Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Downregulation of FOXP3 inhibits invasion and immune escape in cholangiocarcinoma

Q7 Chao Ma [1], Chunyan Peng [1], Xuejia Lu, Xiwei Ding, Shu Zhang, Xiaoping Zou*, Q1 Xiaoqi Zhang*

Q2 *The Drum Tower Hospital Affiliated to Medical School of Nanjing University, Nanjing, Jiangsu Province 210008, PR China*

ARTICLE INFO

Article history:
Received 24 December 2014
Available online xxx

Keywords:
FOXP3
Cholangiocarcinoma
Invasion
Immune escape

ABSTRACT

FOXP3 is known as a master control of regulatory T cells with recently studies indicating its expression in several tumor cells. In order to study the precise role of FOXP3 in cholangiocarcinoma, FOXP3 was knocked down in cholangiocarcinoma cell lines. Down regulation of FOXP3 inhibits tumor cell invasion by reducing the quantity of MMP-9 and MMP-2. With FOXP3 knocking down, IL-10 and TGF-β1 secreted by cancer cells diminishes and the cell survival of T cells is significant up-regulation. These results suggest that FOXP3 plays an important role in tumor malignant phenotype, especially the invasion and immune escape.

1. Introduction

Cholangiocarcinoma (CCA) is a malignant tumor arising from the epithelial cells in intrahepatic and extrahepatic bile ducts with diagnosis difficulty and poor prognosis [1]. Even though surgical resection and liver transplantation are potentially curative treatments [2], most of the patients present with unresectable tumors and die within a year of diagnosis [3]. As the aggressive nature of CCA and its resistance to chemotherapy, both the monotherapy and combination chemotherapy are disappointing [4]. Regarding to molecular targeted therapy [5], sorafenib which was developed as a multiple kinase inhibitor has a tumor suppression role in cholangiocarcinoma in part though inhibition of STAT3 signaling pathway [6]. Unfortunately, a phase II study showed that the addition of sorafenib to gemcitabine and cisplatin has no efficacy increase while with toxicity increasing in CCA [7]. Therefore, there is an urgent need to find new targets in CCA treatments.

Fork head-box protein 3(FOXP3) has been identified as a master control mediating immunosuppression of regulatory T cells (Treg) [8]. Genome-wide analysis of Treg indicated that FOXP3 can bind to 700 genes and several microRNAs [9,10]. Recent papers have validated that FOXP3 can also be expressed by carcinoma cells including pancreatic and gastric carcinomas [11,12], and participate in immune evasion. It may be a candidate for tumor-specific biological and immune therapy. However, the role of FOXP3 in CCA remains unknown. This study was to investigate the biological significance and clinic pathological parameters of FOXP3 in CCA.

2. Materials and methods

2.1. Patient samples

Tissue samples were obtained from CCA patients enrolled in Nanjing Drum Tower Hospital from 2006 to 2012 (30 males, 22 females, mean age = 60.12 ± 11.23 years, range 37–80 years). A total of 85 surgically resected specimens were included in this study, comprising 52 primary CCA specimens and 33 paracarcinoma normal tissues. The survival time was defined from date of operation to date of death or December 31, 2013. In this study, patients who received chemotherapy before surgery, suffered from other cancers or had distant metastasis identified by CT were excluded. The project was conducted with the approval of ethics committee of Nanjing Drum Tower Hospital.

2.2. Immunohistochemistry (IHC)

Immunohistochemical staining of formalin-fixed and paraffin-embedded tissues were done according to standard protocol using FOXP3 antibody (Rat monoclonal; diluted 1:500; eBioscience).

Q3 * Corresponding authors.
E-mail addresses: 13770771661@163.com (X. Zou), zhangxiaoqinj@163.com (X. Zhang).
[1] Chao Ma and Chunyan Peng contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.01.067
0006-291X/

The normal thyroid tissues were used as positive control. MMPs protein levels were examined by IHC with antibodies against MMP9(Rabbit polyclonal; diluted 1:1000; Cell signaling) and MMP2(Rabbit monoclonal; diluted 1:500; Bioworld Technology). The quantification of FOXP3-positive tumor cells were as follows: the percentage of positive cells was scored as 0 (<5%), 1 (5%–24%), 2 (25%–49%), 3 (50%–75%), 4(75%–100%). The staining intensities were scored as 0 (achromatic), 1(light yellow), 2(brownish yellow), 3(brown). The total score was the combination of the two scores; the final score <5 was defined as low expression and ≥5 was defined as high expression. All of these were determined independently by two pathologists who were unknown of clinical data.

2.3. Cells and cell culture

Two CCA cell lines, HuCCT1 and QBC939 were routinely cultured in DMEM supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. RNA interference and gene transfection

The synthesized human FOXP3 siRNA or negative control together with Lipofectamine RNAiMAX Reagent was diluted in Opti-MEM Medium. After incubation for 15 min at room temperature, the siRNA-lipid complex was added to six-well plates with 2×10^5 cells in each well. The following siRNA sequences were used (5′–3′): FOXP3 sense: CCA CAA CAU GGA CUA CUU CAA UUC; FOXP3 antisense: GAA CUU GAA GUA GUC CAU GUU GUGG. Both the transfection reagent and the stealth RNAi negative control duplexes were purchased from Invitrogen.

