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Original Article

A cancer-promoting role of miR-144 in esophageal cancer by targeting PTEN

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Abstract: PTEN inhibits the activity of PI3K/AKT and is a tumor suppressor in several tumors. Elevated miR-144 expression is related to esophageal cancer. Bioinformatics analysis showed a relationship between miR-144 and PTEN mRNA. This study investigated miR-144's role in esophageal cancer cells. The tumor tissues and adjacent tissues of esophageal cancer patients were collected. The dual luciferase reporter gene assay assessed the interaction between miR-144 and PTEN. miR-144 and PTEN expression in normal esophageal epithelial HEEC cells, esophageal cancer Eca109 and KYSE150 cells were measured by real-time PCR. Eca109 cells were divided into miR-NC group and miR-144 inhibitor group followed by analysis of PTEN and p-AKT expression by western blot, cell apoptosis and proliferation by flow cytometry. Compared with adjacent tissues, miR-144 expression was elevated in esophageal cancer tissues, while PTEN expression was decreased. There was a targeted relationship between miR-144 and PTEN. Compared with HEEC cells, miR-144 expression was elevated in Eca109 and KYSE150 cells, and PTEN expression was decreased. Transfection of miR-144 inhibitor increased PTEN expression in Eca109 cells, decreased p-AKT expression, inhibited cell proliferation and increased apoptosis. Increased miR-144 expression reduces PTEN expression and promotes the pathogenesis of esophageal cancer. Inhibition of miR-144 up-regulates PTEN expression, inhibits PI3K/AKT signal pathway, and reduces the proliferation and increased apoptosis of esophageal cancer cells.

Keywords: miR-144, PTEN, PI3K/AKT, esophageal cancer

Introduction

Esophageal cancer (EC) has a complicated pathogenesis, and with a lack of early diagnosis it has high morbidity and mortality, and poor prognosis [1].

Phospholipase and tensin homologue deleted on chromosome ten (PTEN) negatively regulates the PI3K/AKT signaling pathway and is involved in breast cancer, bladder cancer, prostate cancer and other tumors [2-4]. Several studies demonstrated that decreased PTEN expression is related to the progression and pathogenesis of esophageal cancer [5-7].

MicroRNA is a non-coding single-stranded RNA of about 22-25 nucleotides in length discovered in eukaryotes in recent years. It can negatively regulate the expression of target gene mRNA through degradation of mRNA or inhibition of the translation of target gene mRNA, thus regulating cell proliferation, apoptosis, and migration. Abnormal expression and function of microRNA is associated with a variety of tumorigenesis, progression, metastasis and prognosis of several tumors, such as non-small cell lung cancer [8], gastric cancer [9], prostate cancer [10], and bladder cancer [11]. The abnormal expression and function of miR-144 is involved in the development, progression, metastasis and drug resistance of cervical cancer, lung cancer, breast cancer and intestinal cancer [12-15]. Studies have shown that abnormal miR-144 expression was related to the occurrence, progression and metastasis of es-

ophageal cancer [16-18]. Bioinformatics analysis showed that miR-144 has a targeted complementarity relationship with the 3'-UTR of PTEN mRNA. This study investigated whether miR-144 regulates PTEN expression heterogeneity and affects proliferation and apoptosis of esophageal cancer cells.

Materials and methods

Main reagents and materials

Human normal esophageal epithelial HEEC cells and esophageal cancer Eca109 cells were purchased from Beijing Beina; esophageal cancer KYSE150 cells were purchased from Wuhan Boster Bio; DMEM medium, Opti-MEM purchased from Gibco (USA); FBS purchased from Biowest (USA); Invitrogen; RNA iso plus, PrimeScriptTM RT reagent Kit purchased from Dalian Takara; Annexin V/PI apoptosis kit purchased from BioLegend (USA); luciferase reporter plasmid pMIR, luciferase activity assay kit Dual-Luciferase Report Assay System was from Promega in the United States; rabbit anti-PTEN and p-AKT antibodies were purchased from CST in the US; rabbit anti-β-actin antibody and HRP secondary antibody were purchased from Shanghai Biotechnology; miR-144 mimic, miR-144 inhibitor, miR-NC was designed and synthesized by Guangzhou Ruibo Biotechnology; EdU detection kit was purchased from Molecular Probes in the United States; RIPA lysate was purchased from Beijing Suobao Bio.

