Gene 493 (2012) 44–51

Contents lists available at SciVerse ScienceDirect

Gene

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

MicroRNA-421 functions as an oncogenic miRNA in biliary tract cancer through down-regulating farnesoid X receptor expression

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ARTICLE INFO

Article history:
Accepted 15 November 2011
Available online 25 November 2011

Received by Astrid M. Engel

Keywords:
miR-421
Biliary malignancy
Tumor suppressor gene
Gallbladder cancer
Cholangiocarcinoma
Cell cycle arrest

ABSTRACT

MicroRNAs (miRNAs) are involved in the development of most cancers. However, few studies have been conducted to determine their relationship to biliary tract cancer (BTC). Farnesoid X receptor (FXR) has been reported to be a tumor suppressor for hepatocellular carcinoma and breast cancer; but few studies have focused on its correlation with BTC. In this study, we identified miR-421 as a potential regulator of FXR expression. We found that their expression amount was inversely correlated as FXR was aberrantly down-regulated in both primary tumor specimens and cell lines; while miR-421 was significantly up-regulated. Ectopic expression of miR-421 significantly decreased FXR protein concentration in BTC cells and promoted cell proliferation, colony formation and migration *in vitro*. Furthermore, a decrease in miR-421 expression induced G_0/G_1 cell cycle arrest. In conclusion, our study identified microRNA-421 functions as an oncomiR in BTC by targeting FXR. This finding may provide a novel therapeutic strategy for treatment of biliary tract cancer.

1. Introduction

Biliary tract cancer (BTC), arising from the ductal epithelium of biliary tree, is the second most common primary hepatobiliary malignancy (de Groen et al., 1999), and has a rising incidence rate and a dismal prognosis (de Groen et al., 1999; Shaib and El-Serag, 2004; Khan et al., 2005). Traditionally, BTC is divided into cholangiocarcinoma (CCA) and gallbladder cancer (GBC), which have similar pathogenesis and clinical characteristics (de Groen et al., 1999). Furthermore, CCA can be subdivided according to the site of tumor origin into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) (Nakeeb et al., 1996; de Groen et al., 1999). ECC can be subdivided into perihilar cholangiocarcinoma (PCC) and distal extrahepatic cholangiocarcinoma (DECC) (de Groen et al., 1999). The most effective treatment for BTC continues to be surgical resection (Blechacz and Gores, 2008). However, most patients are not candidates for the procedure because they are diagnosed at advanced stages (Malhi and Gores, 2006). Therefore, finding effective biomarkers for early diagnosis and clarifying the molecular mechanisms associated with pathogenesis are required to improve prognosis (Jarnagin and Shoup, 2004; Blechacz and Gores, 2008).

The farnesoid X receptor (FXR or NR1H4), a member of the nuclear receptor superfamily, plays crucial roles in bile acid, cholesterol, lipid and glucose metabolism (Wang et al., 2008). Recently, FXR function was found to extend beyond metabolism, and included inflammation and carcinogenesis (Wang et al., 2008). FXR dysfunction has been identified as an important determinant of carcinogenesis for breast cancer, colon cancer and hepatocellular carcinoma (De Gottardi et al., 2004; Swales et al., 2006; Yang et al., 2007). Although FXR was reported as a down-regulated gene in specimens from ICC patients (Obama et al., 2005), the correlation between FXR and BTC is not clear. Given that aberrant bile acid secretion has been linked to BTC (Werneburg et al., 2003; Blechacz and Gores, 2008), it is interesting to investigate if FXR plays a role in biliary tract tumorigenesis.

MicroRNAs (miRNA) are key negative regulators of gene expression in eukaryotes (Lee and Ambros, 2001) and abundant evidences indicated that miRNAs are involved in human cancers (Stefani and Slack, 2008). MiR-421 is located on X chromosome, and was first implicated in gastric cancer oncogenesis (Guo et al., 2009; Chureau et al., 2011). Moreover, high expression amounts of miR-421 have been found in various tumors, including neuroblastoma, pancreatic cancer and prostate cancer (Hu et al., 2010; Hao et al., 2011; Ostling et al., 2011). These evidences demonstrated that miR-421 functions as an oncogenic miRNA (oncomiR) in human cancers (Jiang et al., 2010). OncomiRs function as an oncogene by repressing the expression of

Abbreviations: miR-421, microRNA-421; FXR, farnesoid X receptor; BTC, biliary tract cancer; CCA, cholangiocarcinoma; GBC, gallbladder cancer; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; PCC, perihilar cholangiocarcinoma; DECC, distal extrahepatic cholangiocarcinoma; BSEP, bile salt export pump; PCNA, proliferating cell nuclear antigen; UTR, untranslated regions.

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doi:10.1016/j.gene.2011.11.028

tumor suppressor genes (Esquela-Kerscher and Slack, 2006). In a previous research, ataxia-telangiectasia mutated (ATM) protein and DPC4/Smad4 were reported to be target genes of miR-421 in pancreatic tumors (Hu et al., 2010; Hao et al., 2011). Given that miRNAs usually regulate a large set of targets, more validated target genes of miR-421 have not been implicated in miR-421-mediated oncogenesis, we speculated that there may be more miR-421 targets that are involved in oncogenesis, including FXR.

In this study, we found that FXR was expressed significantly lower in BTC than normal tissues. Then, we predicted miR-421 as the targeting miRNA to explain the mechanism for FXR down-regulation. In order to support our proposals, we systematically examined the expression of miR-421 in both human BTC tissues and BTC cell lines, and the correlation between miR-421 and FXR was evaluated. Furthermore, the impact of miR-421 on BTC cells biology was evaluated by either over-expression or down-expression. Collectively, our results suggest that miR-421 functions as an oncomiR in BTC by targeting FXR, may be a potential target for BTC therapy.

