Biochemical and Biophysical Research Communications 458 (2015) 234–239

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

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ARTICLE INFO

Article history:
Received 24 December 2014
Available online 23 January 2015

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).

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http://dx.doi.org/10.1016/j.bbrc.2015.01.067
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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.

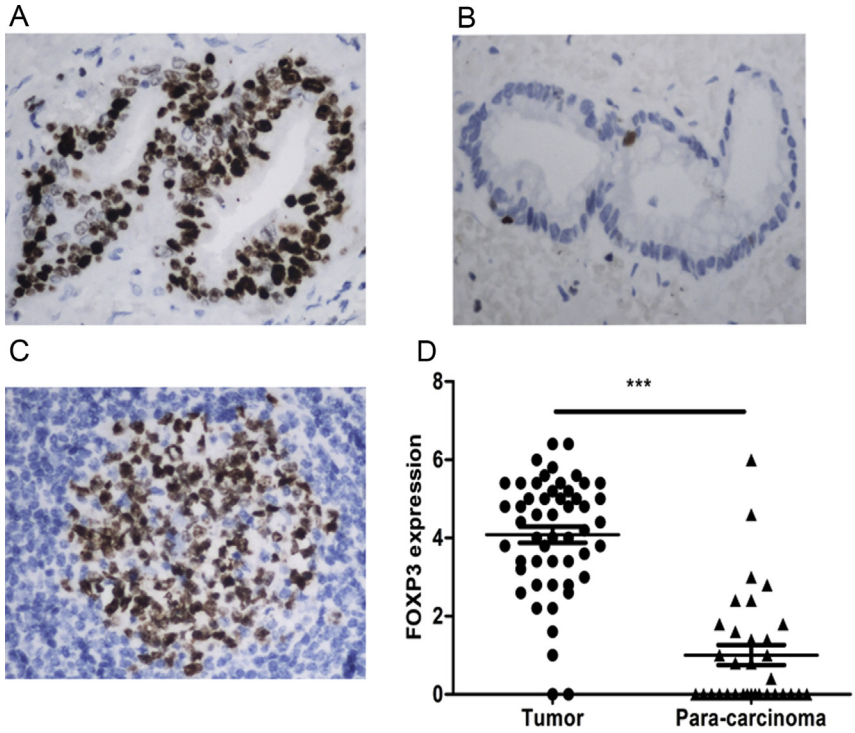

Fig. 1. FOXP3 protein expression demonstrated by immunochemistry was significantly higher in tumor cells than that in para-carcinoma area. (A) FOXP3-positive staining in CCA cells. (B) FOXP3-negative staining in para-carcinoma cells. (C) FOXP3-positive staining in lymphocytes. (D) FOXP3 protein levels 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.

2.7. Cell invasion assay

The upper chamber of the insert (BD, 8 μm) was coated with Matrigel Matrix (BD) and cultured in 37 °C for 5 h. Following re-suspended in medium without serum, 5×10^4 cells were seeded in the upper chamber, while medium containing 10% of serum was added into the lower chamber. After 24 h incubation, cells on the upper side were removed, whereas, cells on the lower side were fixed with formaldehyde and stained with crystal violet. The cell number in five separated high power fields was counted.

2.8. Cell growth assay

Cell Counting Kit-8 (DOJINDO) was used to assess cell proliferation. Cells with different treatments were seeded in 96-well plates at 5×10^3 cells/well and cultured for 48 h–96 h, respectively. Following incubated for 1 h at 37 °C with CCK-8 solution, the plate was measured under the wavelength of 450 nm using microplate reader. All experiments were performed in triplicate.

2.9. Flow cytometry analysis

Cells were washed twice with cold PBS and suspended in buffer. After transferring buffer to tube, PI and Annexin V-FITC were added. Cells were incubated at room temperature in the dark for 15 min and then analyzed. The percentage of cells was analyzed by flow cytometry in each stage of cell cycle.

