Biomedicine & Pharmacotherapy 74 (2015) 89–94

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Original Article

FOXQ1 promotes esophageal cancer proliferation and metastasis by negatively modulating CDH1

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ARTICLE INFO

Article history:
Received 28 May 2015
Accepted 9 July 2015

Keywords:
FOXQ1
CDH1
ESCC
Tumor metastasis

ABSTRACT

Background: Esophageal squamous cell carcinoma (ESCC) is an aggressive tumor with rapid progression, high recurrence and metastasis rate. Besides, the 5-year survival rate of ESCC remains dismal despite improvements in treatments having developed a lot. That is because the cellular basis of ESCC has not yet been fully understood. Forkhead box family membranes possess various kinds of properties as they mediate apoptosis, cell cycle arrest, autophagy, senescence and so on. Foxhead box Q1 (FOXQ1), which has a major impact on several kinds of tumor forming and metastasis, while whether it triggers ESCC remains largely obscure.

Methods: To determine whether aberrant FOXQ1 expression in esophageal cancer, protein level and mRNA level of specimens of cancerous tissues and adjacent non-cancerous tissues were determined by western blot and real-time PCR. Then we overexpressed or knockdowned FOXQ1 in EC9706 cell; cell growth curve and colony formation were analyzed. Cell invasion ability was analyzed by migration chambers. Reporter gene assay was used to study the transcriptional regulation activity.

Results: FOXQ1 was highly expressed in esophageal cancerous tissues compared with adjacent non-cancerous tissues. FOXQ1 overexpression promotes ESCC tumor cell proliferation, whereas FOXQ1 silencing prevents ESCC tumor cell proliferation. FOXQ1 promotes ESCC metastasis via negatively modulation CDH1. CDH1 silencing could rescue the migratory ability which was blemished by FOXQ1 silencing. FOXQ1 could act as transcriptional repressor which binds to the promoter of CDH1 and blocks its transcription.

Conclusions: In this study, we identified FOXQ1 as an oncogene to promote ESCC tumor cell proliferation and metastasis by negatively regulating CDH1 in EC9706 cell. Besides, we deciphered a previously unidentified mechanism of ESCC progression and metastasis.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant cancers, ranking as the sixth most common cancer worldwide [1]. Although esophageal adenocarcinoma (EAC) has become the predominant histological subtype in some western countries, ESCC remains dominant in China. Due to difficulties in early diagnosis and poor efficacy of treatment of ESCC, the 5-year survival rate remains dismal [2], despite improvements having been made in treatments such as surgical resection and adjuvant chemoradiation. Epidemiological studies have suggested tobacco, alcohol, nitrosamines, physical injury and chronic inflammation as major risk factors in ESCC [3–6]. Previous studies manifested that tumor suppressor p53 and Rb frequently altered, including mutation of *p*53, loss of heterozygosity of *R*b [4,7]. The $p14^{ARF}$ and $p15^{INK4b}$ genes were also inactivated by homozygous deletion and hypermethylation [7]. Hypermathylation and inactivation of HLA class I gene, hMLH1, retinoic acid receptor beta (RARβ) and other genes were observed in biopsy esophageal samples [8,9]. Some miRNAs have already been demonstrated in oesophageal cancer. Some miRNAs including miR-21 and miR-92a can act as oncogenes, while others such as let-7 and miR-34a may play roles of tumor suppressors [10–14]. Overall, though great progresses have been made in investigating the molecular mechanisms of ESCC, there is still a long way to go, considering clinical treatment effect.

Forkhead family protein is a transcription factor which plays an important role in embryonic development, carbohydrate and lipid

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http://dx.doi.org/10.1016/j.biopha.2015.07.010
0753-3322/

metabolism, cell cycle regulation, aging, immune regulation and other biological processes [15–17]. Initial studies indicated that FOXQ1 took part in hair follicle development in mammals [18], hereafter FOXQ1 overexpression was found in colorectal cancer, gastric cancer, bladder cancer, liver cancer, breast cancer, non-small cell lung cancer, and ovarian cancer [19–21]. FOXQ1 can activate transcription ZEB2 and VersicanV1, thus promoting metastasis of hepatocellular carcinoma [22]. Besides, as a transcription factor FOXQ1 could combine with the p21 promoter, thus facilitating its expression, resisting apoptosis [23]. However, currently whether FOXQ1 could regulate ESCC remains obscure.

In this present study, we show that FOXQ1 overexpresses in ESCC cancerous specimens on comparing with adjacent non-cancerous tissues and overexpression of FOXQ1 in ESCC cell line EC9706 can promote cell growth and colonies formation. FOXQ1 could also promote EC9706 cell metastasis by negatively regulating CDH1, and concomitantly silence FOXQ1 and CDH1 that could attenuate the metastasis ability interfered by FOXQ1 silence. FOXQ1 modulates CDH1 mainly through transcriptional regulation authenticated by reporter gene assays. Our findings uncover a previously unidentified mechanism of ESCC and may provide a new target for ESCC treatment.

