European Review for Medical and Pharmacological Sciences 2018; 22: 1891-1898

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

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Abstract. – **OBJECTIVE: PI3K/AKT/mTOR pathway plays important roles in tumor pathogenesis. mTOR is up-regulated and miR-214 is down-regulated in cervical can-*cers. 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 ass performed to evaluate the targeted relations tween miR-214 and mTOR. *In vitro* cultured a cells were treated with miR-214 mimic or si-m followed by measuring mTOR, p-mTOR and Bc expression. Cell apoptosis, proli and inv sion were measured by flow me nd tran swell assay.

RESULTS: Bioinforma alysi dtar-geted binding sites be ee of mTOR mRNA. D ucifer porter assay confirmed this re tory relati ip between miR-214 and m NA. Compa o normal cervical epith m, c r tissues h lower expression o iR-214 a her mTOR, both of which w orrelated wit stage and tissue patho grade. Compared Ect1/E6E7 cells, SiH s had er level of miR-214 and higher mT mT nd Bcl-2 expression. Transfection o mimic mTOR significantly de-ased R/p-m or Bcl-2 expression, in-d ce ation or invasion, and en-ha d cell a sis.

CONCLUSIONS: miR-214 down-regulation elevating mTOR expression and in cilitating ervical cancer pathogenesis. Over-ex-ession of miR-214 inhibits cervical cancer cell iferation or invasion, and facilitates apoptosis v geted inhibition of mTOR expression.

Key Words

MiR-214, PI3K/AKT/mTOR, Cervical cancer, Proliferation, Apoptosis, Invasion.

oduction

Ce carcinoma is a common female re ive cancer in c , and is the second p ar malignant tumors in females, only next t east cance cidence of CC is increasing g ally with y ger population age, severely thr ng fe health and life[2,3].

Ph nositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) is a widely expressed signal ay across multiple tissues and cells, and ay 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[4-6]. 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[7]. 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[6,8]. 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[6], prostate cancer[9], gastric carcinoma[10] and colorectal carcinoma[11].

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,

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which occupies only 1% of human genome, can regulate the expression of more than 30% of human target genes[12]. Various studies showed significantly decreased miR-214 expression in CC tumor tissues, indicating its potential tumor-suppressing role in CC pathogenesis[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 chemo- or radiotherapy before surgery. CC tumor tissues were collected d g the surgery and divided into 11 cases of cases of G2 and 13 cases of G3 by histo-pa -gy grade. Based on TNM criteria, there wer cases in stage I-II and 17 cases in stage III- Another cohort of 17 normal mucos epithelial tissue samples wa lecte om surgery and recruited as the trol gro average age = 56.8 ± 13.2 years). N ific of age or sex ratio obs etween two groups. This stud as approv the Ethics Committee of nd Affiliat spital of Harbin Medi Uni y and int med consents wer tained fro participants before enrollm

Me Reac and Materials

Hu epithe cell line SiHa and nor- cer epith cell line Ect1/E6E7 were p ased nghai Cell Banks of CAS (Sh ghai, C). Roswell Park Memorial In- st 640 (RPMI-1640) culture medium, fetal ne (FBS) and penicillin-streptomycin ere purchased from Gibco (Grand Island, NY, A). RNA extraction kit GenElute total RNA P ication Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Transfection reagent FuGENE6 was purchased from Roche (Basel, Switzerland). QuantiTech 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 Te (Danvers, MA, USA). Rabbit anti- an Bcl 2, β-actin antibody and horserad eroxidase (HRP)-conjugated secondary antib re purchased from Abcam (Cam ge, M SA). EdU cell proliferation ki s purchase m Molecular Probes (Euge OR, US Luci activity assay kit wa chase om Promega (Madison, WI, US p ferase orter plasmid was pu sed fro Vect eijing, China). Ann V/PI cell a assay kit was purcha bioscience Diego, CA, USA). Tra swell c er was purchased from Corni rning, N A).