Patients

There were 35 patients with esophageal cancer who were treated in our hospital from July 2018 to December 2018 that were enrolled. Inclusion criteria were: (1) over 6 months survival and no complications; (2) essentially normal renal, hepatic and cardiac function, and blood cell numbers, urine and stool tests; (3) primary ESCC; (4) not received preoperative surgery, chemotherapy or radiotherapy before sampling; (5) aged 30~65 years; (6) no major organ dysfunctions or other diseases. Exclusion criteria included: (1) secondary ESCC; (2) received preoperative surgery, chemotherapy or radiotherapy; (3) complicated with other tumors. The esophageal cancer tissues, confirmed by pathological examination, were collected and the adjacent tissues which were located at least 2 cm away from the tumor tissues were collected as a control. The collection of tissue specimens has been reviewed by the ethics committee of our hospital and the patient's informed consent has been obtained.

Cell culture

HEEC, Eca109 and KYSE150 cells were cultured in DMEM medium at 37°C with 5% CO_2. After the cells reached 80% confluence, cells were collected after enzymatic digestion with 0.25% pancreas, subcultured at a ratio of 1:4 to 1:5, and cells in logarithmic growth phase were subjected to experiments.

Dual luciferase reporter gene assay

Total RNA was extracted using RNA iso plus and was used as a template to amplify the fragment containing the targeted binding site or its mutant fragment in the 3'-UTR region of PTEN gene, and the PCR product was obtained by gelatinization followed by transfection into the pMIR vector and transformed into the DH5α competent cells. The positive clones were confirmed by colony PCR, and plasmids with correct sequences were selected and named as pMIR-PTEN-WT and pMIR-PTEN-MUT, respectively. A total of 100 ng of pMIR-PTEN-WT (or pMIR-PTEN-MUT) and 50 pmol of miR-144 mimic (or miR-NC, miR-144 inhibitor) were co-transfected into HEK293T cells with Lipo 2000, and the relative luciferase activity was detected after 48 hours of culture.

Cell transfection

Eca109 cells were divided into miR-NC group and miR-144 inhibitor group. The above two nucleotide fragments and Lip 2000 were diluted with Opti-MEM for 5 min incubation, then the nucleotide fragment was mixed with Lip 2000 and gently mixed by inversion for a 30 min incubation followed by removal of the culture medium and addition of Opti-MEM medium, and subsequent addition of transfection complex to the medium and culture for 6 hours. Then, DMEM medium was added and cells were collected after 72 h transfection for relevant analysis.

qRT-PCR detection of gene expression

RNA extracted from cells using RNA iso plus was used for qRT-PCR analysis using the PrimeScriptTM RT reagent Kit. The reverse tran-

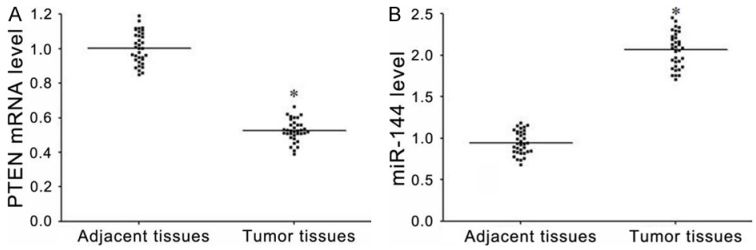

Figure 1. Abnormal expression of miR-144 and PTEN in esophageal carcinoma. A. qRT-PCR detection of PTEN mRNA expression in esophageal cancer tissues; B. qRT-PCR detection of miR-144 expression in esophageal cancer tissues. * represents $P < 0.05$ compared with adjacent tissues.

