Effects of miR-214 on cervical cancer cell proliferation, apoptosis and invasion via modulating PI3K/AKT/mTOR signal pathway


Department of Obstetrics and Gynecology, the Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China

Fei Wang and Wenhua Tan contributed equally to this work

Abstract. – OBJECTIVE: PI3K/AKT/mTOR pathway plays important roles in tumor pathogenesis. mTOR is up-regulated and miR-214 is down-regulated in cervical cancers. This study investigated whether miR-214 regulated mTOR expression and affected cervical cancer cell proliferation, apoptosis or invasion.

PATIENTS AND METHODS: Cervical cancer tissues were collected in parallel with normal epithelium for measuring the expression of miR-214 and mTOR. Dual luciferase expression assay was performed to evaluate the targeted relationship between miR-214 and mTOR. In vitro cultured SiHa cells were treated with miR-214 mimic or si-mTOR followed by measuring mTOR, p-mTOR and Bcl-2 expression. Cell apoptosis, proliferation and invasion were measured by flow cytometry and transwell assay.

RESULTS: Bioinformatics analysis showed targeted binding sites between miR-214 and 3' UTR of mTOR mRNA. Dual luciferase reporter assay confirmed this regulatory relationship between miR-214 and mTOR mRNA. Compared to normal cervical epithelium, cancer tissues had lower expression of miR-214 and higher mTOR, both of which were correlated with TNM stage and tissue pathology grade. Compared to Ect1/E6E7 cells, SiHa cells had lower level of miR-214 and higher mTOR/p-mTOR and Bcl-2 expression. Transfection of miR-214 mimic or si-mTOR significantly decreased mTOR/p-mTOR or Bcl-2 expression, inhibited cell proliferation or invasion, and enhanced cell apoptosis.

CONCLUSIONS: miR-214 down-regulation plays a role in elevating mTOR expression and in facilitating cervical cancer pathogenesis. Over-expression of miR-214 inhibits cervical cancer cell proliferation or invasion, and facilitates apoptosis via targeted inhibition of mTOR expression.

Key Words
miR-214, PI3K/AKT/mTOR, Cervical cancer, Proliferation, Apoptosis, Invasion.

Introduction

Cervical carcinoma (CC) is a common female reproductive cancer in clinic, and is the second popular malignant tumors in females, only next to breast cancer. Incidence of CC is increasing gradually with younger population age, severely threatening female health and life.

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) is a widely expressed signal pathway across multiple tissues and cells, and plays a crucial role in regulating cell survival, cycle, proliferation and apoptosis. Thus, it is correlated with the occurrence, progression, metastasis and drug resistance of multiple tumors. Mammalian target of rapamycin (mTOR) is a highly conserved protein during evolution, and is the member of PI3K protein kinase family as a serine/threonine protein kinase. Although mTOR can respond to various extracellular stimuli or regulatory factors such as cytokines, mitogen, ATP, nutrient status and energy metabolism, its function or activity is mainly regulated by PI3K/ATK signal pathway. Abnormally elevated mTOR expression induces over-expression of PI3K/AKT-mTOR signal pathway, which promotes cell proliferation, migration, invasion and inhibits apoptosis. In addition, mTOR expression is correlated with the onset, progression, metastasis and drug resistance of multiple tumors including breast cancer, prostate cancer, gastric carcinoma and colorectal carcinoma.

MicroRNA (miR) is a group of small molecule non-coding RNA with a length of 22-25 nt in eukaryotes, and regulates gene expression through binding to 3'-untranslated region (3'-UTR) of target gene mRNA in a complete or incomplete complementary binding manner. MiR,
which occupies only 1% of human genome, can regulate the expression of more than 30% of human target genes\textsuperscript{12}. Various studies showed significantly decreased miR-214 expression in CC tumor tissues, indicating its potential tumor-suppressing role in CC pathogenesis\textsuperscript{13-15}. Bioinformatics analysis revealed the existence of complementary binding sites between miR-214 and 3'-UTR of mTOR mRNA. This work investigated if miR-214 played a role in mediating mTOR expression and PI3K/AKT/mTOR pathway activity, as well as in affecting CC cell proliferation, apoptosis and invasion.