Cell Culture

SiHa and E E6E7 cells were inoculated i swell Park emorial Institute-1640 (RPMI) medi containing 10% fetal bovine serum s 1% penicillin-streptomycin in a 37°C chamber with 5% CO_2. Cells were passed at tio when reaching 70-80% confluence, and ls 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. 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), RNA template, 0.5 μL QuantiTech RT M distilled water. Reverse transcription cond were: 50°C for 30 min. PCR conditions w 95°C 15 min pre-denature, followed by 40 cyc each at 94°C for 15 s for denat for 30 for annealing, and 72°C for for ngation. Gene expression was m ed on BI ViiA 7 Real-time fluorescent were designed as ws: P_F: 5'-GG-ACA GGACG AG TC miR-214P_R: 5'-CAGAC G TCCGT ': U6P_F: 5'-ATTGG GA AG AGA ATT-3'; U6P_R: 5'- AAC GC ACGAA TTTG-3'; mTOR -TCCGA GAC AGTC AAGAG G-3' ORP 5'-CACCT TCCAC TCCTA TG C- Bcl-2P_F: 5'-GGTGG GGTCA TGT -3'; B P_R: 5'-CGGTT CAGGT TCA AT ; β-actinP_F: 5'-GAACC G -3'; β-actinP_R: 5'-TGTCA C C GA C-3'.

Western Blot

Cells from all groups were lysed by RIPA lysis fer, and concentration of protein supernatant w 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) conjug ondary antibody (1:25000) was adde 60 mi room temperature incubation. Wit e times of PBST rinsing, ECL approach was us develop the membrane for 2-3 min a om te ture. After exposure and visua ion, the f scanning for data proce g.

Cell Apoptosis Assay

Cells were d ed by in an en collected. Cell di n was que g culture medium c % FBS, fo ed by 300 x g centrifug ion fo in. The supernatant was mixed 5 mL PB d was centrifuged at 30 g for 5 min for w g. 100 μL Binding B er were mixed with every 1 X 10^6 cells. After r spension, 5 Annexin V-FITC were added f min dark ubation at room temperature, foll by 5 propidium iodide (PI) staining for 5 m poptosis was measured by Beckman Coulter FC 500 MCL (Brea, CA, USA) flow etry.

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 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 cells showed significantly elevated 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 cancer tissues compared to those in normal cervical mucosal epithelial cells. With advanced TNM stage and higher histo-pathology grade, mTOR expression level was further elevated (Figure 2B).

Table I. MiR-214 expression in CC cancer tissues with different clinical features.

Age	N	miR-214 expression level	*p*-value
Age			
≤45 yrs	20	1.47±0.11	
>45 yrs	18	1.52±0.13	
Histopathology grade			<0.05
G1	11	1.[illegible]14	
G2	14	1.[illegible]0.13	
G3	13	1.[illegible]	
TNM stage			0.001
I-II	21	1.31±0.14	
III-IV	17	1.[illegible]9±0.12	

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).