scription reaction system (10.0 μL) included: oligdT Primer (50 μM), 0.5 μL; Random 6 mers (100 μM), 0.5 μL; PrimeScript RT Enzyme Mix, 0.5 μL; RNA, 1.0 μg; 5× PrimeScript Buffer, 2 μL; and RNase Free dH_2O; qPCR reaction system: SYBR Fast qPCR Mix, 10.0 μL; Forward Primer (10 μM), 0.8 μL; Reverse Primer (10 μM), 0.8 μL; cDNA, 2.0 μL; RNase Free dH_2O, 6.4 μL with conditions: pre-denaturation 95°C, 10 min; denaturation 95°C 10 s; annealing 60°C 20 s; extension 72°C 15 s; cycle 40 cycle. Primer sequences were: PTEN-F: 5'-TGGATTCGACTTAGACTTGACCT-3', PTEN-R: 5'-GGTGGGTTATGGTCTTCAAAAGG-3'; β-actin-F: 5'-CATGTACGTTGCTATCCAGGC-3', β-actin-R: 5'-CTCCTTAATGTCACGCACGAT-3'.

Western blot

The cells were lysed by RIPA lysate on ice for 15 min and protein was extracted followed by being quantified by BCA method. In total, 40 μg of protein was electrophoresed on 12% SDS-PAGE and the gel was transferred to PVDF membrane followed by blockage with 5% skim milk powder and incubation with antibody (1:2000 dilution for PTEN, 1:800 for p-AKT, 1:10000 for β-actin) at 4°C overnight followed by incubation with HRP-conjugated secondary antibody (1:5000 dilution) for 60 min at room temperature followed by PBST washing 3 times and addition of enhanced chemiluminescence to detect protein expression.

Cell proliferation assay

The miR-NC and miR-144 inhibitor cells were digested with trypsin. The cells were harvested by centrifugation, incubated with 10 μM EdU for 2 h, inoculated into 6-well plates, cultured for 48 h, and collected by trypsin digestion. Cells were fixed for 20 min, washed once and centrifuged for 15 min followed by addition of 500 μL of reaction solution for 30 min incubation under dark and subsequent analysis of cell proliferation by Beckman FC 500 MCL.

Flow cytometry detection of cell apoptosis

The miR-NC groups and miR-144 inhibitor cells were collected by trypsinization. After washing twice with PBS, 100 μL Binding Buffer was added, followed by addition of 5 μL of Annexin V-FITC and 5 μL of PI and incubation for 10 min in the dark. After that, 400 μL of Binding Buffer was used to resuspend cells and cell apoptosis was analyzed by flow cytometry.

Statistical analysis

Data were analyzed by SPSS 18.0 software. The measurement data were displayed as mean ± standard deviation (SD). The comparison between groups was analyzed by student t test. $P < 0.05$ revealed a significant difference.

Results

Abnormal miR-144 and PTEN expression in esophageal carcinoma

Compared with adjacent tissues, tumor tissues showed significantly lower PTEN mRNA expression (**Figure 1A**). Compared with adjacent tissues, miR-144 expression in tumor tissues with esophageal cancer was significantly elevated (**Figure 1B**).

A targeted regulation relationship between miR-144 and PTEN

Bioinformatics analysis revealed a binding site between miR-144 and PTEN mRNA (**Figure 2A**). The dual luciferase gene reporter assay found significantly reduced relative luciferase activity in pMIR-PTEN-WT-transfected HEK293T cells after miR-144 mimic transfection, and miR-144 inhibitor significantly increased the luciferase activity in pMIR-PTEN-WT-transfected HEK293T cells. Whereas transfection of miR-144 mimic or miR-144 inhibitor did not affect the relative