2. Materials and methods

2.1. Specimens

Tissue specimens from the human esophageal cancer tumor were collected together with adjacent non-cancerous tissues in each case and stored in liquid nitrogen pending experimental use. All selected patients were biopsied to confirm the diagnosis of human esophageal cancer by histopathologic evaluation. The study protocol was approved by the Ethical Committee of The First Affiliated Hospital of Zhengzhou University. Informed consents were obtained from all patients.

2.2. Plasmids, antibodies, and reagents

Full-length Forkhead box Q1 (FOXQ1) was cloned from the cDNA of normal human fibroblast using a PCR-based approach and was placed into pLPC-puro vector. The shRNA was designed according to the pMSCV instruction manual (Clontech). The double DNA strand were synthesized by GENEray Biotechnology of Shanghai Corporation and then inserted into the pMCV-puro-miR30 vector. The following gene-specific sequence was used successfully: FOXQ1, 5′-AGATCAACGAGTACCTCAT-3′.

The primary antibodies used for Western blot analysis were as follows: anti-FOXQ1 and anti-CDH1 were from Santa Cruz Biotechnology. Anti-GAPDH was purchased from Tianjin Sungene Biotech.

Boyden chamber was obtained from Haimen City Qilin Medical Instrument Factory.

2.3. Cell lines, cell culture and viral infections

Human esophageal cancer EC9706 cells were purchased from National Institute of Biological Products, Beijing, China. HEK293 cells were from our laboratory. All cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Cultures at 80% confluence were detached with 0.25% trypsin after washing with a PBS solution and then split at a ratio of 1:2. The infections were performed as described [24].

2.4. Real-time PCR

RNA was isolated from EC9706 cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand DNA was synthesized using the StarScript first-strand cDNA synthesis kit (TaKaRa). Real-time PCR was performed using SYBR Green PCR Master Mix (Invitrogen) on an ABI Prism 7300 Sequence Detector (Applied Biosystems). Each test was done in triplicate and the experiment was repeated three times. Gene-specific primers were as follows:

FOXQ1 forward: 5′-CGCGGACTTTGCACTTTGAA-3′,
FOXQ1 reverse: 5′-AGCTTTAAGGCACGTTTGATGGAG-3′;
CDH1 forward: 5′-AGCTACCCCAGGACACCCAA-3′,
CDH1 reverse: 5′-GCAACGCAATCAGAGTCAACG-3′;
β-Actin forward: 5′-GTGGACATCCGCAAAGAC-3′,
β-Actin reverse: 5′-AAAGGGTGTAACGCAACTAA-3′.

2.5. Immunoblot analysis

Cells were scraped from the plate and lysed on ice for 30 min with 300 ul of radioimmune precipitation assay buffer containing a protease inhibitor mixture. Cells lysates were then centrifuged for 15 min at 15,000 × *g* at 4 °C, and the insoluble debris was discarded. Protein concentration of each sample was determined by BCA Protein Assay Reagent (Pierce). Cell lysate (20–40 ug) was subjected to SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose membranes (Millipore). After blocking in 5% nonfat dry milk in TBST (10 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), the membranes were incubated with primary antibodies overnight at 4 °C. The membranes were then washed four times with TBST and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized using chemiluminescent substrate (Millipore).

2.6. Growth curves and colony formation

Cell proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were seeded into 96-well plate at a density of 2×10^3 cells per well and cultured for periods ranging from 1 to 7 d. The medium was changed every 24 h. At the indicated times, an aliquot of cells were stained with 25 ul of MTT solution (5 mg/ml in 1× PBS; Sigma) for 4 h and then dissolved with DMSO. The optical density at 570 nm was determined.

To determine the colony formation, 1×10^4 indicated cells were cultured in six-well plate. Several days later, cells were fixed in 3% (wt/vol) formaldehyde at 37 °C for 30 min and washed twice with 1× PBS, then stained with crystal violet for 1 h and washed with 1× PBS twice.

2.7. Cell invasion assay

The stable expressed EC9706 cells were harvested and resuspended in media at 5×10^5 cells/ml. An aliquot of 25 ul cell suspension was loaded in the upper wells, and the media containing 20% FBS was placed in the lower wells as a chemoattractant stimulus. After 72 h at 37 °C, 5% CO2 incubation, cells that had migrated to the bottom surface of the filter were fixed with 70% methanol, stained with hematoxylin and counted under a microscope in ten randomly selected fields at a magnification of 400×.

2.8. Reporter gene assays

The CDH1 promoter region (from −450 to +193 bp) has been cloned into pGL3-basic promoter vector (Promega). HEK293 cells were plated in 12-well plates at a density of 1×10^5 cells/well and transfected with CDH1 promoter plasmid plus PRL-null plasmid