**Patients and Methods**

**Clinical Information**
A total of 38 CC patients who received radical surgery resection in the Second Affiliated Hospital of Harbin Medical University from August 2016 to January 2017 were recruited in this study (average age = 48.6 ± 10.3 years). All patients did not receive any chemotherapeutic or radiotherapy before surgery. CC tumor tissues were collected during the surgery and divided into 11 cases of G1, 14 cases of G2 and 13 cases of G3 by histopathology grade. Based on TNM criteria, there were 21 cases in stage I-II and 17 cases in stage III-IV. Another cohort of 17 normal cervical mucosal epithelial tissue samples was collected from surgery and recruited as the control group (average age = 56.8 ± 13.2 years). No significant difference of age or sex ratio was observed between two groups. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and informed consents were obtained from all participants before enrollment.

**Major Reagent and Materials**
Human CC epithelial cell line SiHa and normal cervical epithelial cell line Ect1/E6E7 were purchased from Shanghai Cell Banks of CAS (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). RNA extraction kit GenElute total RNA Purification Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Transfection reagent FuGENE6 was purchased from Roche (Basel, Switzerland). QuantTech SYBR Green qRT-PCR Kit was purchased from Qiagen (Hilden, Germany). MiR-214 mimic, miR-214 inhibitor and miR-NC were purchased from RiboBio (Guangzhou, China). Si-NC and si-mTOR were purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse anti-human mTOR and p-mTOR antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-human Bcl-2, β-actin antibody and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Abcam (Cambridge, MA, USA). EdU cell proliferation kit was purchased from Molecular Probes (Eugene, OR, USA). Luciferase activity assay kit was purchased from Promega (Madison, WI, USA). pMIR luciferase reporter plasmid was purchased from BioVector (Beijing, China). Annexin V/PI cell apoptosis assay kit was purchased from Ebioscience (San Diego, CA, USA). Transwell chamber was purchased from Corning (Corning, NY, USA).

**Cell Culture**
SiHa and Ect1/E6E7 cells were inoculated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) plus 1% penicillin-streptomycin in a 37°C chamber with 5% CO\textsubscript{2}. Cells were passed at a 1:4 ratio when reaching 70-80% confluence, and those cells at log-growth phase with satisfactory growth status were used for experiments.

**Dual Luciferase Activity Assay**
Using HEK293T cell genome as the template, full-length or mutant fragment of 3'-UTR of mTOR gene was amplified and sub-cloned into pMIR plasmid for transforming DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and named as pMIR-mTOR-UTR-wt and pMIR-mTOR-UTR-mut. FuGENE6 was used to co-transfect pMIR-mTOR-UTR-wt (or pMIR-mTOR-UTR-mut) and miR-214 mimic (or miR-214 inhibitor or miR-NC) into HEK293T cells. After 48 h incubation, cells were rinsed twice in phosphate-buffered saline (PBS). Passive Lysis Buffer was added to complete lysis on ice. 10 μL lysate were added into 96-well plate for mixture with Stop&Go buffer. Dual luciferase activity was measured at 560 nm wavelength using a micro-plate reader.

