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The clinical significance and biological function of interferon regulatory factor 1 in cholangiocarcinoma

Peiqi Wan[a,b], Junhong Zhang[a,c], Qiang Du[a], David A. Geller[a,*]

[a] Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213, USA
[b] Department of infectious diseases, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, PR China
[c] Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, PR China

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ABSTRACT

Interferon regulatory factor 1 (IRF1) has been suggested to act as a tumor suppressor in human cancers. However, the clinical significance and biological function of IRF1 in cholangiocarcinoma is poorly understood. In our results, IRF1 mRNA and protein expressions were decreased in cholangiocarcinoma tissues and cell lines compared with paired normal hepatic tissues and intrahepatic bile duct epithelial cell line. IRF1 protein low-expression was associated with tumor stage, tumor size, vascular invasion and metastasis and served as a poor independent prognostic parameter in cholangiocarcinoma patients. Up-regulation of IRF1 expression suppressed cholangiocarcinoma cells proliferation, migration and invasion, and blocked cell cycle progression, but has no effect on apoptosis. In conclusion, IRF1 is low-expressed in cholangiocarcinoma tissues and cell lines, and correlated with malignant status and prognosis in cholangiocarcinoma patients. IRF1 served as tumor suppressor in the regulation of cholangiocarcinoma cells proliferation, cell cycle, migration and invasion.

1. Introduction

Cholangiocarcinoma originates from the epithelium of bile duct and is the second most common hepatobiliary cancer after hepatocellular carcinoma [1]. Cholangiocarcinoma is relatively uncommon in Western countries, but has high incidence rates in Eastern Asia [2]. Unfortunately, the incidence and mortality rate of cholangiocarcinoma had a rising incidence worldwide in the past four decades [3,4]. Although recent advances in surgical techniques, chemotherapy and radiotherapy, the prognosis of cholangiocarcinoma patients remains poor and the 5-year survival rate is only 5%–20% [5–7]. Due to limited therapeutic options and poor prognosis, it is urgent to screen and identify biomarkers for predicting clinical outcome and developing therapeutic target in cholangiocarcinoma patients.

Interferon regulatory factor 1 (IRF1) is a member of interferon regulatory factor family, and the first transcription factor identified in the interferon (IFN) system [8]. IRF1 has been suggested to act as an important role in hepatocellular carcinoma. In hepatocellular carcinoma patients, IRF1 expression was decreased in tumor tissues, and IRF1 low-expression was associated with early recurrence and shorter overall survival [9,10]. Moreover, IRF1 was involved in suppressing hepatocellular carcinoma cells growth and invasion, and inducing autophagy [10–12]. However, the clinical significance and biological function of IRF1 in cholangiocarcinoma is still unknown. Based on published studies about IRF1 in hepatocellular carcinoma, we suppose that IRF1 serves as tumor-suppressor in the regulation of cholangiocarcinoma cells proliferation, migration and invasion, and as prognostic factor in predicting cholangiocarcinoma clinical outcome.

In order to verify our guess, the clinical and prognostic significance of IRF1 is analyzed in cholangiocarcinoma patients, and the biological function of IRF1 on proliferation, migration, invasion, cell cycle and apoptosis are explored in cholangiocarcinoma cell lines.

2. Materials and methods

2.1. Clinical samples

A total of ninety-six intrahepatic cholangiocarcinoma tissues and twenty adjacent normal hepatic tissues were gained from American Tissue and Tumor Banks. Clinical samples were respectively stored in liquid nitrogen for qRT-PCR and formaldehyde solution for immunohistochemistry. Clinical staging and system treatment were based on the 7th edition of AJCC Cancer Staging Manual and NCCN guideline, respectively. None of patients in this study had received neoadjuvant anti-tumor treatment. An informed consent was obtained from all the participants before enrollment in the study. The entire study was

* Corresponding authors at: Department of Surgery, University of Pittsburgh, 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, USA.
E-mail addresses: wan_peiqi@163.com (P. Wan), gellerda@upmc.edu (D.A. Geller).

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performed based on the Declaration of Helsinki.

2.2. Quantitative real-time PCR

Total RNA was extracted by using RNAiso Plus (Takara), according to the manufacturer's instruction. A total of 500 ng RNA was converted into cDNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara). Real-time PCR was performed using SYBR Green (TaKaRa) and Light Cycler Roche 480 PCR instrument. The primers are as follows: IRF1, forward primer: 5′- GCTTTGTATCGGCCTGTGTG-3′; reverse primer: 5′-ACCCTGGCTAGAGATGCAGA-3′. GAPDH, forward primer: 5′-GGGAAGCTTGTCATCAATGG-3′; reverse primer: 5′-CATCGCCCCACTTGATTTTG-3′. GAPDH was used as an internal control. The relative expression was analyzed by the $2^{-\Delta Ct}$ method.

2.3. Western blot

Total protein was extracted using cell lysis buffer (Beyotime) for Western blot. Equal amounts of protein were denatured and then separated by 10% SDS-PAGE. The target proteins were incubated with the following primary antibodies: IRF1 (Abcam) or GAPDH Rabbit anti-human antibody (CWBIO). Then the proteins were incubated with homologous secondary antibodies (CWBIO). For HRP detection, an ECL chemiluminescence kit (CWBIO) was used. Intensity of blots was performed by Quantity One Software (Bio-Rad).

