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MiR-214 promotes cell meastasis and inhibites apoptosis of esophageal squamous cell carcinoma via PI3K/AKT/mTOR signaling pathway

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ABSTRACT

There is growing evidence shown that microRNAs (miRNAs) are associated with cancer and can play a role in human cancers as oncogenes or tumor suppressor genes. MicroRNA-214 (miR-214) shows carcinogenesis in various tumor types, but little is known about biological functions of miR-214 in esophageal squamous cell carcinoma (ESCC). In this study, we observe that the expression of miR-214 is not only increased in human ESCC tissues, but also remarkably increased in cell lines correlates with LZTS1. In addition, the expression of miR-214 inhibited proliferation of ESCC cells in vitro and inhibit the growth of xenograft tumor in vivo. The results show miR-214 serve as a tumor promoter regulating cells migration, invasion and apoptosis in ESCC. Ferthermore, LZTS1 has been proved to as a functional target for miR-214 to regulate cells poliferation and apoptosis. In summary, these results suggest that miR-214 serves as tumor promoter to promote proliferation, migration, invasion and inhibit apoptosis of ESCC cells by targeting LZTS1 via PI3K/AKT/mTOR signaling pathway. The miR-214/LZTS1 pathway provides a new insight into the molecular mechanisms that the occurrence and development of ESCC and it provides a novel therapeutic target for ESCC.

1. Introduction

Esophageal cancer is one of the leading causes of death in both developed and developing countries [1], especially in the East Asia, Africa and North America [2]. More than 90% of esophageal cancers are esophageal squamous cell carcinoma (ESCC) [3]. Although considerable progress has been made in diagnosis and treatment, esophageal cancer remains a devastating malignancy due to late diagnosis and rapidly aggressive progression. Patients with esophageal cancer are found to have high terminal metastasis and poor prognosis [4–6]. Therefore, exploration about the development mechanism of ESCC will be helpful for the detection, diagnosis and treatment of ESCC.

MicroRNAs (miRNAs) are small non-coding RNA, usually 21–23 nucleotides long which serve as important regulators of gene expression by binding to the 3′ untranslated region (UTR) of target mRNAs [7,8], including in physiological and pathological processes, including cell proliferation, differentiation, migration and apoptosis [9–12]. In ESCC, many mirnas have been identified as effective tumor biomarkers for cancer diagnosis, or (and) key oncogenes (or tumor suppressor modulation [13–16]. However, few miRNAs are reported to be related to the occurrence and development of ESCC. Accumulating studies have show that miR-214 may induce a wide variety of human malignancies, including pancreatic, hepatoblastoma, hepatocellular, lung, breast, gastric, osteosarcoma, esophageal squamous cell carcinoma, prostate, ovarian, bladder melanoma and cervical cancers [17–21].

The phosphatidylinositol-3－OH kinase (PI3K)-Akt pathway is a major signaling cascade that is activated in a large variety of human cancers [22]. The phosphoinositide 3-kinase (PI3K)/AKT pathway enhances not only cell proliferation, but also cell invasion and migration [23]. PI3K regulates a large number of basic cellular responses and plays a key role in controlling cell survival and apoptosis. PI3K pathway may be the best characterized pathway to promote the growth or survival of cancer cells and inhibit apoptosis in cancer cells [24]. AKT, the key effect of cell survival, phosphorylation in many different growth factors and interleukin-activated responses via phosphatidylinol conversion through PI3K [25]. mTOR plays an important role in the

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downstream signal transduction of AKT pathway and participates in cell proliferation, differentiation and apoptosis.

In this study, our objective is to assess the molecular mechanism about miR-214 on ESCC cell proliferation and apoptosis and analyse the collective signaling pathways enriched by the predicted targets of the down-regulated miRNAs in ESCC carcinogenesis. In this study, we confirmed that miR-214 is negatively correlated with the expression level of LZTS1 inESCC. Our collective results indicated that miR-214 directly targets 3′-UTR of the LZTS1 transcript and inhibited its expression. The downregulation of miR-214 expression suppressed cancer cell viability, proliferation and tumorigenesis by PI3K/AKT/mTOR signaling pathway. This approach helps to clarify the role of miRNA in esophageal cancer and may be a new therapeutic target for ESCC.

2. Materials and methods

2.1. Human tissue samples and cell line

Thirty patients with esophageal squamous cell carcinoma and adjacent normal tissues were obtained from Handan First Hospital. The tissues were surgical removal and immediately stored at -80℃. The collection of human tissue samples was approved by the Ethics Committee of Handan First Hospital. The human esophageal squamous cell carcinoma cell line (KYSE150; ECA-109; KYSE450) and the normal cell line HET-1 A were provided by Institute of Biochemistry and Cell Biology of Chinese Academy of Science (China). These cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum under a humidified atmosphere of 95% atmosphere and 5% CO_2 at 37 ℃.

