Original Article
MiR-424 plays a tumor suppressor role in cervical cancer via regulating ERK/MAPK signaling pathways

Bin Yu¹, Juan Juan², Sunita Byanjanker²

¹Department of Western Medicine Gynaecology, Affiliated Hospital of Inner Mongolia University for The Nationalities, Tongliao, Inner Mongolia, China; ²Clinical College, Inner Mongolian University of Nationalities, Tongliao, Inner Mongolia, China

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Abstract: Objective: ERK1 plays an important role in ERK/MAPK signaling pathways. Expression levels and function enhancement of ERK1 are involved in the pathogenesis of cervical cancer. Previous studies have found that miR-424 is significantly decreased in cervical cancer tissues, suggesting its anti-cancer effects. The current study investigated the roles of miR-424 in regulating ERK1 expression, ERK/MAPK pathway activity, and cervical cancer cell proliferation and apoptosis. Methods: Tumor tissues of patients with cervical cancer were collected. Adjacent tissues were used as controls, detecting miR-424, ERK1, and p-ERK1 expression levels. Dual luciferase reporter gene assays were adopted to validate the targeted relationship between miR-424 and ERK1, predicted by bioinformatics analysis. Cervical cancer SiHa, Caski, and HeLa cells and normal cervical epithelial Ect1/E6E7 cells were cultured in vitro. Caski and HeLa cells were divided into the miR-NC group and miR-424 mimic group. Cell apoptosis was detected by flow cytometry. Cell proliferation activity was detected by EdU staining and CCK-8 assays. Results: Compared with adjacent tissues, miR-424 was significantly decreased, while ERK1 and p-ERK1 were obviously upregulated in cervical cancer tissues. Double luciferase reporter gene assays confirmed a targeted regulation relationship between miR-424 and ERK1. Compared with Ect1/E6E7 cells, miR-424 levels were reduced. ERK1 and p-ERK1 expression levels were enhanced in cervical cancer SiHa, Caski, and HeLa cells. Transfection of miR-424 mimic markedly downregulated ERK1 and p-ERK1 expression levels in Caski and HeLa cells, resulting in a significant increase in cell apoptosis and a marked decrease in cell proliferation. Conclusion: Elevation of miR-424 may inhibit ERK1 expression, attenuate ERK/MAPK pathway activity, suppress cervical cancer cell proliferation, and induce cell apoptosis.

Keywords: MiR-424, ERK1, ERK/MAPK, cervical cancer, proliferation, apoptosis

Introduction
Cervical carcinoma (CC) is a type of common malignant tumor in the reproductive system, ranking second to breast cancer among female malignant tumors [1]. In recent years, incidence rates of cervical cancer have continually increased, presenting with a younger trend and seriously threatening the life and health of female patients [2, 3].

Extracellular signal regulated kinase 1 (ERK1) represents a key protein in ERK/mitogen activated protein kinase (MAPK) signaling pathways. ERK1 expression levels and functional enhancement play a crucial role in occurrence, progression, metastasis, and drug resistance of various tumors, including rectal cancer including rectal cancer [4], prostate cancer [5], and breast cancer [6]. Activity levels of ERK1 in tumor tissues of patients with cervical cancer have been shown to be significantly enhanced, suggesting that ERK1 contributes to oncogene effects in the development of cervical cancer [7-10]. Numerous studies have demonstrated that miR-424 levels were significantly decreased in cervical cancer patients, indicating that miR-424 functions as a tumor suppressor in cervical cancer [11-13]. The current study aimed to explore the roles of miR-424 in regulating ERK1 expression, ERK/MAPK pathway activity, and cervical cancer cell proliferation and apoptosis.
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Materials and methods

Main reagents and materials

Human normal cervical epithelial cells Ect1/E6E7 were acquired from Shanghai Yantian Biotechnology Co., Ltd. (Shanghai, China). Cervical cancer SiHa, Caski, and HeLa cells were obtained from Jennio (Guangzhou, Guangzhou, China). RPMI 1640 medium was purchased from Gibco (Waltham, MA, USA). FBS was purchased from Lonza (Basel, Switzerland). Lip 2000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). ReverTra Ace qPCR RT Kit and SYBR were purchased from Toyobo (Osaka, Japan). Moreover, miR-NC and miR-424 mimics were purchased from Ribobio (Guangzhou, Guangdong, China). Rabbit anti-human ERK1 and p-ERK1 antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit anti-human β-actin antibody was collected from Santa Cruz (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit secondary antibody was purchased from Sangon (Shanghai, China). RIPA protein extract, BCA protein quantification kit, and ECL chemiluminescence reagent were obtained from Beyotime (Beijing, China). Annexin V/PI apoptosis detection reagent and CCK-8 cell proliferation detection reagent were purchased from Dojindo (Rockville, MD, USA). Dual-Luciferase Reporter Assay System and pMIR plasmid were purchased from Promega (Fitchburg, WI, USA). EdU cell proliferation assay kit was purchased from Molecular Probes (Eugene, OR, USA).