2.4. Immunohistochemistry

Immunohistochemical analysis was performed to measure IRF1 protein expression in cholangiocarcinoma tissue samples. In brief, slides were baked at 60 °C for 1 h, followed by deparaffinized in 100% xylene and re-hydrated in descending ethanol series (100%,95%, 90%,80%, 70% ethanol). The sections were submerged in EDTA antigenic retrieval buffer and microwaved for antigen retrieval. They were then treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 5% bovine serum albumin to block nonspecific binding. Sections were incubated with Rabbit anti-human IRF1 antibody (1:100 dilution by antibody diluent, Abcam) overnight at 4 °C. After washing, tissue sections were treated with biotin-labeled goat anti- rabbit antibody for 10 min at room temperature, followed by incubation with conjugated horseradish peroxidase streptavidin. The peroxidase reaction was developed using 3, 3-diaminobenzidine chromogen solution in DAB buffer substrate. Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum. Finally, sections were viewed under a bright-field microscope.

2.5. Evaluation system

The tissue sections stained immunohistochemically for IRF1 were reviewed, and scored separately by two pathologists blinded to the clinical parameters. Any disagreements were arbitrated by the third pathologists. For IRF1 assessment [13], staining intensity was scored as 0, negative; 1, weak; 2, moderate; or 3, strong, and staining extent was scored as 0, 0%-10%; 1, 10%-50%; 2, 50%-75%; 3, greater than 75% positive cells. The final score was calculated by multiplication of these two variables. Low expression of IRF1 was defined as 0 to 4 scores; high expression of IRF1 was defined as more than 4 scores.

2.6. Cell lines

Two human cholangiocarcinoma cell lines (HuCCT1 and CCLP) were provided by Prof. Anthony J Demetris of University of Pittsburgh Medical Center, Pittsburgh, PA, USA. Two human cholangiocarcinoma cell lines (RBE and QBC939) and the human intrahepatic bile duct epithelial cells line (HIBEpic) was obtained from American Type Culture Collection (ATCC). HuCCT1, RBE, QBC939 and HIBEpic were cultured in RPMI 1640 (Gibico). CCLP was cultured in DMEM (Gibico). The media for the cell lines were supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.7. Cell transfection

The coding sequence region of human IRF1 gene was amplified from cDNA and cloned into pcDNA3.1 express vector, which was used to up-regulate IRF1 expression in vitro. The construction was confirmed by DNA sequencing. Cells were transfected using lipofectamineTM 3000 reagent (Invitrogen) in Opti-MEM (Gibco), according to the manufacturer's instructions.

2.8. MTT assay

Cholangiocarcinoma cells were plated in a 96-well plate. After incubated at 37 °C for 24 h, 72 h, and 120 h, MTT (10 μl, 5 mg/ml, Sigma) was added, and then incubated at 37 °C for 4 h. After that, the supernatant was removed, and 100 μl DMSO (Sigma) was added. The absorbance value was detected at 490 nm.

2.9. Colony formation assay

Cholangiocarcinoma cells were plated in six-well culture plates at 100cells/well. Each cell group had three wells. After incubation for ten days at 37 °C, cells were washed twice with PBS and stained with the Giemsa solution (Beyotime). The number of colonies containing > 50 cells was counted under a microscope.

2.10. Cell migration and invasion assays

Cell migration and invasion experiments were performed using 12 plates with 8.0 μm pore diameter polycarbonate membrane insert in a transwell apparatus (Corning). Prior to migration and invasion experiments, cells were starved of FBS for 24 h. 2×10^4 cells were suspended in serum-free medium and seeded in the upper wells. Lower chambers contained media with FBS to assess the migratory behavior of cultured cells, or without FBS as a negative control accordingly. For the invasion assay, the surface of the upper chamber was covered with a monolayer of 5% growth factor-reduced matrigel (BD Biosciences). Cells adhering to the lower surface were fixed with methanol and stained with 0.1% crystal violet (Beyotime). The number of cells in the membrane was counted from five randomly selected visual fields with a microscope.

2.11. Flow cytometry analysis

For cell-cycle analyses, cells were harvested 48 h after transfection with pcDNA-NC or pcDNA-IRF1, washed with phosphate-buffered saline, and fixed in 75% ethanol at 4 °C overnight. RNA was removed from the preparations by incubating with RNaseA (Sigma) at 37 °C for 30 min. Cells were then stained with propidium iodide (PI) solution (Sigma) for 30 min at room temperature and analyzed on flow cytometry (BD Biosciences).

2.12. Apoptosis assay

The apoptosis ratio was analyzed using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen). At 72 h after transfection cells were harvested and re-suspended in binding buffer containing Annexin V-FITC and PI according to the manufacturer's instructions. The samples were analyzed by flow cytometry (FACScan; BD Biosciences, USA). Cells were discriminated into viable cells, necrotic cells, and apoptotic cells by using BD FACSDiva 6.1.3 software (BD Biosciences), and then the percentages of apoptotic cells from each