2.5. Real-time PCR

Total RNA was isolated from tumor cells with different treatments, using TRIzol Reagent (Ambion). After quantified to 500 ng, RNA was reverse transcribed to cDNA with Prime Script RT Master Mix (TaKaRa). Relative mRNA levels of FOXP3, Actin, MMP9, MMP2, P21, and cMYC were determined by quantitative RT-PCR (qRT-PCR) with SYBR Premix Ex Taq II(TaKaRa). The following primers were used (5′–3′): FOXP3 forward: TCC CAG AGT TCC TCC ACA AC; FOXP3 reverse: ATT GAG TGT CCG CTG CTT CT; Actin forward: AGC GAG CAT CCC CCA AAG TT; Actin reverse: GGG CAC GAA GGC TCA TCA TT; MMP9 forward: CCA ACT ACG ACA CCG ACG AC; MMP9 reverse: TGG AAG ATG AAT GGA AAC TGG; MMP2 forward: ATG AAG CAC AGC AGG TCT CA; MMP2 reverse: TGA AGC CAA GCG GTC TAA GT; p21 forward: TTA GCA GCG GAA CAA GGA GT; p21 reverse: CGT TAG TGC CAG GAA AGA CA.

2.6. Western blot

The total protein was extracted from cells plated in six-well plates with different treatments. After washing with cold PBS, cells were lysed with RIPA (P0013B, Beyotime) and protease & phosphatase inhibitor cocktail (P1861281, Thermo Scientific). 20 μg protein was loaded into each lane of SDS-PAGE gel and then transferred to polyvinylidene fluoride membranes. Membranes were probed with primary antibodies at concentration of 1:1000 for anti-FOXP3 (eBioscience), anti-MMP9 (Cell Signaling), anti-MMP2 (Bioworld), anti-p21 (Santa Cruz) and anti-Actin (Sigma) respectively. Anti-mouse or rabbit horseradish peroxidase-linked antibody (Cell Signaling, 1:1000) was used as secondary antibody. After incubation with enhanced chemiluminescent reagents, membranes were exposed to X-ray film for 1 min to 3 min.

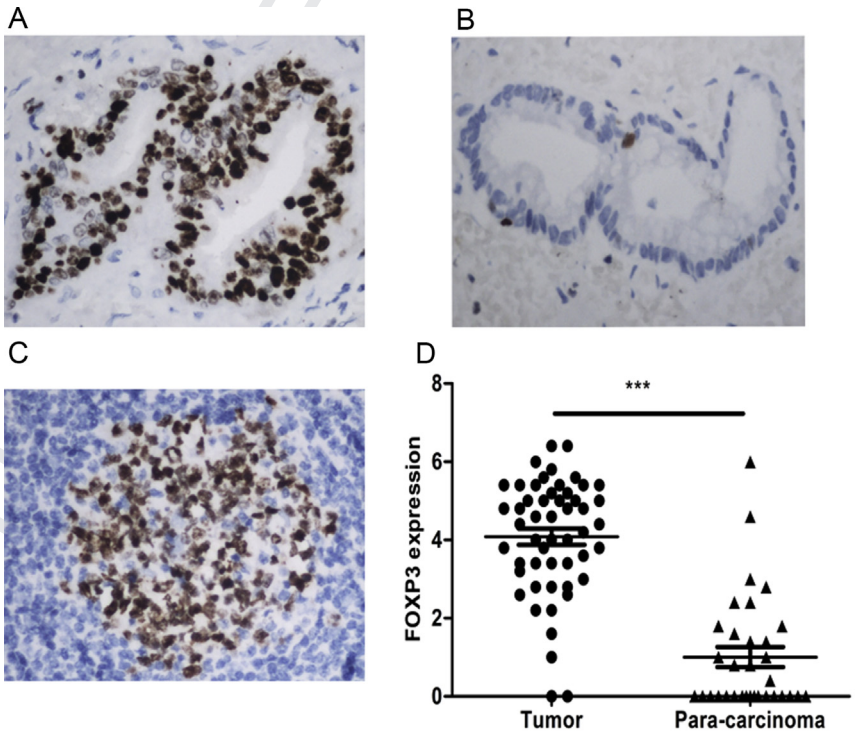

Fig. 1. FOXP3 protein expression demonstrated by immunochemistry was significantly higher in tumor cells than that in para-carcinoma area. (A) FOXP3-positive staining in CCA cells. (B) FOXP3-negative staining in para-carcinoma cells. (C) FOXP3-positive staining in lymphocytes. (D) FOXP3 protein levels were significantly higher in tumor than those in para-carcinoma. (n1 = 52, n2 = 33, Unpaired t test, $p < 0.0001$). A, B and C images are shown at 400X.

Please cite this article in press as: C. Ma, et al., Downregulation of FOXP3 inhibits invasion and immune escape in cholangiocarcinoma, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.01.067