2.10. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood donors by density gradient centrifugation. Cells were cultured for 24 h to allow adherence, the un-adherent cells were collected with centrifugation for further co-culture with tumor supernatants.

2.11. Enzyme-linked immunosorbent assay

Cells with different processing were cultured in six-well plates for 48 h. Supernatants were harvested to test human IL-10 and TGF-β1 via enzyme-linked immunosorbent assay (Multi science).

2.12. Statistical analysis

Each experiment was independently done for three times. The non-paired t-test, Chi-square test and one-way ANOVA were used to perform statistical analysis. $P < 0.05$ was considered to indicate statistical significance.

3. Result

3.1. FOXP3 protein is overexpressed in CCA tissue

We assessed the expression of FOXP3 in human CCA specimens ($n = 52$) and para-carcinoma tissues ($n = 33$) by IHC. FOXP3 was detected in both cancer cells and lymphocytes. FOXP3 was observed both in cytoplasm and nucleus of tumor cells (Fig. 1A),

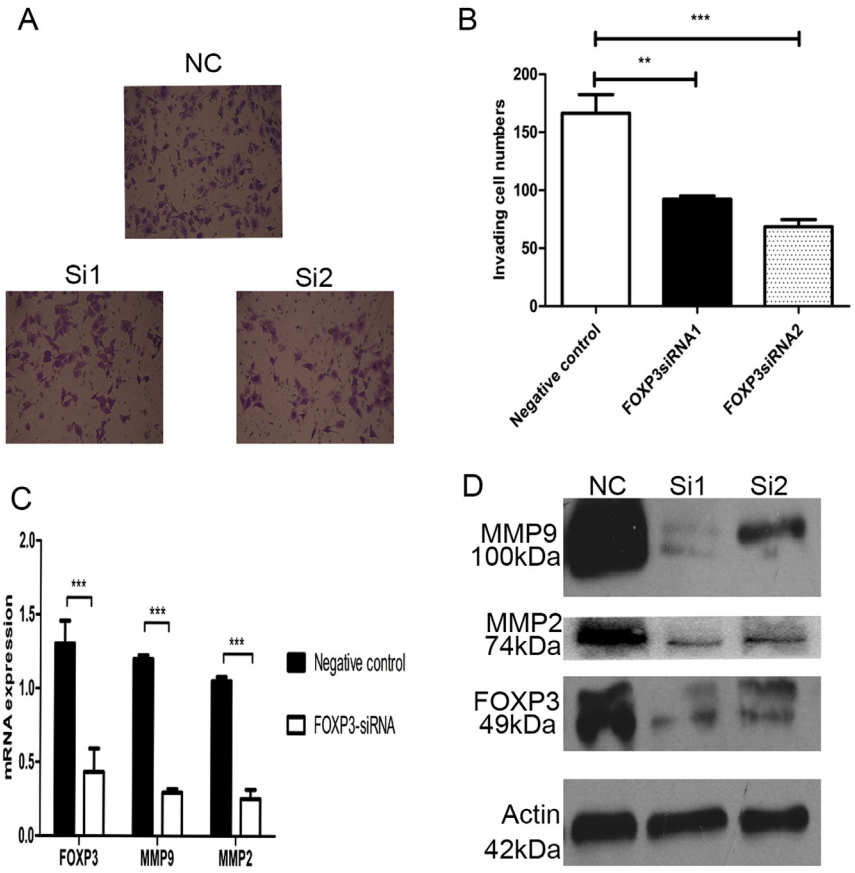

Fig. 2. The mRNA and protein levels of MMP9 and MMP2 in CCA tumor cell lines were both significantly down regulated with the weakened tumor invasion ability after transfecting with FOXP3-siRNA. (A) The representative pictures of Transwell bottom stained by eosin. Cells were plated in the six-well plates and invaded in Transwell with negative control and FOXP3-siRNA. (B) Statistical data of (A) ($p < 0.001$). (C) The mRNA levels of FOXP3, MMP2 and MMP9 were decreased by siRNA targeting FOXP3. (D) The expressions of MMP9 and MMP2 in tumor cells were down-regulated by using FOXP3-siRNA.

with low expression in para-carcinoma tissues (Fig. 1B) and strong nuclear staining in T cells (Fig. 1C). We found that FOXP3 expression was significantly higher in cancer cells than that in para-carcinoma tissues (Fig. 1D).

