Biomedicine & Pharmacotherapy 105 (2018) 582–589

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

MicroRNA-873 acts as a tumor suppressor in esophageal cancer by inhibiting differentiated embryonic chondrocyte expressed gene 2

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ARTICLE INFO

Keywords:
microRNA-873
Esophageal cancer
Differentiated embryonic chondrocyte expressed gene 2

ABSTRACT

Esophageal cancer is one of the most common digestive malignant diseases worldwide and emerging evidences revealed that microRNAs (miRNAs) were implicated in the development and progression of esophageal cancer. However, the expression level and biological function of microRNA-873(miR-873) in esophageal cancer are still largely elusive. In this study, we investigated the expression and biological roles of miR-873 in human esophageal cancer. Our results revealed that miR-873 was significantly underexpressed in esophageal cancer tissues and cell lines when compared with the para-tumor tissue and primary human esophageal epithelial cells. Furthermore, overexpression of miR-873 could remarkably inhibit esophageal cancer cell growth, migration and invasion. Moreover, we validated differentiated embryonic chondrocyte expressed gene 2 (DEC2) as a direct target of miR-873 which could reverse the repressive effects of miR-873 on esophageal cancer cell. In summary, our investigation demonstrated that miR-873 was underexpressed in esophageal cancer and might act as a tumor suppressor gene by directly targeting DEC2.

1. Introduction

Esophageal cancer is one of the most common digestive malignancy and the sixth most common cause of cancer-related mortality worldwide [1]. Globally, there are about 455,800 newly diagnosed esophageal cancer cases and 400,200 deaths every year. Eastern Asia, Eastern Africa and Southern Africa are the areas that have the highest incidence rates of esophageal cancer and it causes major public health problems [2]. Even the diagnosis and treatment strategies for esophageal cancer have improved in the past three decades, the five-year survival rate is still less than 21% [3]. Therefore, it is of great importance to elucidate the underlying pathogenesis and explore therapeutic targets for esophageal cancer.

MicroRNAs(miRNAs) are single-stranded, small non-coding RNA molecules with about 22 nucleotides in length [4]. MiRNAs exert function by binding to the 3′-untranslated regions (3′-UTRs) of their targeted messenger RNAs (mRNAs), which could induce transcriptional repression or degradation [5]. It has been widely accepted that miRNAs play important roles in most physiological and pathological processes [6]. The aberrant expression of miRNAs has been found in almost every kind of cancer, including esophageal cancer, gastric cancer and colon cancer [7–9]. It was also showed that miRNAs acted as pivotal regulators in the processes of cancer development and progression, including cancer cell proliferation, apoptosis, migration and signal transduction [9,10].

The transcription factor differentiated embryonic chondrocyte expressed gene 2 (DEC2) is known as basic helix-loop-helix family member e41 (BHLHE41) and has 482 amino acid residues with alanine and glycine-rich regions in the C-terminus [11,12]. The studies have illustrated that DEC2 is crucial to regulate circadian rhythm [13,14]. Moreover, DEC2 was also reported to be dysregulated in cancer cells [15]. However, the expression and phenotype of DEC2 in esophageal cancer are largely elusive.

Recently miR-873 has been showed to be downregulated or upregulated in some types of cancers and acts as important molecule involved in cancer pathogenesis. However, the biological function of miR-

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https://doi.org/10.1016/j.biopha.2018.05.152
Received 2 March 2018; Received in revised form 28 May 2018; Accepted 29 May 2018

873 in esophageal cancer and the molecular mechanisms remain unclear. In the present study, we explored the expression of miR-873 in esophageal cancer tissues and cell lines. The results demonstrated that miR-873 was remarkably downregulated in esophageal cancer. Moreover, overexpression of miR-873 could observably inhibit esophageal cancer cell growth, migration and invasion. Additionally, DEC2 was firstly verified as a direct target of miR-873 which could rescue the effects of miR-873 on esophageal cancer. Our exploration indicated that miR-873 might act as a tumor suppressor in esophageal cancer and it may serve as a potential therapeutic target for esophageal cancer in the future.

2. Materials and methods

2.1. Tissue samples and ethics

A total of 36 esophageal cancer patients who underwent surgery in Maoming People's Hospital were enrolled in this study. The patients received no anticancer therapy before surgery. After surgical resection, the esophageal cancer tissues and paired adjacent normal tissues were collected and stored in liquid nitrogen container immediately. This study was discussed and approved by the Ethics Committee of Maoming People's Hospital. Written informed consent was got from each recruited participant.