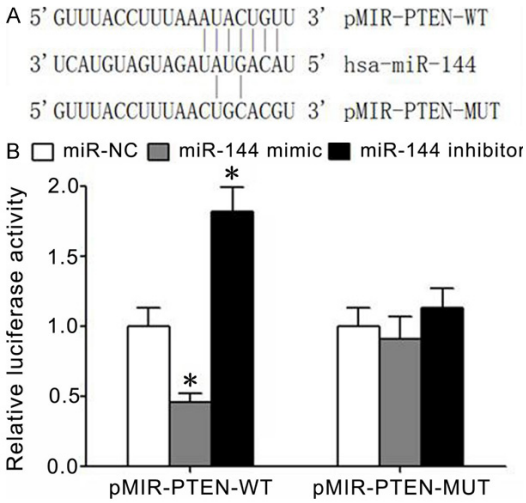

Figure 2. There is a targeted regulation relationship between miR-144 and PTEN. A. A binding site between miR-144 and the 3'-UTR of PTEN mRNA; B. Dual luciferase gene reporter assay. *P < 0.05 compared to the two groups.

luciferase activity in pMIR-PTEN-MUT-transfected HEK293T cells (**Figure 2B**), indicating a relationship between miR-144 and the PTEN mRNA.

Increased miR-144 and decreased PTEN expression in esophageal cancer cells

miR-144 expression in Eca109 and KYSE150 cells was significantly higher than that in human normal esophageal epithelial HEEC cells (**Figure 3A**), while PTEN mRNA expression was significantly decreased (**Figure 3B**). Additionally, PTEN protein expression in Eca109 and KYSE150 cells was significantly lower than that in human normal esophageal epithelial HEEC cells (**Figure 3C**).

Inhibition of miR-144 inhibits the proliferation and induces apoptosis of esophageal cancer Eca109 cells

miR-144 expression in Eca109 cells was significantly decreased in miR-144 inhibitor transfected group (**Figure 4A**), while PTEN mRNA expression was elevated (**Figure 4B**). Western blot analysis showed that compared with miR-NC transfection group, PTEN protein expression in Eca109 cells of miR-144 inhibitor transfection group was significantly elevated, while p-AKT protein expression was significantly reduced (**Figure 4C**). Flow cytometry analysis showed significantly reduced cell proliferation (**Figure 4D**) and increased cell apoptosis (**Figure 4E**) in the miR-144 inhibitor transfection group.

Discussion

Due to the lack of sensitive and specific early diagnosis indicators, most patients with esophageal cancer are already in the advanced stage at the time of being diagnosed, and the treatment efficacy and prognosis are very poor [19-21]. Therefore, exploring the signal molecules of abnormal changes in the pathogenesis of esophageal cancer is of great significance to improve diagnosis, the therapeutic effect and the prognosis.

When the PI3K/AKT signaling pathway is activated, PI3K can catalyze the phosphorylation of its substrate PIP2 to form PIP3, and PIP3 recruits AKT from the cytoplasm to the membrane, leading to phosphorylation of Ser473 and Thr308 sites of Ser/Thr protein kinase AKT in the phosphoinositide dependent protein kinase (phosphinositide dependent protein kinase, PDK). Phosphorylation-activated AKT continues to transmit signals downstream, thereby regulating cell proliferation, cycle, apoptosis, migration and other organism learning process [22-24]. The most important substrate for PTEN is phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which maintains low levels of PIP3 by dephosphorylation of PIP3 and antagonizes the effect of PI3K on the phosphorylation of PIP2, thus inhibiting AKT signaling pathways. Decreased expression or function of PTEN is related to the progression of various tumors such as lung cancer, bladder cancer, and breast cancer [4, 25, 26]. Decreased PTEN expression has been shown to be associated with the occurrence, progression, metastasis and prognosis of esophageal cancer [5-7].

Abnormal miR-144 expression participates in the progression and metastasis of esophageal cancer [16-18]. This study investigated whether miR-144 regulates PTEN expression heterogeneity and affects proliferation and apoptosis of esophageal cancer cells.