3.2. FOXP3 expression is positively correlated with lymphatic metastasis and neoplasm staging

To explore the role of FOXP3 in tumor progression, we analyzed the relationship between FOXP3 expression in tumor and clinical pathological features (Supplement 1). We found that FOXP3 expression was positively correlated with lymph node metastasis. IHC showed that the expression of MMP9 and MMP2 in tumor was respectively higher than that in para-carcinoma tissues (Supplement 2A). There was also a positive correlation between FOXP3 expression and those of MMP9 and MMP2 (Supplement 2B and 2C). Interestingly, all the FOXP3-negative patients were in stageIand II.The average survival time of patients with low FOXP3 expression was 16 months which was longer than that of patients with high FOXP3 expression (median survival time = 12 months), although no significant difference was detected (Supplement 2D).

3.3. FOXP3 gene promotes cell invasion in CCA

For more about the feature of FOXP3 in neoplasm invasiveness, FOXP3 expression was detected in a series of cholangiocarcinoma cell lines (HuCCT1 and QBC939) with Real-Time PCR and Western Blot (data not show). FOXP3 was knockdown by siRNA and the effectiveness was verified at mRNA and protein level respectively by Real-Time PCR and Western Blot. Transwell cultured with Matrigel was used to assess neoplasm invasion. Results reflect that down regulation of tumor FOXP3 leads to less invasive power (Fig. 2A and B) together with MMP9 and MMP2 decrease (Fig. 2C and D). The in vitro data were consistent with the in vivo data.

3.4. FOXP3 expression in CCA cells up-regulates cell proliferation

As shown (Fig. 3A), the down regulation of FOXP3 in CCA cells inhibited its growth and proliferation which also increased the rate of apoptosis, especially at early stage (Fig. 3B). Matched with negative control, cell cycle in FOXP3 down regulated group was significantly arrested in the S phase (Fig. 3C). The western blot analysis detected that the level of p21 which involved in cell cycle control was upregulated by FOXP3 knockdown (Fig. 3D). All of these implied that FOXP3 played a positive role in tumor growth.

3.5. FOXP3 in tumor cells inhibits cell survival of T cell and increases secretion of IL-10 and TGF-β1

The immunosuppression of Treg is maintained by stable FOXP3 expression [13]. To answer the question whether FOXP3 expression participants in CCA tumor immune escape, T cells were isolated from peripheral blood and co-cultured with tumor supernatant. The cell survival of T cells in tumor supernatant of FOXP3-knockout cells was significantly higher than that of negative control (Fig. 4A). As FOXP3 was indispensable for IL-10 transcription in T cells [14], and to test whether FOXP3 regulates immunosuppressive cytokine secretion in CCA cells, we used ELISA to test supernatant. The data elucidated that IL-10 and TGF-β1 were diminished by FOXP3 down-regulation (Fig. 4B and C). In summary, we suggested that the expression of FOXP3 in CCA cells reflected evasion from immune-surveillance.