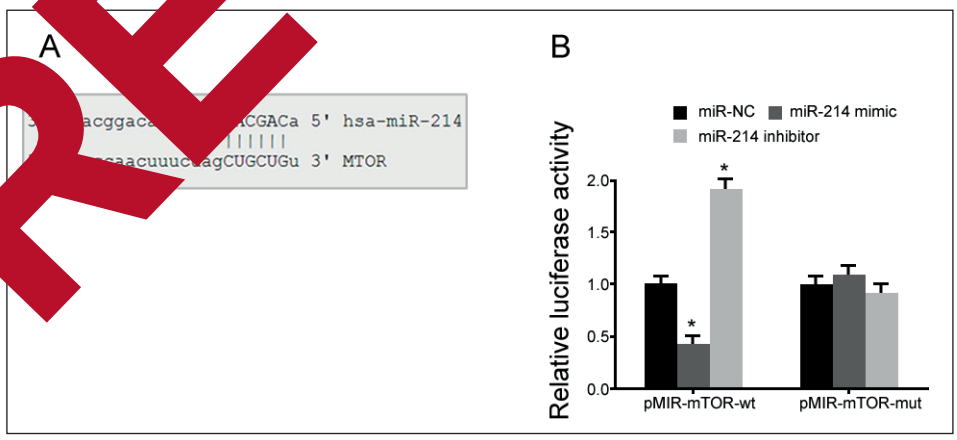


Figure 1. Targeted regulation between miR-214 and mTOR. **A**, Functioning sites between miR-214 and 3'-UTR of mTOR mRNA; **B**, Dual luciferase gene reporter assay. *, $p<0.05$ comparing to miR-NC.

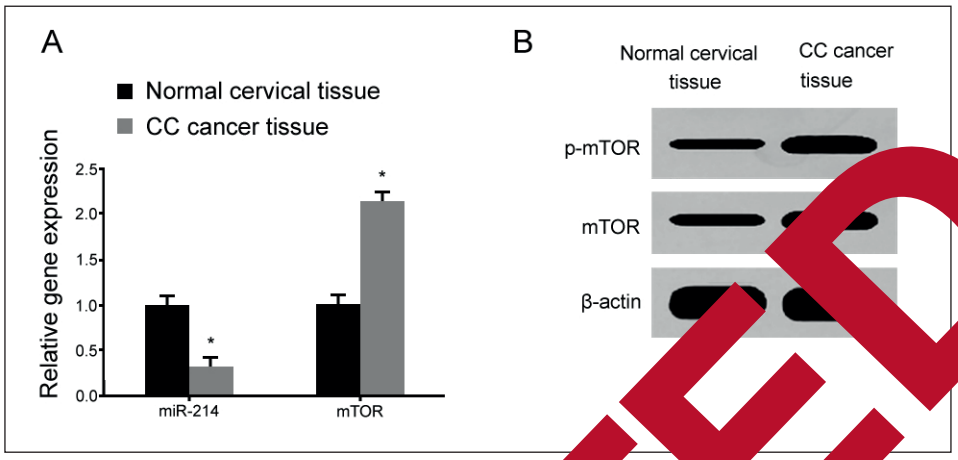


Figure 2. MiR-214 down-regulation and mTOR/p-mTOR up-regulation in CC cancer tissues. **A**, qRT-PCR for miR-214 and mTOR mRNA expression in cervical tissues; **B**, Western blot for mTOR and p-mTOR expression in cervical tissues. *, $p<0.05$ compared to normal cervical mucosal tissues.

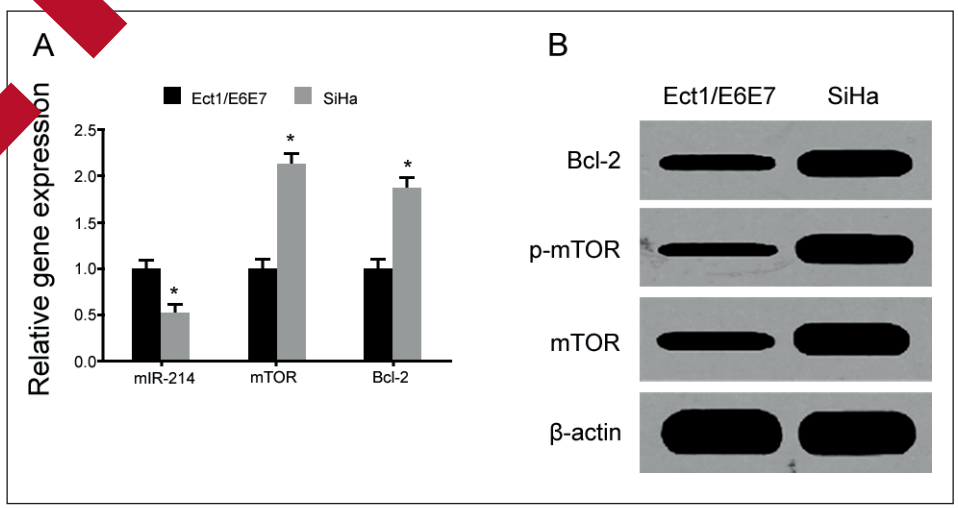
MiR-214 Over-Expression Inhibited CC Cell Proliferation or Invasion, and Facilitated Cell Apoptosis

