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Fibroblast growth factor receptor 4 promotes progression and correlates to poor prognosis in cholangiocarcinoma

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ABSTRACT

Fibroblast growth factor receptor 4 (FGFR4) is related to poor prognosis of several cancers, but the correlation between FGFR4 expression and cholangiocarcinoma (CCA) has not been well elucidated. We investigated the expression of FGFR4 in 83 intrahepatic cholangiocarcinomas (IHCCs), 75 perihilar cholangiocarcinomas (PHCCs) and 41 distal cholangiocarcinomas (DCCs) by immunohistochemistry (IHC), and subsequently evaluated association of FGFR4 with clinicopathologic parameters and survival rate. The rate of FGFR4 higher expression was 61.4% (51/83) in IHCCs, 53.3% (40/75) in PHCCs and 56.1% (23/41) in DCCs. FGFR4 expression was significantly related to poor prognosis of IHCC ($P = 0.002$) and PHCC ($P = 0.019$) with univariate analysis, and also identified as an independent prognostic factor in IHCC ($P = 0.045$) and PHCC ($P = 0.049$) with multivariate analysis. Additionally, with functional assays in vitro, we found FGFR4 can induce proliferation, invasion and epithelial–mesenchymal transition (EMT) of CCA cell lines with FGF19 stimulation. Moreover, FGFR4 inhibitor AP24354 can suppress proliferation, invasion and induce apoptosis of CCA cells. In conclusion, FGFR4 expression can be identified as a significant independent prognostic biomarker of IHCC and PHCC. FGFR4 played a pivotal role in proliferation, invasion and EMT of CCA. FGFR4 inhibitor can suppress proliferation, invasion and induce apoptosis of CCA, indicating that FGFR4 may act as a potential therapeutic target.

1. Introduction

Cholangiocarcinoma is a relatively rare tumor but the most common malignancy of the biliary tract [1]. The incidence and mortality of CCAs are increasing worldwide [2]. According to the 7th edition of AJCC/UICC TNM staging classification [3], CCAs are classified to intrahepatic, perihilar and distal cholangiocarcinomas, which are identified to have different biological and neoplastic features [4]. PHCC is the most common type of cholangiocarcinoma, followed by the DCC and then IHCC [5]. Incidence of CCAs is low with approximately 9760 new cases diagnosed annually in the United States [6]. However, the mortality of CCAs is very high. Moreover, the radical resection and liver transplantation are the only known curative ways but more than 65% patients lose surgical indications when diagnosed because of early lymph node and distant metastases. In addition, the recurrence of CCAs is high and few data support that adjuvant approach can benefit survival outcomes [7,8]. Consequently, the prognosis of cholangiocarcinoma is very poor, with 5-year survival rate ranging from 5% to 15% [9,10]. In summary, CCAs are difficult to be treated successfully and have a poor prognosis as a result of difficulty of early diagnosis, early metastasis and easy recurrence [11]. Hence, there is an urgent need for the predictive, prognostic and therapeutic markers. Unfortunately, few studies on CCAs aimed at identifying prognostic factors have been reported and the researches on new biomarkers of CCAs make progress slowly because that the majority of patients present are usually at an unresectable stage which makes it difficult to obtain samples and perform a large randomized trial.

Abbreviations: FGFR4, fibroblast growth factor receptor 4; IHCC, intrahepatic cholangicolangcinoma; PHCC, perihilar cholangiocarcinoma; EMT, epithelial–mesenchymal transition; CCA, cholangiocarcinoma.

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Fibroblast growth factors (FGFs) are heparin-binding proteins involved in many cellular processes like proliferation, differentiation and angiogenesis. They have a large family consisting of 18 members in human, which are essential for signal transduction through interacting with cell-surface-associated heparan sulfate proteoglycans [12]. FGF signaling is induced by FGFs and transduced through FGF receptors. Fibroblast growth factor receptors (FGFRs) comprise of five members, including four tyrosine kinase receptors (FGFR1, FGFR2, FGFR3 and FGFR4) and a non-tyrosine kinase receptor (FGFR5, also known as FGFRL1) [13]. FGFRs overexpression has been reported to correlate with the progression and poor prognosis in a wide variety of tumors including prostate, breast, bladder, and gastric cancer [14]. Moreover, different types of FGFRs are overexpressed depending on the tumor types [15]. Among all the FGFRs, FGFR4 upregulation was observed in hepatocellular carcinoma, prostate, breast, pancreatic, gynecologic gastric cancers and rhabdomyosarcomas [16–18]. FGFR4 is highly expressed in hepatocytes and can regulate metabolism of bile acid and glucose [19]. In addition, FGF19, the specific FGFR4 ligand, is demonstrated to induce hepatocytes proliferation and hepatocellular carcinoma progression via stimulating FGFR4 [20,21].