2.2. Cell lines and culture

Human esophageal cancer cell lines, including EC-109, EC-1, TE-1, TE-10, and KYSE-150, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HEEC cells were purchased from Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China) and used as normal human esophageal epithelial cell line. The Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) and 0.1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) were used for cultivation. The cell incubator was humidified and maintained at 37 °C with 5% CO2.

2.3. RNA extraction and quantitative analysis

The total RNA of clinical tissues and cell lines were extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Real-time quantitative reverse transcription PCR was used for the detection of RNA expression. The detection was performed using standard SYBR Green PCR kit (TaKaRa, Dalian,China) on the detective machine of LightCycler 480 (Roche, Meylan, France). Results of miRNAs were normalized to the expression of U6 and mRNAs were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specific sequences of primers were: DEC2, 5′-AACGAGACGACACCAAGGAT-3′ (Forward primer), 5′-CGCTCCCCATTCTGTAAAGC-3′ (Reverse primer); GAPGH, 5′−CCAAGGAGTAAGACCCCTGG-3′ (Forward primer), 5′-TGGTTGAGCACAGGGTACTT-3′ (Reverse primer). The data were collected and analyzed using $2^{-\triangle\triangle Ct}$ method.

2.4. Cell transfection

MiR-873 mimic and miRNA mimic control (miR-NC) were purchased from Ribobio (Guangzhou, China). The pcDNA3.1-DEC2 (pc-DEC2) and pcDNA3.1-control (pc-NC) were obtained from Obio (Shanghai, China). The siRNA-DEC2 (si-DEC2) and siRNA-control (si-NC) were synthesized by Ribobio (Guangzhou, China). According to the manufacturer's protocols, lipofectamine 2000 (Invitrogen Life Technologies) was used for transfection and the transfection efficiency was detected. The transfection efficiency must be above 80% then the cells could be used for further experiments.

2.5. Cell counting kit-8(CCK8) assays

CCK8 reagent (Dojindo, Kumamoto, Japan) was used for the measurement of cell proliferation. Briefly, cells were planted into the 96-well plates after the transfection of indicated miRNA or pcDNA. Then the CCK8 solution was put into the plates and incubated in incubator for 2 h. The absorbance at 460 nm of each well was detected every 24 h according to the manufacturer's instructions. The assay was conducted three times independently.

2.6. Cell migration and invasion assays

Cell migration and invasion assays were performed using 8 μm pores transwell inserts (Coring, NY, USA). For cell migration analysis, 300 μl serum free medium with 3.0×10^5 cells were plated into the upper chambers of transwell inserts. For cell invasion analysis, 300 μl serum free medium with 6.0×10^5 cells were added into the upper transwell insert chambers which were covered with matrigel matrix (BD, NJ, USA). The bottom chambers were filled with 10% FBS DMEM which acted as chemoattractant. With a 24 h incubation, non-migration and non-invasion cells were wiped by cotton buds. Then methanol and 0.1% crystal violet were used for the fixation and staining of migrated or invaded cells. We selected five randomized fields under microscope for the calculation of migrated or invaded cells. Each assay was repeated in triplicate independently.

2.7. Western blot assays

Cells were harvested and lysed by strong RIPA lysis (Beyotime, Beijing, China) with PMSF. Standard protein analysis tool BCA Protein Assay Kit (Beyotime, Beijing, China) was then applied for the measurement of protein concentrations. Afterwards, approximately 80 μg protein extracts were loaded onto the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separation by gel electrophoresis. After electrophoresis, the extracts were blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked by 5% non-fat milk (BD, NJ, USA). The blots were probed with primary mouse antibody against DEC2 (1:1000, Abcam, Cambridge, MA, USA) and primary mouse antibody against GAPDH (1:5000, Abcam, Cambridge, MA, USA). Membranes were finally incubated with secondary antibody (1:5000, IgG-HRP, Biogot technology, Nanjing, China). Visualization was conducted using ECL Substrates (Millipore, MA, USA).

2.8. Luciferase reporter assays

Cells were planted into the 96-well plates and cultured for transfection. The psiCHECK-2 vectors (Promega, Madison, WI, USA) were cloned with wild-type DEC2 -3′-UTR or mutant DEC2 -3′-UTR fragment. Then the cells were cotransfected with the reporter plasmids with miR-873 or miR-NC. The luciferase activity was determined by Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase activity was measured for normalization. Each assay was repeated in triplicate independently.

2.9. Statistics

Statistical analysis was performed by using SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). All the data were expressed as mean ± standard error of mean (SEMs). The difference between 2 groups were determined by two tailed Student' s *t*-test. Survival curves were presented using Kaplan-Meier's method and the difference was determined by Log-rank test. $P < 0.05$ was defined as statistically significant.