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 [illegible] NC transfection group, miR-214 mimic tra[illegible]ed SiHa cells had significantly reduced p[illegible]f-eration potency (Figure 4C), and increased [illegible] apoptosis (Figure 4D). Transwell assay show[illegible] that, compared to miR-NC t[illegible]n grou[illegible] miR-214 mimic transfected [illegible]a ce[illegible]had de-creased invasion potency ([illegible]re 4B)[illegible]er using siRNA to interfere with m[illegible]exp[illegible]2 expression was signi[illegible]tly d[illegible]d, accompanied with lower c[illegible]oliferatio[illegible]nvasion, but higher apoptosi[illegible]n cells.

Discussion

CC [illegible]elatively l[illegible]ous disease onset, as no[illegible]ous symptom ca[illegible]e observed at early [illegible]age. It is frequently being misdiagnosed, l[illegible]ng to lower[illegible]rly diagnostic rate. Although c[illegible]ined thera[illegible]including surgical resection and[illegible]mo-/ra[illegible]therapy, have achieved a big progre[illegible]atment efficacy is still not satisfactory for patients at the terminal stage. Therefore, investigation and identification of critical [illegible]g[illegible]y 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



[illegible]gure 3. Abnormal ex[illegible]ion of miR-214 and [illegible]n CC cancer tissues. **A**, qRT-PCR for cell gene expression; **B**, Western blot for protein expression. *, $p<0.05$ compared to Ect1/E6E7 cells.