To identify the role of FGFR4 in CCAs, we detected the expression of FGFR4 and VEGF in 83 samples of IHCC, 75 samples of PHCC and 41 samples of DCC by immunohistochemistry, subsequently evaluated the association between FGFR4 expression and clinicopathologic parameters with Chi-Square test. Furthermore, we investigated the correlation between FGFR4 and survival rate with univariate and multivariate analysis. To confirm the observation acquired from clinical investigation, FGFR4 function assays in CCA cells were evaluated subsequently, including proliferation, invasion and EMT. Moreover, significance of FGFR4 inhibitor AP24354 on proliferation, invasion and apoptosis of CCA cells were also detected. As a result, we inferred that FGFR4 expression correlated to poor prognosis through inducing cancer progression and could be a potential therapeutic drug target in CCAs.

2. Materials and methods

2.1. Patients and follow-up

All the 83 patients with IHCC, 75 patients with PHCC and 41 patients with DCC underwent tumor resection during the period from 2002 to 2010. The diagnosis was confirmed by the routine pathology. All samples were obtained from the Department of Pathology of Qilu Hospital and Central Hospital of Yishui, Shandong Province, with prior patient consents and the approval of the Institutional Clinical Ethics Review Board. Clinical data, including age, gender and other clinicopahtologic features were abstracted from the patients' medical records. Pathologic tumor-node-metastasis (pTNM) staging is based on the 7th staging classification of AJCC/UICC (2009).

In our study, 252 patients were diagnosed to have cholangiocarcinoma by routine pathology and 199 patients (83 patients with IHCC, 75 patients with PHCC and 41 patients with DCC) were enrolled in validation cohort following the criteria: (i) available formalin-fixed tumor tissues, (ii) available clinical follow-up data and complete medical records and (iii) no history of previous anticancer therapy and other malignancies. The information of primary and validation cohorts is provided in Supplemental Table S1.

2.2. Tissue microarrays

The tissue microarrays (TMA) were made using buffered formalin-fixed and paraffin-embedded tissue sections from all the 199 patients (83 IHCCs, 75 PHCCs and 41 DCCs). Before immunohistochemistry detection, hematoxylin and eosin staining were performed to confirm the histological characterization of all samples. Two 1 mm cylinders were used for representing each sample in the TMA slide (164 cores per slide) [22].

2.3. Immunohistochemistry and evaluation

The protocol of IHC staining was described before [23]. Detail for IHC can be seen in Supplemental Materials and methods. Immunohistochemical staining was evaluated independently by two senior pathologists unaware of the clinical information. Both the intensity of the color reaction and the percentage of stained cells were considered when evaluated. Positive (normal bile duct control) and negative controls (absence of primary or secondary antibody) were applied for quality control. Both FGFR4 and VEGF staining intensity were scored as negative (0), weak (1), moderate (2) and strong (3). Criteria percentage of FGFR4 positive tumor cells were defined as follows: (1) <10% of cells were positive, (2) 10–50% of cells were positive and (3) >50% of cells were positive. Both scores were multiplied and the mean resulting score was used to define the lower and higher expressions. The cut-off scores of FGFR4 in IHCCs, PHCCs and DCCs were 3.69, 3.71 and 3.34 respectively. Cut-off of VEGF in IHCCs, PHCCs and DCCs were 5.85, 5.51 and 5.49 respectively.

2.4. Cell culture and reagents

The IHCC cell line RBE, PHCC cell line QBC939 and FRH0201, HepG2 and HEK-293 cells were all purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). IHCC cell line HUCCT-1 was obtained from RIKEN Bioresourse Center (Koyadai, Japan). All CCA cells were cultured in the RPMI-1640 medium, while HepG2 and HEK-293 were cultured in DMEM medium, supplemented with 10% fetal bovine serum (Gibco, USA) and 1% ampicillin/streptomycin (HyClone, USA) in 5% CO_2 resuscitation. FGF19 was purchased from PeproTech Company. Inhibitor AP24534 was purchased from Selleck Chemicals. All other reagents were purchased from Sigma Company.

The following antibodies were used: anti-FGFR4, anti-VEGF, and anti-actin (Santa Cruz Biotechnology, USA), EMT antibody sampler kit (Cell Signaling Technology, USA) and anti-FLAG (Sigma, USA).

2.5. FGFR4 knockdown and transfection

Human FGFR4 (transcript variant 1) open-reading-frame plasmid was purchased from Sino Biological Incorporation (Beijing, China), then subcloned into the pFLAG-CMV-2 vector by double-enzyme digestion. FGFR4 knockdown is accomplished by small interfering RNA (siRNA) purchased from the Invitrogen Company. The siRNA sequences were designed as reported by Ye et al previously [21], with sense sequence 5′-GCCGACACAAGAACAUCAUTT-3′, and antisense sequence 5′-AUGAUGUUCUUGUGUCGGCTT-3′. As to the scramble oligo RNA, the sense sequence was 5′-UUCUCCGAACGUGUCACGUTT-3′, and the anti-sense sequence was 5′-ACGU GACACGUUCGGAGAATT-3′. Transfection of siRNA or vector was accomplished by Lipofectamine 2000 (Invitrogen, USA) according to the reverse transfection manual. Results of knockdown and overexpression were detected by Western blotting 48 h after transfection.

2.6. Matrigel invasion assay

Detailed in Supplementary material.