**Cell Transfection and Grouping**
*In vitro* cultured SiHa cells were assigned into four transfection groups: miR-NC transfection group, miR-214 mimic transfection group, si-NC transfection group, and si-mTOR transfection group.
group. One day before transfection, cells were inoculated into culture plate to reach 60-70% confluence at transfection. During transfection, 100 μL serum- or antibiotics- free basic medium were used to dilute 10 μL FuGENE6. After gentle mixture, 30 nmol. miR-NC, miR-214 mimic, si-NC or si-mTOR were added for gentle mixture and incubation at room temperature for 20 min. Medium containing fetal bovine serum (FBS) and dual antibiotics were removed. Transfection complex was added for 6 h incubation after complete mixture. Then, normal complete medium containing FBS and dual antibiotics was used for 72 h of continuous incubation. Cells were collected for analysis of proliferation, invasion or apoptosis.

qRT-PCR for Gene Expression
QuantiTech SYBR Green RT-PCR Kit was used to test relative expression level of target genes in one-step qRT-PCR using RNA extracted by GenElute total RNA extraction kit. In a 20 μL reaction system, there were 10 μL 2 X QuantiTech SYBR Green Master Mix, 1.0 μL forward and reverse primers (0.5 μm/L each), 2 μg RNA template, 0.5 μL QuantiTech RT Mix, and distilled water. Reverse transcription conditions were: 50°C for 30 min. PCR conditions were: 95°C 15 min pre-denature, followed by 40 cycles each at 94°C for 15 s for denature, 60°C for 30 s for annealing, and 72°C for 30 s for elongation. Gene expression was measured on ABI ViiA 7 Real-time fluorescent qPCR cycler. Primers were designed as follows: miR-214PF: 5’-GGACA GGACG CACAG TCA-3’; miR-214P R: 5’-CAGAC GAGGC TCCGT GGT-3’; U6PF: 5’-ATTGGAAC GAACAT GCAG AT-3’; U6PP: 5’-GGAAA CTCGCT TTTG-3’; mTORPF: 5’-TCCGAGGAGAT GAGTC AAGAG G-3’; mTORP R: 5’-CACCT TCCAC TCTCTC TCTAT-3’; Bcl-2PF: 5’-GGTGG GGTCA TGTGTT GTGG-3’; Bcl-2PR: 5’-CGGTT CAGGCA TCTCA GTCAT CC-3’; β-actinPF: 5’-GAACC CTAAG GCCAA C-3’; β-actinPR: 5’-TGTCCT CCGCAG GATTTC C-3’.

Western Blot
Cells from all groups were lysed by RIPA lysis buffer, and concentration of protein supernatant was measured. 50 μg samples were loaded and separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to the membrane, which was blocked in 5% defatted milk powder at room temperature. Primary antibody (mTOR at 1:3000, p-mTOR at 1:1000, Bcl-2 at 1:2000, and β-actin at 1:18000) was added at 4°C incubation overnight. The membrane was rinsed in phosphate-buffered saline and Tween (PBST) for three times, and horseradish-peroxidase (HRP) conjugated secondary antibody (1:25000) was added for 60 min room temperature incubation. With three times of PBST rinsing, ECL approach was used to develop the membrane for 2-3 min at room temperature. After exposure and visualization, the film was scanning for data processing.

Cell Apoptosis Assay
Cells were digested by trypsin and then collected. Cell digestion was quenched using culture medium containing 10% FBS, followed by 300 x g centrifugation for 5 min. The supernatant was mixed with 5 mL PBS and was centrifuged at 300 x g for 5 min for washing. 100 μL Binding Buffer were mixed with every 1 X 10⁶ cells. After re-suspension, 5 μL Annexin V-FITC were added for 10 min dark incubation at room temperature, followed by 5 μL propidium iodide (PI) staining for 5 min. Cell apoptosis was measured by Beckman Coulter FC 500 MCL (Brea, CA, USA) flow cytometry.

Flow Cytometry for Cell Proliferation
Cells were re-suspended in complete medium. Cell proliferation was measured by Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kits. In brief, after incubation in 10 μM EdU for 2 h, cells were continuously incubated for 48 h, and digested by trypsin. After centrifugation, fixation and permeabilization, reaction buffer with Alexa Fluor 488 labels was added for 30 min dark incubation at room temperature. By centrifugation and washing, Beckman Coulter FC500MCL flow cytometry was used to measure cell proliferation.