Analysis of patients' samples showed significantly higher miR-144 and lower PTEN expression in the tumor tissues, suggesting that miR-144 and PTEN abnormalities might play a role

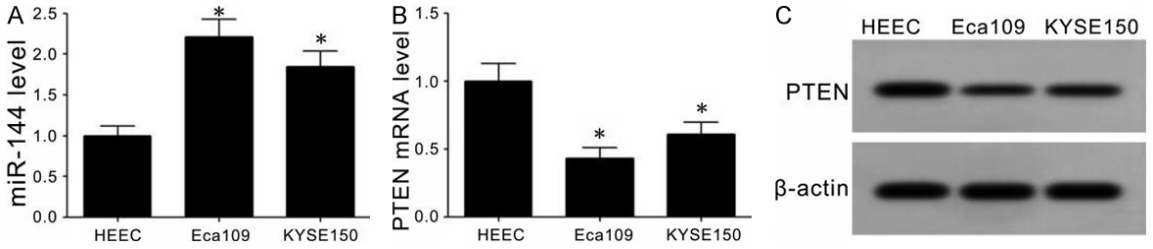

Figure 3. Increased expression of miR-155 in esophageal cancer cells and decreased expression of PTEN. A. qRT-PCR detection of miR-144 expression in esophageal cancer cells; B. qRT-PCR detection of PTEN mRNA expression in esophageal cancer cells; C. Western blot was used to detect the expression of PTEN protein in esophageal cancer cells. * represents $P < 0.05$ compared to HEEC cells.

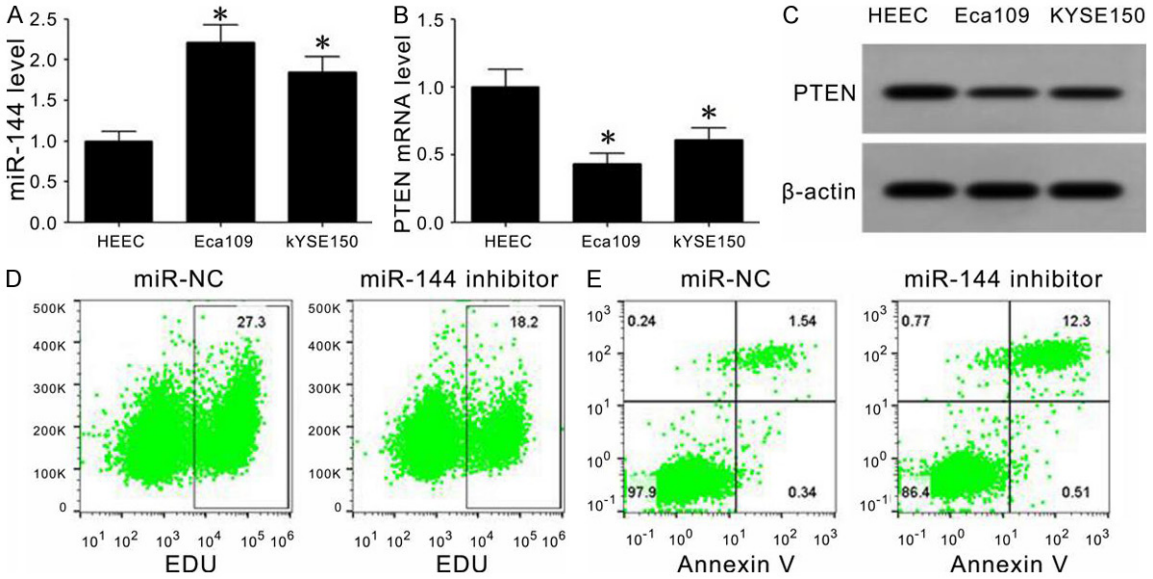

Figure 4. Inhibition of miR-144 expression can significantly inhibit the proliferation of esophageal cancer Eca109 cells and induce apoptosis. A. qRT-PCR detection of miR-144 expression in Eca109 cells; B. qRT-PCR detection of PTEN mRNA expression in Eca109 cells; C. Western blot was used to detect the expression of PTEN and p-AKT protein in Eca109 cells; D. Flow cytometry to detect apoptosis in Eca109 cells; E. EdU staining for detection of Eca109 cell proliferation. * represents $P < 0.05$ compared to miR-NC.