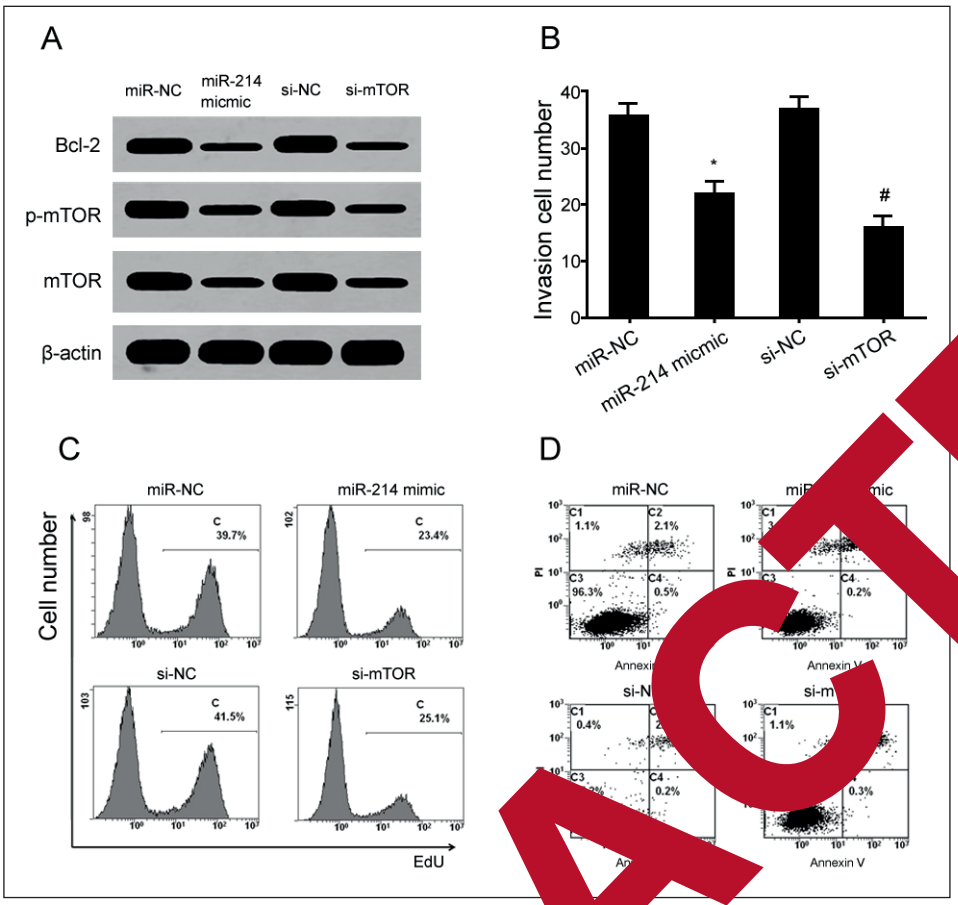


Figure 4. MiR-214 overexpression weakened proliferation or invasion potency of CC cancer cells and facilitated their apoptosis. **A**, Western blot for protein expression; **B**, assay for cell invasion potency; **C**, staining for cell proliferation potency; **D**, Flow cytometry for cell apoptosis. *, # comparing between miR-214 mimic and miR-NC, $p<0.05$ comparing to mTOR and si-NC group.

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 signal transduction[18]. Phosphorylated and activated mTOR can interact with eukaryotic initiation factor-4E (eIF-4E) and eIF-4E-binding protein (4E-BP), facilitate gene transcription and expression, leading to facilitation of cell proliferation and decrease of cell apoptosis[19]. 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 cancer[6], prostate cancer[9], gastric carcinoma[10] and colorectal carcinoma[11].

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 cancer[20], gastric carcinoma[21] and colorectal cancer[22]. Various studies[13-15] 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

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[13] found significantly lower miR-214 expression in CC tissues compared to normal cervical tissues. Chandrasekaran et al[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[15] showed significantly lower miR-214 expression in CC tissues than normal cervical mucosal tissues. Qiang et al[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 met In this investigation, CC tissues had rema ly lower miR-214 expression, consistently with et al[13], Chandrasekaran et al[23] and Yang et a Cong et al[24] showed the correla normal elevated mTOR expression w e ab mal proliferation or lower apoptosi CC cell eisching et al[25] showed signific vat pression in CC tiss All observations were similar wit findings, ving higher mTOR expressi C pathogen Transfection of miR-21 imic ificantly do n-regulated mTOR l p-mTOR ssion in SiHa cells, decreas ownstream anti totic factor Bcl-2 ex ion, r ced cell in sion potency and pr tion enhanced cell apoptosis. After inter OR ex sion by siRNA, similar cts w btain those cells with miR-214 xpres et al[13] revealed that over-expr n of mi 4 inhibited proliferation of CC c or C33A, and decreased their migration inva potency via suppression of ARL2 ene expression. Chandrasekaran et al[23] showed miR-214 up-regulation could decrease cervical c r cell proliferation, migration or invasion via targeted inhibition on HMGA1 expression. Yang et al[15] 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[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 *in vivo* growth of HeLa c BALB/c nude mice. Wang et al[26] also wed tha miR-214 over-expression inhibited 2 expression, proliferation or survival of CC c eLa and C-33A, induced cell apoptosis well a nced their sensitivity against ch therapy dr s platin. Wen et al[27] show correl on bet miR-214 down-regula and nced malignant biological fe es ells. M over, over-expression iR-214 regul TFAM expression in ative feed er to inhibit prolif cle progre of CC cells HeLa or Caski, lea to reduced clonal formation a ration pot Li et al[28] showed that aft AZD8055 to s ess mTOR function, C ell proliferation potency was significantly i ited but ap sis was enhanced. All these s s illustrate e role of miR-214 down-regula mTO p-regulation in facilitating CC pathog enhancing malignant biological features of CC cells, suggesting that up-regulation of miR-214 or decrease of mTOR expression 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.

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