Original Article
Upregulation of miR-494 promotes cervical cancer cell proliferation, migration and invasion via the MAPK/ERK pathway

Nina Yu¹, Jing Jin², Jing Liu², Ming Ma³

Departments of ¹Gynecology, ³Oncology, Linyi People’s Hospital, Linyi, Shandong, China; ²Department of Gynaecology, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China

Received July 10, 2016; Accepted September 1, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Cervical cancer is a common malignant tumor in women, and aberrant miR-494 expression has been identified to be associated with the initiation and progression of various cancers. However, the functional role of miR-494 in cervical cancer remains unknown to a large extent. The aim of this study was to investigate the biological function and potential mechanism of miR-494 in cervical cancer. miR-494 expression levels in plasma of healthy control subjects and cervical cancer patients were measured by qRT-PCR. miR-494 mimic, inhibitor and negative control were transfected with lipofectamine 2000. Cell proliferation was detected by CCK-8 assay and cell migration and invasion were performed by wound healing and transwell assay, respectively. Western blotting was adopted to determine pERK1/2, tERK1/2 and SOX9 levels. Dual luciferase assay was carried out to verify the target of miR-494. miR-494 was up-regulated in cervical cancer plasma and correlated with invasion degree. Increased miR-494 expression promoted HeLa cell proliferation, migration and invasion, while decreased miR-494 expression inhibited HT-3 cell proliferation, migration and invasion. Besides, the MAPK/ERK pathway was involved in miR-494-induced HeLa cell proliferation, migration and invasion. Dual Luciferase assay indicated that SOX9 was a target of miR-494. To summarize, these results indicate that upregulation of miR-494 promotes cell proliferation, migration and invasion through the MAPK/ERK pathway, and SOX9 is a direct target of miR-494.

Keywords: miR-494, proliferation, migration, invasion, SOX9, cervical cancer

Introduction
Cervical cancer is the fourth most commonly diagnosed cancer in women, and there were 528,000 new cases occurred worldwide in 2012 [1]. In China, cervical cancer accounts for 5% of the cancer incidence and 3.8% of the mortality in women [2]. Numerous studies have suggested that aberrant miRNAs expression contributes to tumorigenesis [3], and thus investigation concerning cancer-related miRNAs is needed.

miRNAs are small, noncoding RNAs that play important roles in post-transcriptional regulation [4], and aberrant miRNAs expression is one of the most common changes observed in human cancers [5, 6]. The expression of mirR-494 in cancer has been reported to be tissue-dependent. Decreased expression of mir-494 has been found in gastric carcinoma [7], cholangiocarcinoma [8], whereas miR-494 up-regulation has been observed in retinoblastoma [9], gastrointestinal stromal tumors [10]. By targeting different genes, miR-494 may act as an oncogene or tumor suppressor gene and regulate cell proliferation, apoptosis, migration and invasion of numerous cancers [10-15]. However, the biological role of mir-494 is still needed to explore in cervical cancer.

In this study, we detected miR-494 expression levels in cervical cancer plasma, and evaluated the functional role and potential molecular mechanism of miR-494 in cervical cancer cell lines. It was initially showed that miR-494 was up-regulated in cervical cancer plasma. Furthermore, MAPK/ERK pathway was involved in miR-494-induced cervical cancer cell proliferation, migration and invasion, and SOX9 was a direct target of miR-494.
miR-494 promotes cell proliferation, migration and invasion

Materials and methods

Blood samples and cell culture

This study was approved by the Committee for the Ethical Review of Research at the Linyi People's Hospital. Blood of 40 cervical cancer patients and 40 healthy control subjects were collected, and consent forms were obtained.

The cervical cancer cell lines were purchased in Chinese Academy of Sciences Institute of Cell Resource Center (Shanghai, China). HeLa cell line was cultured in the Eagle's Minimum Essential Medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA), and HT-3 cell line was cultured in the McCoy's 5a Medium (Gibco-BRL, Invitrogen Life Technologies). All the mediums contain 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco-BRL, Invitrogen Life Technologies), and all the cells were cultured in a humidified incubator with 5% CO$_2$ at 37°C.