in esophageal cancer. In addition, the relative luciferase activity was significantly reduced after miR-144 mimic transfection, whereas it was increased after transfection of miR-144 inhibitor. However miR-144 mimic or miR-144 inhibitor had no significant effect on relative luciferase activity in pKIR-PTEN-MUT transfected HEK293T cells, indicating the presence of targeting regulatory relationship between miR-144 and PTEN. The results of a comparative study showed that compared with normal esophageal epithelial HEEC cells, miR-144 expression in esophageal cancer cells Eca109 and KYSE150 cells was abnormally increased, while PTEN mRNA and protein expression was abnormally decreased, suggesting that abnormal elevation of miR-144 reduces PTEN expression and promotes the onset of esophageal cancer. In a study of the relationship between miR-144 and esophageal cancer, the results of Du et al [27] showed significantly elevated miR-144 expression in the saliva of patients with esophageal cancer. The results of Sharma et al [17] showed significantly increased miR-144 expression in tumor tissues of patients with esophageal cancer compared with adjacent tissues. Compared with healthy controls, miR-144 expression was elevated in serum of esophageal cancer patients, and detection of miR-144 expression had a certain diagnostic value for esophageal cancer (AUC=0.731, P= 0.015). Sharma et al [28] showed significantly

higher miR-144 level in esophageal cancer patients, and the expression of miR-144 could be used to distinguish between esophageal cancer and healthy people (AUC=0.677, P= 0.043). The results of Xie et al [29] showed significantly higher miR-144 in the saliva of esophageal cancer patients. In this study, miR-144 was significantly elevated in tumor tissues and esophageal cancer cell lines of esophageal cancer patients, indicating that abnormal miR-144 expression might exert a role in esophageal cancer, and is a cancer-promoting factor in the pathogenesis of esophageal cancer. Which were in accordance with the results of Sharma et al [28], Xie et al [29].

This study further explored miR-144's effect on esophageal cancer cells and found that miR-144 inhibitor transfection significantly upregulated PTEN expression in esophageal cancer Eca109 cells, and decreased the expression of p-AKT protein. The proliferative capacity is weakened and the number of cells undergoing apoptosis increased. The results showed that in esophageal cancer, miR-144 inhibits PTEN expression, enhances the activity of PI3K/AKT pathway, and exerts a role in regulating cell proliferation and cell apoptosis. In the study of miR-144 regulating the biological effects of esophageal cancer cells, Sharma et al [18] showed a targeted relationship between miR-144 and PURA in esophageal cancer KYSE-410 cells, down-regulating miR-144 expression can significantly increase the expression of PURA, inhibit the proliferation of esophageal cancer cells, and attenuate the migration, invasion and clonal formation of esophageal cancer KYSE-410 cells, indicating that miR-144 plays a cancer-promoting role in esophageal cancer. This is similar to the promotion of esophageal cancer by miR-144 observed in this study. Regarding the role of PTEN in esophageal cancer, the results of Li et al [7] found that the decrease of PTEN expression is related to the pathogenesis of esophageal cancer. Increasing the expression of PTEN can inhibit the proliferation of esophageal cancer cells and reduce the migration and invasive ability of cells. Jin et al [6] demonstrated that PTEN decreased the drug resistance of esophageal cancer EC9706 cells to 5-FU, and the decrease of PTEN expression was caused by the abnormal increase of miR-141 expression. This study combines the targeting relationship between miR-144 and PTEN, and reveals that elevated expression of miR-144 down-regulates PTEN expression and promotes the development of esophageal cancer.

Conclusion

Increased miR-144 is involved in reducing PTEN expression and promoting the pathogenesis of esophageal cancer. Inhibition of miR-144 up-regulates PTEN, inhibits PI3K/AKT signaling, and reduces the proliferation and increases apoptosis of esophageal cancer cells.

Acknowledgements

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Disclosure of conflict of interest

None.

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