Transwell for Cell Invasion Potency
100 μL Matrigel were paved on the upper surface of transwell chamber, which was incubated at 37°C incubator for 30 min for complete polymerization. 500 μL complete medium containing 10% FBS were added into 24-well plate, which contained inserting transwell chamber. 200 μL SiHa cells re-suspended in serum-free medium were added into the upper chamber for 48 h of further incubation. Cells were fixed in methanol and stained in 0.1% crystal violet, and the number of perforated cells was measured under an inverted microscope.
Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. All measurement data were presented as mean ± standard deviation (SD). Student t-test was performed to compare measurement data between groups. A statistical significance was considered when \( p < 0.05 \).

Results

Targeted Regulatory Relationship Between miR-214 and mTOR

Online prediction from microRNA.org showed the existence of complementary binding sites between miR-214 and 3'-UTR of mTOR mRNA (Figure 1A). Dual luciferase gene reporter assay showed that transfection of miR-214 mimic significantly suppressed the relative luciferase activity in HEK293T cells transfected with pMIR-mTOR-wt plasmid. However, those cells transfected with miR-214 inhibitor had elevated luciferase activity (Figure 1B). Both of them, however, had no significant effect on the relative luciferase activity in HEK293T cells with pMIR-mTOR-mut plasmid transfection, indicating the targeted regulation between miR-214 and mTOR.

MiR-214 Down-Regulation and mTOR/p-mTOR Up-Regulation in CC Cancer Tissues

qRT-PCR showed that, compared to normal cervical epithelial tissues, CC cancer tissues showed significantly elevated mTOR mRNA expression and lower miR-214 expression (Figure 2A). With advanced TNM stage and higher tissue pathology grade, miR-214 expression was lower (Table I). Western blot results showed significantly higher mTOR and p-mTOR protein expression in CC cancer tissues compared to those in normal cervical mucosal epithelial cells. With advanced TNM stage and higher histo-pathology grade, expression level was further elevated (Figure 2B).

Abnormal Expression of miR-214 and mTOR in CC Cancer Tissues

qRT-PCR results showed that, compared to normal cervical epithelial cells Ect1/E6E7, CC SiHa cells showed significantly lower miR-214 expression (\( p < 0.05 \)) and higher expression of mTOR, Bcl-2 mRNA (\( p < 0.05 \), Figure 3A). Western blot results showed that SiHa cells had significantly higher mTOR, p-mTOR and Bcl-2 protein expression than Ect1/E6E7 cells (Figure 3B).

<p>| Table I. MiR-214 expression in CC cancer tissues with different clinical features. |</p>
<table>
<thead>
<tr>
<th>Age</th>
<th>N</th>
<th>miR-214 expression level</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 45 ) yrs</td>
<td>20</td>
<td>1.47±0.11</td>
<td>0.104</td>
</tr>
<tr>
<td>&gt;45 yrs</td>
<td>18</td>
<td>1.52±0.13</td>
<td></td>
</tr>
</tbody>
</table>
MiR-214 Over-Expression Inhibited CC Cell Proliferation or Invasion, and Facilitated Cell Apoptosis

Western blot showed that miR-214 mimic transfection significantly suppressed mTOR and p-mTOR expression in SiHa cells, as well as decreased the expression of downstream anti-apoptotic factor Bcl-2 expression (Figure 4A). Flow cytometry results showed that, compared to miR-NC transfection group, miR-214 mimic transfected SiHa cells had significantly reduced proliferation potency (Figure 4C), and increased cell apoptosis (Figure 4D). Transwell assay showed that, compared to miR-NC transfection group, miR-214 mimic transfected SiHa cells had decreased invasion potency (Figure 4B). After using siRNA to interfere with mTOR expression, Bcl-2 expression was significantly decreased, accompanied with lower cell proliferation or invasion, but higher apoptosis in SiHa cells.