2.2. Cell transfections

The lentivirus expressing human miR-214 inhibitor and an empty lentivirus were purchased from RiboBio (RiboBio Biotech, China). Transfections were performed using Lipofectamine 2000 according to the manufacturer's protocol. Cells were incubated for 72 h. Lentivirus-induced miR-214 downregulation was assessed by RT-PCR, western blot and immunohistochemical.

2.3. RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). TaqMan miRNA assays were performed to assess the expression level of miR-214. Briefly, 3 mg of small RNA extracted from tissue samples or cells was reverse transcribed to cDNA. The cDNA was subsequently used for the amplification of miR-214, U6 was used as an internal control. Real-time PCR was performed using SYBR Premix Ex Taq II. The PCR conditions were performed at 94℃ for 4 min, followed by 40 cycles of amplification: 94℃ for 40 s, 52℃ for 40 s, 72℃ for 40 s. All samples were normalized to control and calculated using the $2^{-\Delta\Delta CT}$ analysis method.

2.4. Western blotting

Cells were lysed with RIPA lysis buffer and proteins were harvested. Total cell protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane which block with 5% non-fat milk. Whereafter the blots were incubated with primary antibodies at 4℃ overnight, followed by incubation with secondary antibody at room temperature for 1 h. Immunoblots were visualized using the ECL detection system and the protein levels were quantified using ImageJ software.

2.5. Immunohistochemistry

Immunohistochemistry was performed according to the methods described previously [26]. Microwave irradiation was performed on the sections which were incubated with polyclonal rabbit anti-human LZTS1 at room temperature of 2 h. Sections were washed with PBS and incubated with secondary antibodies at room temperature for 1 h. Staining intensity was evaluated. All images represent at least three separate experiments.

2.6. Cell proliferation assay

MTT assay and colony formation assay were used to explore the cell viability and cell proliferation ability. The cells were seeded in 96-well plate and cultured in medium including 10% FBS for 6 days. Then cells were detected by MTT assay at different time points. 10 ml of MTT solution was added to 100 ml medium, following incubated at 37 ℃ for 4 h. The absorbance was read on a spectrophotometer at 570 nm. Cell proliferation was detected by colony formation assay. After 12 days of inoculation, the cells were stained with crystal violet and the number of colonies was measured.

2.7. Cell cycle analysis

Cells were collected and fixed in 70% ethanol at 4℃ overnight. The cells were incubated with RNase A in PBS for 30 min after the ethanol was removed. Whereafter the cells were incubated in 0.5 ml of 50 mg/ml propidium iodide (PI) for 30 min in the dark. The cells throughout the cell cycle was analyzed by Cell Lab Quanta SC flow cytometry (Beckman Coulter, Fullerton, CA) according to the established method as described previously [27] after washed with cold PBS twice.

2.8. TUNEL assays

4×10^3 cells were seeded on a 14-well slide in triplicate for 24 h, then fresh medium containing 10% FBS were replaced and cultured for 48 h. We use an In situ Cell Death Detection Kit with Fluorescein (Roche Applied Science, IN, USA) to detecte cell apoptosis. DNA was stained with DAPI which was used to visualize the nuclei. The TUNEL assays were quantified by counting the DAPI. The apoptotic rate was calculated by dividing the average number of TUNEL-positive cells by that of the DAPI-positive cells in one field. Experiments were performed at least three separate experiments.

2.9. Cell migration and invasion assays

Wound healing assay was used to assessed cell migration ability. Cells were inoculated into 24 well culture plates and cultured until confluent. A P200 pipette tip was used to make a straight line simulation “wound”. In addition, cells were washed with appropriate culture medium for 3 times to remove cell debris. The degree of wound closure was monitored at different times. Futhermore, migration and invasion ability were detected by transwell, a total of 1×10^4 cells were inoculated into the upper part of a transwell chamber (transwell filter inserts with a pore size of 8 μm), which was precoated with or without 30 μl of 1 mg/ml matrigel for 2 h. The number of migrated cells on the lower surface of the membrane was counted under a light microscope with a magnification of ×400 in 10 fields. All assays were performed three times.

2.10. In vivo tumorigenesis assay

Male BALB/c nude mice (6–7 weeks old) were randomly divided into three groups (n = 10). 5×10^6 si-miR-214, Lv-NC or control cells were injected subcutaneously into the right flank of the nude mice axillary. The mice were sacrificed and evaluated after 2 weeks. Tumor growth was analyzed by measuring tumor length and width, and tumor volume was calculated according to $1/2 \times \text{length} \times \text{width}^2$.

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