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

Blood samples were first centrifuged at 3000 rpm for 5 min to isolate the plasma, and then RNA was extracted using TRIzol LS Reagent (Invitrogen). RNA from cells was extracted using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). 1 μg RNA was reverse-transcribed using PrimeScript™ RT reagent Kit (Takara, Japan) according to the manufacture's instruction. qRT-PCR was performed on ABI StepOne Plus using the SYBR Premix Ex Taq Kit (Takara, Japan) according to the manufacturer’s instruction. qRT-PCR was performed on ABI StepOne Plus using the SYBR Premix Ex Taq Kit (Takara, Japan). U6 or β-Actin was identified as the internal control. Primer sequences of miR-494 were: F 5’-UGAAACAUACACGGGA-AACCUC-3’ and R 5’-GGUUUCCCGUGUAUGU-UUCAUU-3’. Primer sequences of U6 were: F 5’-CGCTTCGGCAGCACATATACTAAAATTGGAC-3’ (sense) and R 5’-GCTTCACGAATTTGCGTGTCATCCTTGCC-3’. Primer sequences of SOX9 were: F 5’-ACGGCTCCAGCAAGAAACAG-3’ and R 5’-GCCGGCTTCCAGCAAGAAACAG-3’. Primer sequences of β-actin were: F 5’-AACGACCTGT-ACGCCCAACAC-3’ and R 5’-GTCTACTCCTGGCTTGCTGAT-3’.

Western blotting

Cells were harvested using trypsin-EDTA solution (Gibco-BRL, Invitrogen Life Technologies), and lysed with the RIPA lysis buffer (Beyotime Institute of Biotechnology). 30 μg proteins were separated by 10% SDS-PAGE and transferred into the PVDF membrane (EMD Millipore, Billerica, MA, USA). After blocking in 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies including pERK1/2 (#4370, 1:2000, CST, MA, USA), tERK1/2 (#9102, 1:1000, CST, MA, USA), SOX9 (ab76997, 1:500, Abcam, Cambridge, UK) and β-actin (A5441, 1:8000, sigma, MO, USA) overnight. Subsequent to washing with TBST for 6×10 min, the membranes were incubated with a goat anti-mouse secondary antibody (BA1001; 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China) or a goat anti-rabbit secondary antibody (BA1001; 1:5,000; Wuhan Boster Biological Technology) at 37°C for 2 h. After washing with TBST for 9×10 min, the protein bands were detected using BeyoECL Star kit (Beyotime Institute of Biotechnology) with exposure to X-ray films (Eastman Kodak, Rochester, NY, USA) on dark room. β-Actin was used as an internal control.

Cell transfection

Cells were planted into six-well plates at the density of 3×10$^5$, and transfected using lipofectamine 2000 (Invitrogen Life Technologies) after reaching to 70%-90% confluence according to the manufacturer’s instructions. The concentration for transfection of miRNAs mimic was 100 nM, for miRNAs inhibitor was 200 nM and for plasmids was 2 μg. The RNA and protein were extracted at 48 h and 72 h post transfection, respectively.

CCK-8 assay

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8, Beyotime, China). Cells were seeded in 96-well plates at the density of 5×10$^3$. 24 h later after transfection, the medium was replaced by the mixture of 10 μl CCK-8 and 100 μl fresh medium at 0 h, 24 h, 48 h, 72 h. Then the plate was incubated at 37°C for 2 h, and the absorbance of each well was measured at 450 nm on a microplate reader (Bio-Rad, USA).

Wound healing assay

3×10$^5$ cells were planted into six-well plate and transfected as described above. 24 h later, 20-μl pipette tips were used to scratch the wounds. The images were captured under a microscope (IX3; Olympus Co., Tokyo, Japan) at 0 and 48 h after the scratch.
miR-494 promotes cell proliferation, migration and invasion

**Cell invasion analysis**

Cell invasion was detected using Transwell chambers (Costar, USA). 24 h after transfection, cells were starved for 8 h. $3\times10^5$ cells suspended in 100 μl serum-free medium were planted into the upper chamber pre-coated with matrigel, and 600 μl complete medium was added to the lower chamber. After 48 h incubation, cells invaded to the lower surface of the chamber were stained with crystal violet (Sunshine, China) for 20 min and photographed under an Olympus microscope.

**Dual luciferase assay**

The wild type and mutant 3'-UTR of SOX9 were cloned into the luciferase reporter vector pGL3 (Promega, WI, USA), respectively. HeLa cells were seeded into the 24-well plates, pGL3-SOX9-3'UTR-WT or pGL3-SOX9-3'UTR-Mut together with miR-494 mimics or negative control (NC) were co-transfected. After 48 h, cells were harvested and the luciferase activities were analyzed with the dual-luciferase reporter assay system (Promega). Renilla luciferase activities were served as the internal control.

**Statistical analysis**

All the data were analyzed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Two-tailed Student’s T-test was employed to compare group differences. The correlations between miR-494 expression and pathological features of cervical cancer patient were analyzed by χ² test. P<0.05 was considered to be statistically significant difference.

