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Original article

MiR-96 promotes proliferation and chemo- or radioresistance by down-regulating RECK in esophageal cancer

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ABSTRACT

The involvement of miR-96 in esophageal cancer (EC) remains unclear. The aim of this study is to explore the functional role of miR-96 and determine whether miR-96 could be a potential therapeutic target for human esophageal cancer. MiR-96 up-regulation was demonstrated in 145 EC samples and RECK down-regulation was validated in EC cell lines. Moreover, ectopic overexpression of miR-96 in TE-1 or ECa-109 contributed to tumor growth in xenograft mouse models. Furthermore, up-regulation of miR-96 could reduce the susceptibilities of EC cells to chemotherapy or radiotherapy. RECK was identified as a target of miR-96 and RECK overexpressing could abrogate the growth of EC cells induced by miR-96. Taken together, miR-96 serves as an oncogene role in EC cells through downregulating RECK.

1. Introduction

Esophageal cancer (EC) is the sixth cause of death in the world, which kills about 386,000 people each year [1]. There are two main subtypes of esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), with ESCC being the most frequent type of esophageal malignancy [2]. Despite the rapid advancement in diagnosis and therapy for EC, the average 5-year overall survival has remained at 10–20% because of the proliferation and invasion of cancer cells [3]. Therefore, to improve the survival rate and the life quality of EC patients, it is crucial for us to elucidate the molecular mechanisms underlying EC tumorigenesis.

MicroRNAs (miRNAs) are class of small non-coding RNAs that are evolutionary, conserved and involved in post-transcriptional regulation of gene expression. They modulate gene expression by binding to 3′-UTR target sites of mRNAs and repress their translation or promote cleavage and degradation [4]. In esophageal cancer, miRNAs down-regulated such as miR-375 [5], miR-34a [6], miR-143 and miR-122 [7], miR-145 [8], miR-302b [9] usually function as suppressive miRNAs, while miRNAs up-regulated such as miR-645 [10], miR-100 [11], miR-208 [12], miR-21 [13] usually exert oncogenic roles. The expression of miR-96 has been confirmed up-regulated in non-small cell lung cancer [14], human breast cancer [15], prostate cancer [16], and bladder cancer [17]. Furthermore, latest research has reported miR-96 promotes tumor growth by downregulating RECK expression [14,18].

In our study, we have detected that miR-96 was highly expressed in EC tissues and cell lines. In additional, we found that miR-96 was related to tumor depth, stage, and metastasis of esophageal cancer and up-regulation of miR-96 could reduce the susceptibilities of EC cells to drug or irradiation. Furthermore, we demonstrated that miR-96 regulates its effect by targeting RECK. Therefore, our findings suggest that targeting miR-96 could be a promising treatment for EC patients.

2. Materials and methods

2.1. Cell culture

Three EC cell lines, TE-1, ECa-109, EC-9706, and a normal human esophageal epithelial cell line (HEEC) were cultured in DMEM media supplemented 10% fetal bovine serum (Invitrogen, USA) at 37°C with 5% CO_2.

2.2. Human tissue samples

A total of 145 patients with esophageal cancer had undergone routine surgery at The First Affiliated Hospital of Soochow University from January 2013 to September 2014. Esophageal

Abbreviations: miRNAs, microRNAs; 3′-UTR, 3′-untranslated region; EC, esophageal cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RECK, reversion-inducing-cysteine-rich protein with kazal motifs.

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cancer samples and the corresponding adjacent esophageal tissues taken from the 145 patients were collected and immediately snap-frozen in liquid nitrogen. All patients have not received any therapy prior to surgery. The use of these tissues for all assays was obtained with informed consent and this project was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University.

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cell lines and human tissues with the Trizol (Invitrogen, USA). Using a miR-96 specificity qRT-PCR detection kit (Stratagene, USA), qRT-PCR assays were performed on an ABI 7500 fast real-time PCR system (Applied, Biosystems) according to the manufacturer's instructions. U6 small nuclear RNA was used for normalization. The relative gene expression was calculated via a $2\Delta CT$ method.

2.4. Plasmid construction

MiR-96 mimic and the control were obtained from RiboBio (Guangzhou, China). The 3′-UTR of RECK mRNA was amplified using the following primers: sense 5′-CCCTCGAGGCTGGAAAT GAGATGAC-3′ and antisense 5′-TTGCGGCCGCTATGGCTATT CACCTTCTTC-3′. The PCR product was inserted into psiCHECK2 within XhoI and NotI restriction sites (Promega, Madison, WI, USA). Mutation experiment was performed using a fast mutation kit (NEB, Ipswich, Canada). At last, pCDNA3.1·RECK was constructed by inserting RECK to the specific region of pCDNA3.1.