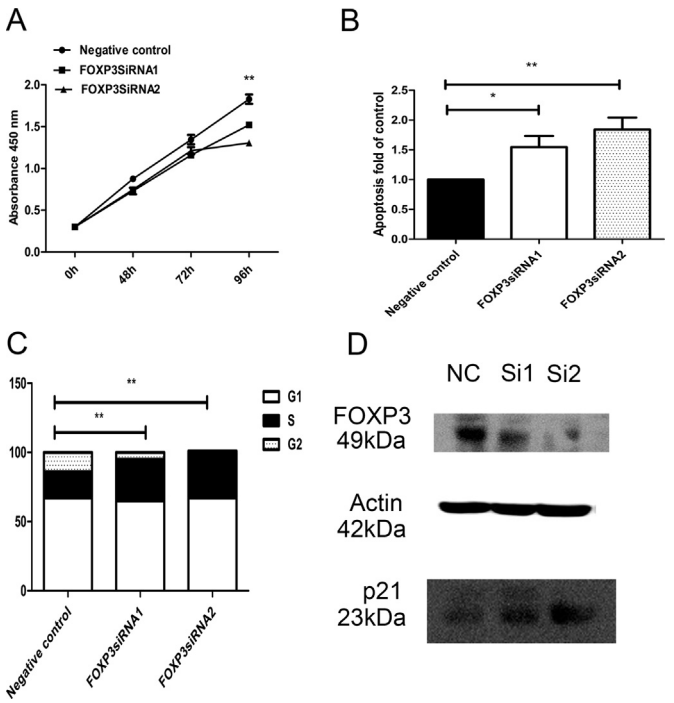

Fig. 3. Knocking down of FOXP3 leads to reduced proliferation, less evasion, increased apoptosis, and arrested in S phase. (A) Cells were treated with FOXP3-siRNA for 96 h and CCK-8 was used to analyze cell proliferation ($p < 0.0010$). (B) Cells cultured with FOXP3-siRNA have increased apoptosis compared to negative control ($p < 0.05$). (C) Cells with FOXP3 knockdown were significantly arrested in S phase ($p < 0.0010$). (D) The protein expression of p21 were significantly higher with FOXP3 down-regulation.

4. Discussion

Our study showed that FOXP3 is expressed in both CCA tumor tissues and cell lines. We also found that downregulation of FOXP3 inhibits proliferation and invasion, promotes apoptosis and weakens immune escape in CCA cells. Therefore, FOXP3 may play an oncogenic role in CCA.

Although FOXP3 has been shown to be expressed in several cancer cells, its role in cancer is controversial. It was first time reported in breast cancer that FOXP3 could repress oncogenes HER-2/ErbB2 and SKP2 [15,16]. Similarly, in prostate cancer, FOXP3 was found to inhibit oncogene c-MYC [17]. Furthermore, overexpression of FOXP3 in ovarian cancer cells could repress cell proliferation, invasion, and migration [18].

On the other hand, some researchers also have found that FOXP3 correlates to poor prognosis and lymphatic metastasis. Patients with high expression of FOXP3 had significantly shorter overall survival time in primary melanoma [19]. While in esophageal cancer, the expression of FOXP3 was positively related to metastasis [20]. Likewise, in hepatoma, FOXP3 could facilitate tumor metastasis through regulating MMP-1 expression both in vitro and in vivo [21].

Recent studies investigated the influence of tumor microenvironment on FXOP3. Inflammation environment could dampen the tumor suppressor role of FOXP3 by induce FOXP3-NFκB interaction [22]. In addition, FOXP3 expression was reported to predict better survival possibility owing to the interaction between cancer cells and lymphocytes in microenvironment [12].

Except for microenvironment, the dual role of FOXP3 in cancer cells may owe to several other reasons [23] including splicing variants, subcellular location, and epigenetic control [24]. We