**Results**

*miR-494 is up-regulated in cervical cancer plasma*

To investigate the possible role of miR-494 in cervical cancer, the expression levels of miR-494 in cervical cancer plasma were measured by qRT-PCR. The data showed that the expression level of miR-494 was much higher in cervi-
miR-494 promotes cell proliferation, migration and invasion

Figure 2. miR-494 promotes cervical cancer cell proliferation, migration and invasion of. (A) The expression of miR-494 was up-regulated after the transfection of mir-494 mimic and down-regulated after transfection of mir-494 inhibitor. miR-494 overexpression promoted the proliferation (B), migration (C) and invasion (D) ability of HeLa cells, while miR-494 downexpression inhibited the proliferation (B), migration (C) and invasion (D) ability of HT-3 cells. NC, Negative control, **P<0.01, when compared with NC; *P<0.05, when compared with NC.

Figure 2. miR-494 promotes cervical cancer cell proliferation, migration and invasion of. (A) The expression of miR-494 was up-regulated after the transfection of mir-494 mimic and down-regulated after transfection of mir-494 inhibitor. miR-494 overexpression promoted the proliferation (B), migration (C) and invasion (D) ability of HeLa cells, while miR-494 downexpression inhibited the proliferation (B), migration (C) and invasion (D) ability of HT-3 cells. NC, Negative control, **P<0.01, when compared with NC; *P<0.05, when compared with NC.

The effects of miR-494 on cell proliferation, migration and invasion were detected to analyze the biological function of miR-494 in cervical cancer cells. miR-494 was over-expressed in HeLa cells and knocked down in HT-3 cells, respectively. The expression levels of miR-494 were examined by qRT-PCR (Figure 2A). The CCK-8 assay showed that miR-494 overexpression accelerated the growth of HeLa cells and knockdown of miR-494 suppressed the growth of HT-3 cells (Figure 2B). The wound healing results revealed that forced expression of miR-494 promotes cervical cancer cell proliferation, migration and invasion.

miR-494 promotes cervical cancer cell proliferation, migration and invasion

The effects of miR-494 on cell proliferation, migration and invasion were detected to analyze the biological function of miR-494 in cervical cancer cells. miR-494 was over-expressed in HeLa cells and knocked down in HT-3 cells, respectively. The expression levels of miR-494 were examined by qRT-PCR (Figure 2A). The CCK-8 assay showed that miR-494 overexpression accelerated the growth of HeLa cells and knockdown of miR-494 suppressed the growth of HT-3 cells (Figure 2B). The wound healing results revealed that forced expression of miR-494 promotes cervical cancer cell proliferation, migration and invasion.

The effects of miR-494 on cell proliferation, migration and invasion were detected to analyze the biological function of miR-494 in cervical cancer cells. miR-494 was over-expressed in HeLa cells and knocked down in HT-3 cells, respectively. The expression levels of miR-494 were examined by qRT-PCR (Figure 2A). The CCK-8 assay showed that miR-494 overexpression accelerated the growth of HeLa cells and knockdown of miR-494 suppressed the growth of HT-3 cells (Figure 2B). The wound healing results revealed that forced expression of miR-494 promotes cervical cancer cell proliferation, migration and invasion.

miR-494 promotes cervical cancer cell proliferation, migration and invasion

The effects of miR-494 on cell proliferation, migration and invasion were detected to analyze the biological function of miR-494 in cervical cancer cells. miR-494 was over-expressed in HeLa cells and knocked down in HT-3 cells, respectively. The expression levels of miR-494 were examined by qRT-PCR (Figure 2A). The CCK-8 assay showed that miR-494 overexpression accelerated the growth of HeLa cells and knockdown of miR-494 suppressed the growth of HT-3 cells (Figure 2B). The wound healing results revealed that forced expression of miR-494 promotes cervical cancer cell proliferation, migration and invasion.

miR-494 promotes cervical cancer cell proliferation, migration and invasion

The effects of miR-494 on cell proliferation, migration and invasion were detected to analyze the biological function of miR-494 in cervical cancer cells. miR-494 was over-expressed in HeLa cells and knocked down in HT-3 cells, respectively. The expression levels of miR-494 were examined by qRT-PCR (Figure 2A). The CCK-8 assay showed that miR-494 overexpression accelerated the growth of HeLa cells and knockdown of miR-494 suppressed the growth of HT-3 cells (Figure 2B). The wound healing results revealed that forced expression of miR-494 promotes cervical cancer cell proliferation, migration and invasion.
miR-494 promotes cell proliferation, migration and invasion

miR-494 promoted HeLa cells migration, and reduced expression of miR-494 inhibited HT-3 cells migration (Figure 2C). Cell invasion assay indicated the invasive ability of HeLa cells transfected with miR-494 mimics was higher than that transfected with negative control, while there were adverse results in cells transfected with miR-494 inhibitor and negative control (Figure 2D).

