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MiR-502 mediates esophageal cancer cell TE1 proliferation by promoting AKT phosphorylation

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ABSTRACT

Esophageal cancer is one of the most common cancers in the world and esophageal squamous cell carcinoma is one of the two main types in esophageal cancer. MicroRNA is a small non-coding RNA molecule functions in many different cancers including esophageal cancer. We found miR-502 was up-regulated in esophageal tissues, which indicated miRNA-502 may play important roles in esophageal cancer. In this study, we used esophageal cancer cell line TE1 as an in vitro model for investigating the role of miR-502 in promoting the proliferation of the cancer cells. We found that overexpressing miR-502 in TE1 cells promoted the proliferation and inhibited the apoptosis induced by dox. Down-regulating miR-502 made the opposite phenomenon. Furthermore, western blot showed that miR-502 enhanced the phosphorylation levels of AKT pathways, which may be the mechanism of the overgrowth for esophageal cancer cell. Our data provide the evidence of a role for miR-502 in the regulation the proliferation of esophageal cancer cell through promoting the phosphorylation of AKT signaling. Due to its ability to promote the overgrowth of esophageal cancer cell, miR-502 may be a novel target for esophageal cancer therapeutic.

1. Introduction

Esophageal cancer (EC) is one of the most common cancers in the world [1]. It stands eighth among the most common cancers in the world [2]. It is also the sixth leading cause of cancer-related mortality in the world [3]. Developing countries make up for more than 80% of the total clinical cases of esophageal cancer [4]. Esophageal cancer is often diagnosed at a very advanced stage and thus carries poor prognosis [5]. Esophageal cancer contains two main types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) [6]. ESCC is the major form of EC in Asian countries while EAC is the most common type of EC in Western countries [7].

More and more evidence shows that many molecular changes are associated with EC tumorigenesis, which including epidermal growth factor receptor (EGFR) amplification [8], phosphoinositide 3-kinase, catalytic subunit alpha (PIK3CA) amplification and mutation [9,10], and mutation or loss of function of phosphatase and tensin homolog (PTEN) [11,12]. Alteration of these molecular events contributes to downstream pathway activation. Inhibitors targeting these pathways were developed and tested in clinical trials with certain success [13,14]. However, transcriptional regulation of gene expression in these well-known pathways may not be the only reason which can cause esophageal cancer, finding more targets or the new mechanisms which in the level differ from transcriptional regulation will be important.

MicroRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) functions in post-transcriptional regulation of gene expression, which can be found in plants, animals, and some viruses. MiRNAs are small, non-coding RNAs that can negatively regulate the protein coding gene. They are associated with approximately all known physiological and pathological processes, especially cancer. MiRNAs can affect cancer pathogenesis, playing a crucial role as either oncogenes or tumor suppressors. The recent emergence of observations on the role of miRNAs in

Abbreviations: EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma; miR, microRNA.

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cancer and their functions has induced many investigations to examine their relevance to esophageal cancer. In esophageal cancer, miRNA dysregulation plays a crucial role in cancer prognosis and in patients' responsiveness to neo-adjuvant and adjuvant therapies.

In this study, we found that miR-502 is up-regulated in esophageal squamous cell carcinoma compared to the normal tissues. This up-regulation will promote the proliferation and inhibit the apoptosis of TE1 cells. We further found that miR-502 regulated the proliferation of esophageal cancer cell through promoting the phosphorylation of AKT signaling. Due to its ability to promote the overgrowth of esophageal cancer cell, miR-502 may be a novel target for esophageal cancer therapeutic.

2. Methods

2.1. Samples

Biospecimens of squamous cell carcinoma and corresponding nontumorous tissues from 13 patients diagnosed with esophageal squamous cell carcinoma were used after institutional review board-approved consents. All the patients were treated at First Affiliated Hospital of Nanjing Medical University during 2009–2011. The pieces of tumor tissue and nontumorous tissues were carefully collected and snap frozen in liquid nitrogen, followed by storage at −80 °C.

2.2. Reagents

Dulbecco's Modified Eagle Medium was bought from GIBCO (USA). The fetal bovine serum (FBS) was purchased from HyClone Laboratories (Inc., Logan, UT, USA). The anti-human AKT, phos-AKT, ERK, phos-ERK and GAPDH for western blot were purchased from Cell Signaling Technology (Inc., Danvers, MA, USA).