2.5. Cells transfection

Cells were divided into six groups according to the treatment and named as follows:

- blank group (untreated);
- vector group (transfected with miR-96 mimic control);
- MiR-96 group (transfected with miR-96 mimic);
- RECK·3′-UTR-wild (transfected with psiCHECK2-RECK·3′-UTR-wild reporter);
- RECK·3′-UTR-mut (transfected with psiCHECK2-RECK·3′-UTR-mut reporter);
- pCDNA3.1·RECK (transfected with RECK);

All the transfection assays were performed using Lipofectamin 2000 (Invitrogen, USA). The cells transfected with miR-96 or RECK were selected with G418 and the stable transfected cells could be obtained.

2.6. Western blot

Total proteins were extracted from cultured cells by using RIPA Lysis Buffer (Beyotime, Shanghai, China). The protein concentration was detected by BCA Protein Assay Kit (Bio-Rad, Italy). Proteins were separated by 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk in TBST for 2 h, the membranes were incubated overnight at 4 °C with diluted primary antibody. The primary antibodies included RECK (1:400, SantaCruz, USA) and β-actin (1:1000, SantaCruz, USA). Followed by washing with TBST solution three times, the membranes were incubated with secondary antibody at 37 °C while shaking on a rotary for 2 h. The signals were visualized using ECL reagents (Pierce, Rockford, IL, USA).

2.7. MTT cell proliferation assay

Cells, seeded on 96-well plates, were stained at various times with MTT dye (0.5 mg/mL, Sigma) for 4 h at 37 °C, followed by removal of the culture medium and addition of 150 μL of dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO, USA). The absorbance values at 490 nm were detected using a MRXII absorbance reader (DYNEX Technologies, Chantilly, VA, USA).

2.8. Tumor formation assay in nude mouse models

Five-week BALB/C nude mice were purchased from Experimental Animal Center of Soochow University, and all animal protocols were approved by the Institutional Animal Care and Treatment Committee of Soochow University. Tumor cells in the logarithmic phase were collected, washed twice with PBS, and resuspended at a concentration of 2×10^7 cells per milliliter of PBS. Then 0.1 ml-suspending cells were subcutaneously injected into either side of the flank of each female nude mouse. After six weeks, animals were sacrificed and solid tumor tissues were removed and weighed.

2.9. Apoptosis assay

Apoptosis was analyzed by flow cytometry using Annexin V/PI double staining. Twelve-four hours after transfection, then cells of blank group, vector group and miR-96 group were cultured in the presence of cisplatin (2 μg/mL) and irradiation (6 Gy) respectively for 48 h. Apoptosis in cultured cells was evaluated using Annexin V/PI double staining solution (Sigma-Aldrich, USA). The number of apoptotic and necrotic cells were calculated by flow cytometry (Becton-Dickinson Co, USA) and analyzed by Cell Quest Software.

2.10. CCK-8 assay

Cell proliferation was assessed by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). Cells were seeded into 96-well plates at 2×10^3 cells/well. The proliferation rate was measured at 0, 24, 48, 72 h after transfection. Absorbance was determined at 450 nm using a microplate reader (Bio-Rad, USA).

2.11. Luciferase assay

Cells were seeded in 24-well plate at a concentration of 2×10^5 cells/per well and co-transfected 24 h later with RECK 3′-UTR-wild or RECK 3′-UTR-mut and miR-96 or vector. 48 h after transfection, cells were collected, and the relative luciferase activity was performed using Dula-Luciferase Reporter Assay Kit (Promega, USA).

2.12. Statistical analysis

All data are presented as mean values ± SD and analyzed by SPSS 19.0. One-way analysis of variance (ANOVA) or Student's *t* test was used to evaluate the statistical significance of differences. The significance was accepted as *P* value was less than 0.05.

3. Results

3.1. MiR-96 is elevated in EC tissues and cell lines, whereas RECK is reduced in EC cell lines

The expression of miR-96 in three EC cell lines (TE-1, ECa-109 and EC-9706) and a normal human esophageal epithelial cell line (HEEC) was analyzed by qRT-PCR. Significantly, miR-96 expression levels in three EC cell lines were higher than that in normal cells (Fig. 1A). Next, miR-96 expression was obviously higher in EC tissues than corresponding adjacent tissues (Fig. 1B). The correlation between miR-96 expression and clinicopathological