MAPK/ERK pathway is involved in miR-494-mediated cell proliferation, migration and invasion

The involvement of MAPK/ERK pathway in miR-494-mediated cell behavior changes was further investigated and miR-494 overexpression was found to significantly increase pERK1/2 protein level. Adverse results were found in miR-494 down-expressed cells. Suppressed miR-494 expression led to decreased p-ERK1/2 expression (Figure 3A). To better confirm the results, miR-494 over-expressed HeLa cells were treated with ERK inhibitor U0126 or DMSO for 1 h, and then subjected to proliferation, migration and invasion assays. Results showed that ERK inhibition significantly suppressed cell proliferation, migration and invasion (Figure 3B-D). These results indicated that MAPK/ERK pathway was involved in miR-494-induced cell proliferation, migration and invasion.

SOX9 is a target of miR-494

According to predictions of TargetScan (www.targetscan.org), Pictar (pictar.mdc-berlin.de/) and miRdb (http://www.mirdb.org/), SOX9 con-
miR-494 promotes cell proliferation, migration and invasion

miR-494 has been reported to play significant roles in various cancers. Previous studies have shown that down-regulation of miR-494 functioned as an independent prognostic factor in nasopharyngeal carcinoma [18] and overexpression of miR-494 suppressed breast cancer progression by regulating the Wnt/β-catenin pathway [15]. In addition, miR-494 activated the Akt/eNOS pathway to promote angiogenesis in non-small cell lung cancer [14]. In the present study, we found miR-494 was up-regulated in plasma of 40 cervical cancer patients and high miR-494 level was correlated with invasion degree, suggesting that miR-494 might participate in cervical cancer progression.

In addition, the role of miR-494 in cervical cancer cell lines was further studied. Cellular experiments indicated that miR-494 promoted cervical cancer cells proliferation, migration and invasion. MAPK/ERK pathway modulated a number of cellular functions and played important roles in tumorgenesis [19]. Therefore, whether MAPK/ERK pathway is involved in miR-494-induced cell proliferation, migration and invasion was identified. The results indicated that overexpression of miR-494 could increase p-ERK1/2 expression level to activate the MAPK/ERK pathway. Furthermore, cell proliferation, migration and invasion were decreased in miR-494 over-expressed cells when treated with ERK inhibitor U0126. These data suggested that miR-494 promoted cell proliferation, migration and invasion by activating that MAPK/ERK pathway.

miRNAs participated in cell biological processes by regulating their targets, thus luciferase assay was performed to verify the target of miR-494. The results indicated that SOX9 was a direct target of miR-494.

Discussion

Aberrant expression of miRNAs has been regularly found in cervical cancer, which is associated with tumor initiation and progression [16, 17]. Therefore, investigation about miRNAs is beneficial to uncover the molecular mechanism of tumorgenesis and develop new diagnosis methods.
miR-494 promotes cell proliferation, migration and invasion

and neural crest [20-26]. Previous studies demonstrated that SOX9 functioned as the oncogene or tumor suppressor to regulate malignant behaviors, such as metastasis and tumorigenicity [20, 27]. Wang HY et al found that SOX9 was a tumor suppressor gene and may serve as a potential therapeutic target in cervical cancer [24]. Whether miR-494 regulates cell proliferation, migration and invasion by targeting SOX9 will be studied in the future.

In conclusion, this study demonstrates that miR-494 is up-regulated in cervical cancer plasma. miR-494 promotes cell proliferation, migration and invasion through the MAPK/ERK pathway and SOX9 is a direct target of miR-494.

Acknowledgements

This work was supported by the Open Project of Jiangsu Traditional Chinese Medical Authority (JD201507).

Disclosure of conflict of interest

None.

Address correspondence to: Ming Ma, Department of Oncology, Linyi People’s Hospital, 27 Jiefang Road, Linyi, Shandong, China. Tel: 0086-539-8078518; E-mail: mamingmax@126.com

References

miR-494 promotes cell proliferation, migration and invasion

[18] Dadpay M, Zarea M, Rabati RG, Rezakhaniba B, Barari B, Behnod V and Ziari K. Upregulation of miR-21 and downregulation of miR-494 may serve as emerging molecular biomarkers for prediagnostic samples of subjects who developed nasopharyngeal carcinoma associates with lymph node metastasis and poor prognosis. Tumour Biol 2015; [Epub ahead of print].