Discussion

CC has relatively insidious disease onset, as no obvious symptom can be observed at early stage. It is frequently being misdiagnosed, leading to lower early diagnostic rate. Although combined therapy, including surgical resection and chem/o-radio-therapy, have achieved a big progress, the treatment efficacy is still not satisfactory for patients at the terminal stage. Therefore, investigation and identification of critical regulatory factors are of critical importance for early diagnosis, treatment efficacy and prognosis.

Although mTOR is under stimuli and regulation by various extracellular factors, its function and activity are mainly regulated by PI3K/AKT/PKB signal pathway. PI3K/AKT/mTOR pathway is the major pathway for mTOR to exert its functions [6, 8]. Under the stimuli of growth factor or mitogen, PI3K is activated through...
conformational change, resulting in promoting the transition of phosphatidylinositol- (4, 5)- bisphosphate (PIP2) into phosphatidylinositol- (3, 4, 5)– trisphosphate (PIP3), which can further phosphorylate AKT protein under the assistance of 3-phosphoinositide dependent protein kinase-1 (PDK1) and 3-phosphoinositide dependent protein kinase-2 (PDK2)16,17. Phosphorylated and activated AKT activates mTOR during PI3K/AKT/mTOR signaling transduction18. Phosphorylated and activated mTOR can interact with eukaryotic initiation factor-4E (eIF-4E) and eIF-4E-binding protein (4E-BP1) to facilitate gene transcription and expression, leading to facilitation of cell proliferation and decrease of cell apoptosis19. Through inducing over-activation of PI3K/AKT/mTOR pathway, abnormally elevated mTOR expression facilitates cell proliferation, migration, invasion and inhibits cell apoptosis, which is correlated with the occurrence, progression, metastasis and drug resistance of various tumors including breast cancer9, prostate cancer1, gastric carcinoma10 and colorectal carcinoma11.

B-cell lymphoma-2 (Bcl-2) is an important anti-apoptotic factor, and plays critical roles in antagonizing cell apoptosis and facilitating cell proliferation. It is correlated with the onset, advancement, metastasis and drug resistance of multiple tumors including lung cancer20, gastric carcinoma21 and colorectal cancer22. Various studies13-15 showed significantly decreased miR-214 expression in CC patient tumor tissues, indicating its potential role as tumor suppressor gene during CC pathogenesis. Bioinformatics analysis showed the existence of complementary binding sites between miR-214 and 3’-UTR of mTOR mRNA. Therefore, this study investigated if miR-214 played a role in regulating mTOR expression as well as in proliferation, apoptosis and invasion of CC cancer cells.

Dual luciferase gene reporter assay showed that transfection of miR-214 mimic significantly decreased the relative luciferase activity in HEK293T cells, whilst miR-214 inhibitor transfection significantly elevated the relative luciferase activity, indicating targeted regulatory relationship