2.3. RNA extraction and q-PCR

For miRNA expression, total RNA was isolated with a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA). All primers for detecting miRNA expression were designed and synthesized by Genscript Co Ltd, Nanjing, China, using the mirVana™ qRT-PCR Primer Sets. U6 was used as an endogenous control to normalize the expression levels of each miRNA. The fold change of miRNA expression was determined by comparative CT method. The agomir or antagomir were purchased from Genepharma Co Ltd, Shanghai, China.

2.4. Western blot analysis

Cells (1×10^7) were lysed in M2 buffer (which containing 20 mM Tris-HCl (pH 7.6), 250 mM NaCl, 0.5%NP-40, 3 mM EDTA and 1.5 mM EGTA with 10 μg/ml Aprotinin, 10 μg/ml leupeptin, 1 mM DTT, 1 mM PNPP and 0.1 mM Na_3VO_4 as protease and phosphatase inhibitor). After centrifugation, cell lysates (100μg/lane) were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Roche, Germany). The membranes were blocked for 1 h in TBST (25 mM Tris-HCl, pH 7.6, 125 mM NaCl, 0.1% Tween-20) containing 5% nonfat dried milk, and then the membrane was incubated with antibodies against AKT or ERK diluted in TBST containing 5% nonfat dried milk at 4 °C overnight. HRP conjugated goat anti-rabbit or anti-mouse antibodies (Beyotime, 1:3000) were used as second antibodies. When detecting the phosphorylated forms of these kinases, membranes were blocked in TBST containing 5% BSA and the antibodies were also diluted in TBST containing 5% BSA.

2.5. Cell culture

TE-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA). TE-1 cells are used as one of the accepted models to study esophageal cancer [15].

2.6. The cell proliferation assay and apoptosis assay

Cell Counting Kit-8 was used for detecting the cell proliferation according to the manufacturer's instructions. The apoptosis was assayed by the Annexin-V-FITC & PI Apoptosis Kit (Biouniquer) according to the manufacturer's instructions. TE1 cells were washed twice in Annexin-V binding buffer and then cells were incubated for 30 min at 4 °C with antibodies according to the standard procedure. Fluorescence was measured by using a FACS-Calibur (Becton Dickinson, San Diego, CA) and data were analyzed by using the CellQuest Software (Becton Dickinson, San Diego, CA).

2.7. Statistical analyses

The results are expressed as the mean ± standard deviation(SD). P values (p) were determined using two-tailed Student's t-test. P values (p) were indicated in each figure.

3. Results

Since microRNAs play the important role in esophageal carcinoma, we compared the expression level of several microRNAs in 3 esophageal carcinoma and their non-tumorous tissues. The q-PCR assay was used to evaluate the expression levels of the microRNAs, and miR-502 was found significantly up-regulated in the esophageal carcinoma samples rather than the normal tissues compared to other microRNAs (Fig. 1A). Then we examined the miR-502 expression level in another 13 esophageal carcinoma and their non-tumorous tissues, q-PCR assay indicated that miR-502 was up-regulated in all these esophageal carcinoma (Fig. 1B).

In order to figure out the role of miR-502 in esophageal carcinoma, we used esophageal cancer cell line TE1 as an in vitro model for investigation. First, we overexpressed miR-502 in TE1 by introducing the miR-502 agomir into the cells and tested the proliferation and apoptosis. The up-regulated cell viability after the cck-8 treatment indicated that miR-502 agomir actually promoted the proliferation of the TE1 cells (Fig. 2A). The treatment of the miR-502 agomir would not change the apoptosis of TE1 cells in the normal culture condition, however, if we treated the cells with 1 μM dox, which would induce the apoptosis, the overexpressed miR-502 group exhibited dramatic decrease of the apoptosis (Fig. 2B).

We next investigated whether knocking down miR-502 would make the opposite phenomenon. So we introduced miR-502 antagomir into the cells to mimic the loss of function of miR-502. CCK-8 assay showed that down-regulating miR-502 would not affect the viability of the TE1 cells (Fig. 3A). The apoptosis of TE1 cells in the normal culture condition would not be changed in the absence of miR-502 either. Then we induced the apoptosis by 1 μM dox, the group of the cells which knocking down the miR-502 showed the increased percent of the apoptosis cells (Fig. 3B).

Then we asked how miR-502 could promote the proliferation and inhibit the apoptosis in TE1 cells. Since aberrant AKT and extracellular signal-regulated kinase (ERK) activation is often observed in various human cancers, this kind of abnormal also exist in TE1 cell lines. So we detected the level of phosphor-AKT and

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