Clinical information

A total of 50 cervical cancer patients undergoing surgical resections from July 2017 to November 2017 were enrolled. The patients had a mean age of 51.7±11.6 years old. Specimens of cervical cancer tumors and adjacent tissues removed during surgery were collected. Collection of tissue specimens was reviewed and approved by the Ethics Committee.

Cell culturing

Ect1/E6E7, SiHa, Caski, and HeLa cells were maintained in RPMI 1640 medium containing 10% FBS and 1% streptomycin. They were cultured in an incubator containing 5% CO₂ at 37°C and passaged in a ratio of 1:3 to 1:4. Cells in the logarithmic phase were used for experiments.

Dual luciferase reporter gene assay

HEK293T cell genomic mRNAs were used as a template to amplify the targeted binding site in the 3'-UTR region of ERK1 gene fragment or its mutant fragment. The collected PCR product was digested and inserted into pMIR vectors. After the recombinant plasmid was transformed to DH5α competent cells, positive clones with correct plasmids were picked and named pMIR-ERK1-WT and pMIR-ERK-MUT.

Moreover, pMIR-ERK-WT (or pMIR-ERK-MUT), miR-424 mimic (or miR-NC), and pRL-TK were co-transfected into HEK293T cells using Lipofectamine 2000. After 48 hours, relative luciferase activity values were determined via the Dual-Luciferase Reporter Assay System kit.

Cell transfection and grouping

Caski and HeLa cells were cultured and divided into the miR-NC transfection group and miR-424 mimic transfection group. Next, 10 μL Lip 2000, 50 nmoL miR-NC, and 50 nmoL miR-424 mimics were diluted with 100 μL serum-free Opti-MEM for 5 minutes at room temperature. Lip 2000 was then mixed with miR-NC and miR-424 mimics and incubated for 20 minutes at room temperature, respectively. Finally, the mixture was added to the cells and incubated for 72 hours.

Cell apoptosis

The cells were digested by trypsin and incubated in 100 μL of binding buffer. After adding with 5 μL Annexin V-FITC and 5 μL PI for 20 minutes in the dark, the cells were added with 400 μL binding buffer and tested using FC 500 MCL flow cytometry.

Cell proliferation

The cells were collected by trypsinization and resuspended in RPMI 1640 complete medium containing 10% FBS. After incubation with 10 μM EdU at 37°C for 2 hours, the cells were further cultured for 48 hours. They were fixed in 100 μL fixative solution at room temperature for 15 minutes. Next, the cells were centrifuged with PBS and penetrated for 15 minutes at
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Room temperature upon 100 μL permeabilization solution. They were then incubated in 500 μL detection reaction solution at room temperature, void of light, for 30 minutes. Finally, the cells were washed using 3 mL washing solution, resuspended in 500 μL washing solution, and measured using a Beckman Coulter FC 500 MCL flow cytometer.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted using TRIzol Reagent and reverse transcribed to cDNA using the ReverTra Ace qPCR RT Kit. The reaction system included 2 μg total RNA, 1 μL dNTP, 4 μL RT Buffer, 1 μL RT primer, 2 μL RT Enzyme, 1 μL RNase inhibitor, and ddH2O. PCR amplification reaction conditions were composed of 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 74°C for 30 seconds on a Bio-Rad CFX96 real-time PCR machine.

Western blotting

For Western blotting, 100 μL radio immunoprecipitation assay (RIPA) protein extract was added to every 5 mg or 1 million cells at 4°C. After centrifugation at 10,000 g for 15 minutes, the protein supernatant was moved to a new eppendorf (EP) tube and quantified by bicinchoninic acid (BCA). Next, 50 μg protein was separated by electrophoresis for 3.5 hours and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skim milk for 60 minutes at room temperature, the membrane was incubated overnight in primary antibody at 4°C (ERK1, p-ERK1, and β-actin for 1:500, 1,1000, and 1:6000, respectively). After washing with PBST 3 times, the membrane was incubated in HRP-labeled goat anti-rabbit secondary antibody (1:10000) for 1 hour at room temperature. Finally, the membrane was added with electrochemiluminescence (ECL) luminescent solution for 1~3 minutes and developed.