Figure 4. MiR-214 over-expression weakened proliferation or invasion potency of CC cancer cells and facilitated their apoptosis. A, Western blot for protein expression; B, Transwell assay for cell invasion potency; C, EdU staining for cell proliferation potency; D, Flow cytometry for cell apoptosis. *, p<0.05 comparing between miR-214 mimic and miR-NC; #, p<0.05 comparing to si-mTOR and si-NC group.
between miR-214 and mTOR. Test results showed that, compared to normal cervical mucosal epithelial tissues, CC cancer tissues had significantly elevated mTOR and p-mTOR expression. However, miR-214 expression was down-regulated and correlated with TNM stage and histo-pathology grade. Moreover, compared to normal cervical epithelial cells Ect1/E6E7, CC cell SiHa had remarkably lower miR-214 expression, accompanied with higher mTOR, p-mTOR and Bcl-2 expression. Results indicated that miR-214 down-regulation might play a role in facilitating mTOR expression and CC pathogenesis. Peng et al.\textsuperscript{13} found significantly lower miR-214 expression in CC tissues compared to normal cervical tissues. Chandrasekaran et al.\textsuperscript{23} found abnormally lower miR-214 expression in CC tumor tissues and its target gene HMGA1 expression was abnormally up-regulated, in addition to the correlation between miR-214 down-regulation and CC progression. Yang et al.\textsuperscript{30} showed significantly lower miR-214 expression in CC tissues than normal cervical mucosal tissues. Qiang et al.\textsuperscript{14} found that, compared to normal cervical tissues, CC tissues had abnormally lower expression of miR-214, which was correlated with peripheral tissue infiltration and distal metastasis. In this investigation, CC tissues had remarkably lower miR-214 expression, consistently with Peng et al.\textsuperscript{13}, Chandrasekaran et al.\textsuperscript{23} and Yang et al.\textsuperscript{15}. Cong et al.\textsuperscript{24} showed the correlation of abnormally elevated mTOR expression with the abnormal proliferation or lower apoptosis of CC cells. Leisching et al.\textsuperscript{35} showed significantly elevated mTOR expression in CC tissues. All these observations were similar with our findings, showing higher mTOR expression in CC pathogenesis. Transfection of miR-214 mimic significantly down-regulated mTOR and p-mTOR expression in SiHa cells, decreased downstream anti-apoptotic factor Bcl-2 expression, reduced cell invasion potency and proliferation but enhanced cell apoptosis. After interfering mTOR expression by siRNA, similar effects were obtained as those cells with miR-214 over-expression. Peng et al.\textsuperscript{13} revealed that over-expression of miR-214 inhibited proliferation of CC cells HeLa or C33A, and decreased their migration or invasion potency via suppression of ARL2 gene expression. Chandrasekaran et al.\textsuperscript{23} showed that miR-214 up-regulation could decrease cervical cancer cell proliferation, migration or invasion via targeted inhibition on HMGA1 expression. Yang et al.\textsuperscript{14} used MTT and clonal formation assays to show that miR-214 up-regulation could reduce CC HeLa cell proliferation or clonal formation potency via inhibition of MEK3 and JNK1 gene expression. Qiang et al.\textsuperscript{14} showed that over-expression of miR-214 in HeLa cells significantly decreased the expression of Plexin-B1, reduced HeLa cell proliferation, migration and invasion, as well as remarkably inhibited \textit{in vivo} growth of HeLa cells inside BALB/c nude mice. Wang et al.\textsuperscript{26} also showed that miR-214 over-expression inhibited Bel2 expression, proliferation or survival of CC cells HeLa and C-33A, induced cell apoptosis, as well as enhanced their sensitivity against chemotherapy drugs cisplatin. Wen et al.\textsuperscript{27} showed a correlation between miR-214 down-regulation and enhanced malignant biological features of CC cells. Moreover, over-expression of miR-214 can regulate TFAM expression in a negative feedback manner to inhibit proliferation or cycle progression of CC cells HeLa or Caski, leading to reduced clonal formation and migration potency. Li et al.\textsuperscript{28} showed that after using AZD8055 to suppress mTOR function, CC cell proliferation potency was significantly inhibited but apoptosis was enhanced. All these studies illustrated the role of miR-214 down-regulation or mTOR up-regulation in facilitating CC pathogenesis and enhancing malignant biological features of CC cells, suggesting that up-regulation of miR-214 or decrease of mTOR expression or function may inhibit malignant biological behaviors of CC cells. Inconsistency, however, still existed as this study connected miR-214 and mTOR, demonstrating the role of miR-214-mTOR targeted regulatory relationship in affecting CC pathogenesis and tumor biology features, which have not been reported before.

Conclusions

We showed that miR-214 down-regulation plays a role in elevating mTOR expression and facilitating CC pathogenesis. Over-expression of miR-214 inhibited CC cell proliferation and invasion via inhibition of mTOR expression, leading to facilitation of cell apoptosis.

Acknowledgments

This work was supported by the National Natural Science Youth Foundation of China (No. 81401502).

Conflict of Interest

The Authors declare that they have no conflict of interests.


