and then centrifuged at 12,000×g for 5 min at 4°C. Samples protein were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% not-fat milk in TBST and incubated with primary antibodies overnight, which was washed with TBST for
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<table>
<thead>
<tr>
<th>Position 329-335 of Rab1 3' UTR</th>
<th>Predicted consequent pairing of target region (top) and miRNA (bottom)</th>
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</thead>
<tbody>
<tr>
<td>hsa-miR-489-3p</td>
<td>5'...AUAAUGGCAUGUUAGAGUCA...</td>
</tr>
<tr>
<td></td>
<td>3'...CGACGCCAUACACUAACAGUG...</td>
</tr>
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three times and incubated with secondary antibodies in TBST at room temperature for 1 h. The proteins were detected using enhanced chemiluminescence (ECL) detection reagents (Roche, Basel, Switzerland) [2].

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (version 13.0). Student’s t test, one-way ANOVA, and Pearson’s chi-square test were used according to the data characteristics. P<0.05 was considered statistically significant. The quantitative data are presented as mean ± SEM.

Results

MiR-489-3p low-expressed in cervical cancer

We used qRT-PCR to detect the expression of miR-489-3p level of 24 couples of cervical cancer tissue and the adjacent normal tissue, the result is shown in Figure 1A. Obviously, miR-489-3p was significantly low-expressed in cervical cancer tissue (P<0.05). Meantime, we used qRT-PCR to construct the detection of the levels of miR-489-3p expression in cervical cancer cell lines of Caski, SiHa, HeLa, HT-3 and normal endothelial cell line HIEC. The results as shown in Figure 1B, compared with normal mucosa cells, miR-489-3p significantly down expressed in cervical cancer cells (P<0.05).

MiR-489-3p inhibits cervical cancer cells migration and invasion

In this part of work, we choses Caski and HeLa for the following research which have low expression of miR-489-3p. In Caski and HeLa cells, the transwell experiment found that over-expression of miR-489-3p could inhibit cervical cancer cell migration and invasion ability, while
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silence of miR-489-3p could promote cell migration and invasion (Figure 2A and 2B). Furtherly, we detected the expression of epithelial mesenchymal cell transformation process related proteins by qRT-PCR and western blot assay. The results shown that overexpression of miR-489-3p could inhibit MET-related protein expression, while silence of miR-489-3p got the inversely results (Figure 2C and 2D).

**RAB1 is the direct target gene of miR-489-3p**

We predicted RAB1 as the potential target genes of miR-489-3p by using Targets can as shown in Figure 3A. We confirmed miR-489-3p target 3’UTR of miR-489-3p by Luciferase reporter vector technology (Figure 3B). At the same time, by using the qRT-PCR and western blot technique, we proved that miR-489-3p negatively regulate the expression of RAB1 (Figure 3C and 3D).

**RAB1 protect miR-489-3p induced cervical cancer cells migration inhibition**

Next, we research function of RAB1 in Hela cells. The results by transwell analysis tell that RAB1 could rescue both of the migration and invasion inhibition as shown in Figure 4A and 4B. Meantime, qRT-PCR and western blot technique also proved RAB1 overexpression can save EMT inhibition caused by miR-489-3p (Figure 4C and 4D).

**MiR-489-3p inhibits Wnt/β-catenin signaling pathway**

Previous studies have shown that Wnt/β-catenin pathway plays an important role in the occurrence and development of cervical cancer [17, 18]. So in this present study, we continue to explore whether the miR-489-3p inhibits the Wnt pathway activation by targeting RAB1 in cervical cancer cells. We detected several important factors’ expression in Hela cells, such as β-catenin, GSK-3β, p-GSK-3β, and Ki-67 gene. The results shown in Figure 5A and 5B that overexpression of miR-489-3p notably inhibits expression of β-catenin and Ki-67 and promotes GSK-3β and p-GSK-3β (P<0.05). We got the opposite results when miR-489-3p expression is inhibited.

In addition, we also found that overexpression of miR-489-3p could significantly inhibit two important transcription factors expression of Wnt/β-catenin signaling pathway, TCF-4 and LEF-1 (Figure 5C and 5D).

Above on, we can believe that miR-489-3p could induce the Wnt/β-catenin signaling pathway inhibition.
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Discussion

Cervical cancer is one of the most prevalent cancer related morbidity and mortality in women worldwide, among them, up to 3% of cases of cervical cancer are found in pregnant women or those who are in the post-birth period [19, 20]. Multiple factors are involved in the mechanism of cervical cancer, and many therapies and treatments were proposed in cervical cancer, according to the reports before. For instance, the study of Boyraz G, et al, has evaluated the clinical and pathological characteristics of the affected patients including age, histologic subtype, tumor size, depth of cervical stromal invasion, lympho-vascular space invasion (LVSI), and lymph node metastasis, and LVSI, deep cervical stromal invasion, lymph node metastasis and tumor size are significantly associated with clinical early-stage cervical cancer [21]. Though effective screening, diagnosis and treatment strategies of cervical cancer have dramatically decreased the morbidity and mortality of this malignancy, cervical cancer remains the second cause of cancer death in females in their 20 s and 30 s [21].

MiRNAs may act as carcinogen or tumor suppressor genes by targeting various biological molecules, and is important to identify significant markers for prognosis, diagnosis treatment strategies of cancers [22]. A large number of studies have shown that miRNA involved in almost all cell biological processes, including individual development, cell proliferation, cell apoptosis, cell differentiation, cell migration and invasion. For instance, the study by Takacs CM, et al, has proposed that miR-430 coordinates the stereotypical cell divisions that instruct neural tube morphogenesis in zebrafish [23]. The study on hepatocellular carcinoma constructed by Zhou X, et al, has proved that miR-625 might function as an anti-metastatic miRNA to have an important role in hepatocellular carcinoma progression by modulating the IGF2BP1/PTEN pathway [24]. Additionally, researchers have found some abnormal expression of miRNA in cervical cancer, including miR-96, miR-144, and miR-126 [25].

In this study, we investigated the role of miR-489-3p in cervical cancer and tried to make clear of the underlying mechanism. Our study found that miR-489-3p low-expressed in cervical cancer, and inhibits cervical cancer cell migration and invasion through targeting RAB1.

Wnt pathways are critical for embryonic development and adult tissue homeostasis in all multicellular animals [26]. Moreover, the Wnt/β-Catenin signaling pathway anomaly is closely related to the occurrence of a wide variety of tumors, including colorectal cancer, gastric cancer, and liver cancer [27-29]. In our study, we found miR-489-3p induced wnt-catenin pathway inhibition by targeting RAB1 in cervical cancer.
In conclusion, all our findings convinced us that miR-489-3p plays a role as tumor suppressor gene in cervical cancer, and miR-489-3p can be used as a potential biomarkers of cervical cancer diagnosis and treatment options.

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Disclosure of conflict of interest

None.

Address correspondence to: Jun Xu, Department of Nursing, Ningbo No. 2 Hospital, No. 41 Xibei Street, Ningbo 315010, Zhejiang, China. E-mail: xujun87-52@126.com

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