Data analyses were performed using SPSS 18.0. Measurement data are expressed as mean ± standard deviation. miR-424 and ERK1 mRNA expression levels were compared using the Mann-Whitney U-test. Correlation analysis of miR-424 and ERK mRNA expression levels in cervical cancer tissues was performed by Spearman's method. Continuous data from multiple groups were analyzed using one-way ANOVA, along with Tukey's post-hoc test. P < 0.05 indicates statistical significance.

Results

miR-424 expression was reduced and ERK1 levels were upregulated in tissues of CC patients

Results of qRT-PCR showed that ERK1 mRNA expression was significantly increased, while miR-424 levels were obviously decreased in CC tissues, compared with adjacent tissues (Figure 1A and 1B). Spearman’s rank correlation analysis showed a significant negative correlation between miR-424 and ERK1 mRNA expression in tumor tissues of CC patients (r = -0.684, P < 0.05) (Figure 1C). Western blot analysis demonstrated that ERK1 and p-ERK1 protein expression levels in CC tissues were apparently higher than those in adjacent tissues (Figure 1D).

Relationship between miR-424 expression and clinical characteristics

CC patients were divided into high-expression miR-424 and low-expression miR-424 groups, investigating the relationship with clinical features based on median miR-424 expression levels. Results showed that miR-424 levels were correlated with TNM stage and histopathological grade (P < 0.05), but not with lymph node metastasis (P > 0.05) (Table 1).

Targeted regulatory relationship between miR-424 and ERK1

In silico analysis using microRNA.org online prediction revealed a targeted complementary binding site between miR-424 and the 3’-UTR of ERK1 mRNA (Figure 2A). Dual luciferase gene reporter assay results further validated that transfection of miR-424 mimic significantly reduced the relative luciferase activity of pMIR-ERK1-WT transfected HEK293T cells. However, there was no significant impact of miR-424 mimic transfection on the relative luciferase activity in pMIR-ERK1-MUT transfected HEK293T cells (Figure 2B), indicating a targeted regulatory relationship between miR-424 and ERK1 mRNA.
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miR-424 expression decreased and ERK1 levels enhanced in CC cells

Results of qRT-PCR revealed that ERK1 mRNA levels in CC SiHa, Caski, and HeLa cells were significantly higher than those in human normal cervical epithelial Ect1/E6E7 cells (Figure 3A). Compared with human normal cervical epithelial Ect1/E6E7 cells, miR-424 expression in CC SiHa, Caski, and HeLa cells was markedly reduced (Figure 3B). Western blot suggested that ERK1 and p-ERK1 protein expression levels in CC SiHa, Caski, and HeLa cells were apparently higher than those in human normal cervical epithelial Ect1/E6E7 cells (Figure 3C).

miR-424 overexpression inhibited CC cell proliferation and induced cell apoptosis

Results of qRT-PCR showed that ERK1 mRNA expression levels were significantly decreased in the miR-424 mimic transfection group, compared with miR-NC group (Figure 4A). Western blot data indicated that ERK1 and p-ERK1 protein levels were apparently downregulated in the miR-424 mimic transfection group, compared with miR-NC group (Figure 4B). According to flow cytometry assays, compared with the miR-NC group, HeLa and Caski cell apoptosis was obviously enhanced (Figure 4C), while the positive rate of EdU was markedly decreased in the miR-424 mimic transfection group (Figure 4D). CCK-8 assays also confirmed that miR-424 mimic transfection significantly suppressed the proliferative activity of HeLa and Caski cells (Figure 4E).

Discussion

Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathways are widely found in various tissues and cells, regulating numerous biological processes, including cell proliferation, cycle, apoptosis, migration, and invasion [14, 15]. Over-activation of ERK/MAPK signaling pathways can cause abnormal cell proliferation, apoptosis, and differentiation disorders. These
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Table 1. Relationship between miR-424 expression and clinical characteristics

<table>
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<th>Clinical characteristics</th>
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<th>$\chi^2$ value</th>
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Accumulative findings have shown that [7-10] expression levels and functional activity of ERK1 are significantly enhanced in tumor tissues of CC patients, suggesting that ERK1 plays an oncogene role in the development of cervical cancer.