2. Materials and methods

2.1. Clinical tissue samples

Tissue samples were obtained from 64 patients at the Second Affiliated Hospital of Harbin Medical University from January 2007 to March 2011. Informed consent was obtained from patients and the tissue acquisition protocol was approved by the Harbin Medical University Institutional Review Board. Among the samples, 47 were from tumor tissues (including ICC, PCC, DECC, GBC, metastasis), and 17 samples were from normal bile ducts or gallbladder. The normal bile ducts were obtained from patients undergoing pancreatoduodenectomy for treatment of pancreatic or duodenal while their bile ducts remained diseases-free. Tumor samples were obtained from CCA or GBC patients undergoing cancer-related surgery. The clinical characteristics of the patients were collected and included tumor location, histological type, differentiation grade, lymph node invasion, TNM staging, or 1-year survival. The fresh tissues were frozen in liquid nitrogen and used for RNA and protein extraction.

2.2. Immunohistochemistry staining

FXR and BSEP (ABCB11) expression were detected immunohistochemically using paraffin-embedded specimens from patients with ICC. Following deparaffinization and rehydration of the sections, endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The sections were incubated with primary antibody (anti-FXR, anti-BSEP, Santa Cruz, CA, USA) overnight at 4 °C, followed by incubation with appropriate HRP-conjugated secondary antibody for 1.5 h. After a thorough washing, the sections were developed in 3,3′-diaminobenzidine and counterstained with hematoxylin. Sections stained without primary antibodies were used as negative controls. Each stained sample was observed under high power magnification (200×).

2.3. Cell culture

HCCC-9180, SSP25, RBE cholangiocarcinoma cell lines, GBC-SD gallbladder cancer line and human embryonic kidney 293 T cell line (HEK293T) were obtained from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). HCCC-9180, SSP25 and RBE cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. GBC-SD were maintained in RPMI 1640 medium supplemented with 20% FBS and antibiotics. HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. Human intrahepatic biliary epithelial cells (BEC) and epithelial cell medium were purchased from ScienCell Research Laboratories (San Diego, CA, USA). BEC were cultured in complete medium containing 10% FBS and antibiotics. In this study, BEC were employed as the control cells to represent normal biliary epithelial cells.

2.4. Luciferase reporter assays

The 3′-untranslated region (3′-UTR) of FXR was amplified using PCR with human genomic DNA as template. The primers used were: 5′-CGACGCGTGGCAAGCCCTGTTT-3′ and 5′-CCCAAGCTTAAGATGAGCAAAATGAGATTTTCCC-3′. The PCR product, which contained a possible binding site for miR-421, was digested with MluI and HindIII and was inserted downstream of the luciferase gene in the pMIR-REPORT system (Applied Biosystem, Foster City, CA, USA). The constructs were verified by DNA sequencing. The correct construct was named as pMIR-FXR-3′UTR. The mutant reporter plasmids were constructed in accordance with the afore-mentioned criteria by annealing the following oligonucleotides: 5′-CGACGCGTGGCAAGCCCTGTTTGCCTAATTAAATTGATTGTTACTTCAATTCTATgTcTTcAACTAGGGAAAATCTCATTTTGCTCATCTTAAGCTTGGG-3′ and 5′-CCCAAGCTTAAGATGAGCAAAATGAGATTTTCCCTAGTTgAAgAcATAGAATTGAAGTAACAATCAATTTAATTAGGCAAACAGGGCTTGCCACGCGTCG-3′ (the bases in miniscule were the site mutations). The mutant reporter plasmids were named as mut-pMIR-FXR-3′UTR.

For luciferase assays, HEK293T cells were cultured in 6-well plates and transfected with plasmids (including pMIR-FXR-3′UTR and β-galactosidase reporter plasmid) and RNA oligonucleotide (miR-421 mimics or control RNA, RiboBio, Guangzhou, China) using Calcium Phosphate Cell Transfection Kit (Beyotime, Nanjing, China). A β-galactosidase reporter plasmid was designed for transfection normalization. Forty-eight hours after transfection, the cells were lysed, the luciferase activity was determined using Luciferase Assay System (Promega, Fitchburg, WI, USA), and β-galactosidase activity was measured with β-galactosidase Enzyme Assay System (Promega, Fitchburg, WI, USA).

2.5. RNA extraction and quantitative real-time PCR

Total RNA samples were isolated from primary tissues or from cell lines using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed to cDNA using PrimeScript Regent Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR green kit (Takara, Tokyo, Japan) and the ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Specific primers were designed for FXR, BSEP and β-actin (Table 1). Human β-actin was employed as the endogenous control. Specific primers for miR-421 and U6 RNA were obtained from RiboBio (RiboBio, Guangzhou, China). U6 RNA was used as a control for microRNA quantification. The procedures for PCR were performed according to the manufacturer's instructions. All assays were performed at least in triplicate. The relative expression levels were then determined using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

Table 1
Primer sequences.

Gene symbol	Primer sequences	
	Forward	Reverse
Actb	5′-CAGCACAATGAAGATCAAGATC-3′	5′-GTGTAACGCAACTAAGTCATAG-3′
NR1H4	5′-CTCCTCACCTCATTGTCTC-3′	5′-CTTCTACGATGTCTTCTACCT-3′
ABCB11	5′-TTACAAGAACTCCAGATTCC-3′	5′-TGATAAGTACTGCGACAGC-3′