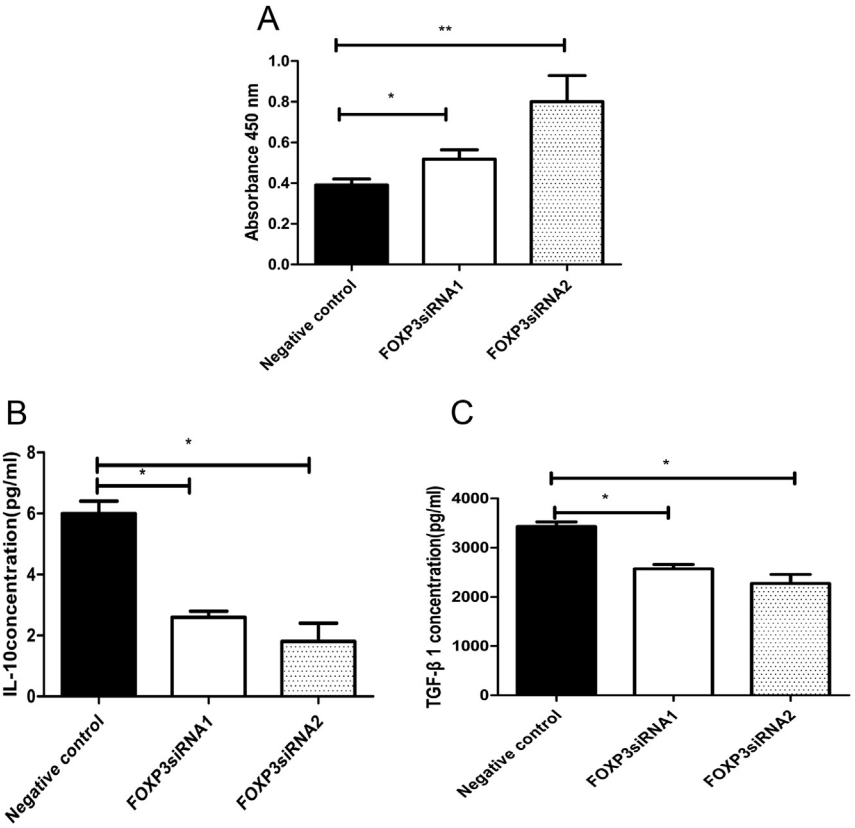

Fig. 4. After FOXP3 knocked down, the immune evasion was weakened. (A) T cells co-cultured with tumor supernatant were tested with CCK-8 and the supernatant with FOXP3 knocked down promoted survival rate of T cell ($p < 0.05$). (B) IL-10 secreted by tumor cells significantly diminished after FOXP3 knocked out ($p < 0.05$). (C)TGF-β1 secreted by cancer cells significantly decreased by FOXP3 down regulation ($p < 0.05$).

assume that the main possible reason might be variant isoforms. There are two isoforms of FOXP3 with different functions in Treg [25]. In melanoma, the FOXP3 variant lacking exons 3 and 4 removes the repressing domain of normal FOXP3 protein and encodes a new one [26]. Moreover, some studies in CCA found that the antibody recognizing C-terminus of wild-type FOXP3 would fail in detect variant protein of FOXP3 lacking exon 3 [27]. This may partly explain the same role of FOXP3 in melanoma and CCA. Importantly, we should not ignore that overexpression of full-length FOXP3 was used in most of previous studies, while the large quantity of full-length FOXP3 may cover the real function of variants of FOXP3 in tumor cells.

Even through there were disputes on the role of FOXP3 in metastasis, its role in immune evasion is beyond question. FOXP3 was detected in twenty five tumor cell lines and its expression was correlated with the level of IL-10 and TGF-β1 [28]. In melanoma and pancreatic cancers, FOXP3 expression in tumor confers the ability of immune evade to cancer cells [11,29]. Here, we have shown that FOXP3 in CCA cells inhibits T cells and this may be through secreting IL-10 and TGF-β1.

Progression of CCA is controlled by multiple signaling pathways, in which the IL-6/STAT3 signaling pathway plays an important role [30]. STAT3 can regulate tumor microenvironment by promoting FOXP3 [31], while FOXP3 acts as co-transcription factor with STAT3 [14], although the exact interaction between them in CCA has not been clearly elucidated yet. This might open new strategies for immunotherapeutic and molecular targeted treatments. As our work focused mainly on the biological features of FOXP3 in CCA and sample size is small, more studies are needed for further exploration.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

This work was supported by a grant from the National Science Foundation; Grant number: 8201908, 81101814.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.067.

Transparency document

The transparency document associated with this article can be found in the online version at 10.1016/j.bbrc.2015.01.067.

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