MicroRNA is a newly discovered non-coding single-stranded small RNA of 19-25 nucleotides in length. It is an extremely important gene regulatory substance. MiRNAs degrade the target mRNA or inhibit post-transcriptional translation through anti-sense complementary binding to the 3′-untranslated region (3′-UTR) of mRNA. Thereby, they participate in the regulation of various biological processes, including cell growth, differentiation, apoptosis, and migration [22, 23]. miR-424 has been found to be related to occurrence, progression, and drug resistance of various tumors, including lung cancer, breast cancer, and endometrial cancer [24-26]. A number of studies have confirmed that miR-424 expression was significantly decreased in cervical cancer patients, suggesting that miR-424 serves as a tumor suppressor in the development of cervical cancer [11-13]. The current study found that, compared with adjacent tissues, expression of miR-424 was significantly decreased, while ERK1 and p-ERK1 levels were obviously upregulated in CC tissues. Also, expression of miR-424 was related to the clinical stage and histopathological grade of CC patients, suggesting that decreasing expression levels of miR-424 may play a role in upregulating ERK1 expression and promoting the pathogenesis of cervical cancer. Spearman’s rank correlation analysis exhibited a negative correlation between miR-424 and...
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ERK1 mRNA expression in CC tissues, revealing a potential targeted relationship between these two. Dual luciferase reporter gene assays further suggested that transfection of miR-424 mimic significantly reduced the relative luciferase activity of pMIR-ERK1-WT transfected HEK293T cells. Results of in vitro testing suggested that expression of miR-424 was significantly lower, while expression of ERK1 and p-ERK1 was markedly higher in CC cells, compared to normal cervical epithelial cells. Consistently, Dong et al. [27] showed that expression of miR-424 was significantly decreased in cervical cancer patients, compared with adjacent tissues, caused by the abnormal expression of long non-coding RNA SNHG12. Gao et al. [11] observed that expression of miR-424 was abnormally reduced in cervical cancer tissues, compared with adjacent tissues, caused by the abnormal enhancement of tumor-promoting gene IncRNA PVT1. Varghese et al. [28] found that expression of miR-424 was abnormally declined in cervical cancer tissues, compared with normal cervical epithelium. Compared with normal cervical tissues, miR-424 expression of cervical cancer cell lines SiHa, Caski, and HeLa was significantly downregulated due to hypermethylation of the gene promoter region. Wang et al. [29] revealed that expression of miR-424 in tumor tissues of radiotherapy resistant CC patients was significantly lower than that of radiotherapy sensitive CC patients. Results demonstrated that miR-424 plays a tumor suppressor role in cervical cancer.

Further investigating the effects of miR-424 on the biological effects of cervical cancer cells, the current study observed changes in cell proliferation and apoptosis after levels of miR-424 in cervical cancer cells were overexpressed in vitro. Results showed that transfection of miR-424 mimics significantly inhibited expression of ERK1 and p-ERK1 in cervical cancer HeLa and Caski cells, obviously reducing cell proliferation and enhanced apoptosis. ERK1 was shown to play a suppression role on malignant biological characteristics of cervical cancer cells. Moreover, Varghese et al. [28] showed that, compared with negative controls, proliferation...
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Figure 4. miR-424 overexpression inhibited CC cell proliferation and induced cell apoptosis. A. qRT-PCR detection of ERK1 mRNA expression; B. Western blot detection of ERK1 and p-ERK1 protein expression; C. Flow cytometry detection of cell apoptosis; D. Flow cytometry detection of cell proliferation; E. CCK-8 assay detection of cell proliferative activity. *P < 0.05, compared with miR-NC.
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of SiHa cells in the miR-424 mimic transfection group was significantly reduced by targeted suppression of target gene RBBP6. Wang et al. [29] showed a targeted regulation between miR-424 and aprataxin in cervical cancer cells. Overexpression of miR-424 can facilitate cervical cancer cell apoptosis, block cell cycle, induce DNA damage, enhance the sensitivity of cervical cancer cells to radiation, and reduce radiotherapy resistance by targeting aprataxin. Compared with normal cervical epithelial cell End1/E6E7, miR-424 levels declined in cervical cancer HeLa and SiHa cells. miR-424 presents anti-cancer effects through targeting KDM5B, inhibiting cell proliferation, and promoting cell apoptosis. An increase of KDM5B can attenuate the anti-cancer effects of miR-424 on cervical cancer. The present study revealed the anti-cancer effects of miR-424 via restraining cervical cancer cell proliferation and inhibiting ERK1 expression. However, the impact of miR-424 on ERK1 in vivo has yet to be confirmed.

Conclusion

Present data suggests that an increase of miR-424 can inhibit ERK1 expression, attenuate ERK/MAPK pathway activity, suppress cervical cancer cell proliferation, and induce cell apoptosis, offering tremendous academic support for anti-cervical cancer therapy.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bin Yu, Department of Western Medicine Gynaecology, Affiliated Hospital of Inner Mongolia University for The Nationalities, 1742 Huolinhe Street, Tongliao 028000, Inner Mongolia, China. Tel: +86-475-8215816; Fax: +86-475-8215816; E-mail: YuBin9gs@163.com

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