

FOXP3 expression and clinical characteristics of hepatocellular carcinoma

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Abstract

AIM: To study the biological and clinical characteristics of transcription factor forkhead box protein 3 (FOXP3) in hepatocellular carcinoma (HCC).

METHODS: We analyzed the expression and localization of FOXP3 in HCC tissues and cell lines to evaluate its biological features. The relationship between FOXP3 staining and clinical risk factors of HCC was assessed

to identify the clinical characteristics of FOXP3 in HCC.

RESULTS: The mRNA and protein expression of FOXP3 were found in some hepatoma cell lines. Immunohistochemical (IHC) analysis of HCC sections revealed that 48% of HCC displayed FOXP3 staining, but we did not find any FOXP3 staining in normal liver tissues and para-tumor tissues. IHC and Confocal analysis showed that the expressions of FOXP3 were mainly present in the nucleus and cytoplasm of tumor cells in tissues or cell lines. In HCC, the distribution of FOXP3 was similar to that of the cirrhosis, but not to the hepatitis B virus. Those findings implicate that FOXP3 staining seems to be associated with the high risk of HCC.

CONCLUSION: The clinical characteristics of FOXP3 in HCC warrants further studies to explore its functions and roles in the cirrhosis and development of HCC.

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Key words: Forkhead box protein 3; Hepatocellular carcinoma; Tumor differentiation; Cirrhosis; Hepatitis B virus

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INTRODUCTION

Hepatocellular carcinoma (HCC), which consists predom-

inantly of primary liver cancer, is the fifth most common malignancy in men and the eighth one in women worldwide. The number of new cases of HCC is about 564 000 per year^[1]. Cirrhosis and virus infection, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), are the major known risk factors for HCC^[2,3]. HCC has a poor prognosis and a low survival rate in the majority of patients^[4]. The current treatment options of HCC include surgical resection, liver transplantation and local ablative therapy, which are effective only in limited tumors^[5]. To improve the treatment of HCC will require a better understanding of the biological development and molecular events in the immune system of HCC.

Forkhead box protein 3 (FOXP3) is a member of the forkhead/winged-helix family of transcriptional regulators and is highly conserved in normal cells. The full-length protein contains 431 amino acids. *Foxp3* is considered to be an important gene of thymically derived and naturally occurring regulatory T cells (Tregs)^[6]. Mutations in human *Foxp3* are associated with immune diseases, such as multi-organ autoimmune disorder, immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX)^[7], in which Tregs from affected patients are greatly reduced in number and suppressive activity^[8-10]. A high prevalence of Tregs is thought to be an unfavorable prognostic indicator for HCC^[11].

Recent publications described the expression of FOXP3 in pancreatic carcinoma cells, melanoma cells and other tumor cells^[12-14]. It has been found that FOXP3 expression was related to the regulation of several cytokines, such as IL-10 and TGF- β 2, and FOXP3 might mediate the inhibiting efficacy of tumor cells to escape immune destruction. Those reports implicated that FOXP3 performs its functions in the regulation of tumor progression by expressing not only in Tregs, but also in tumor cells. We assumed that FOXP3 may also be functional in tumor cells of HCC.

This study was designed to investigate whether expression of FOXP3 transcripts and mature protein is related to HCC. We also evaluated the distribution of FOXP3 in human HCC tissues and its relationship with the diagnosis, differentiation and clinical risk factors of HCC.

MATERIALS AND METHODS

Source of normal and cancerous liver tissue sections and tissue array

Normal (8) and cancerous liver tissues (21) were obtained from HCC patients who underwent resection of liver. The circulating HBV markers and ultrasound examination were performed regularly. The quantitative cirrhosis score was derived from the ultrasonographic evaluation of the liver surface, liver parenchyma, caliber of intrahepatic blood vessels and spleen size. Cirrhosis scores of ≥ 7 were used to define cirrhosis^[15]. Normal controls were histologically normal tissues obtained from patients who underwent partial hepatectomy for metastatic tumor or liver biopsy. Microarray tissues were obtained from Cybrdi, USA. The study protocol conformed to the ethical guidelines of the 1975 Declaration

of Helsinki in a prior approval by the Fourth Military Medical University, China.

Cell culture

Complete medium (RPMI-1640) contained RPMI-1640 supplemented with 2 mmol/L Glutamax, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10 mmol/L HEPES (Invitrogen, USA) and 10% FCS (Thermo Trace, Australia). The following cell lines were obtained from the Biotechnology Center of the Fourth Military Medical University, such as SMMC-7721 and Hepa-G2. All tumor cell lines were maintained in complete RPMI-1640 and passaged using trypsin/EDTA (Invitrogen, USA). *Foxp3* transiently transferred 293 cells were established. Melanocytes were freshly prepared when used (derived from normal human skins from the Department of Dermatology of Xijing Hospital of the Fourth Military Medical University).

Semi-quantitative and reverse-transcription polymerase chain reaction

Total RNA was isolated from HCC cells and melanocyte (as control) using Trizol reagent (Invitrogen, USA). A total of 500 ng RNA was reversely transcribed using the Kit from Takara, Japan. The polymerase chain reaction (PCR) was performed for *Foxp3* fragment amplification. The following primers were used (5'-3'): *Foxp3* sense: CACAA-CATGCGACCCCTTTCACC; *Foxp3* antisense: AG-GTTGTGGCGGATGGCGTTCCTC. β -actin was used as an internal control for normalization (primer sequences available on request). Semi-quantitative PCR of *Foxp3* transcripts was done by comparing the signal intensity of PCR product of *Foxp3* gene with that of β -actin gene from the same RNA sample using agarose gel electrophoresis. The intensity of the product band was quantified by densitometric scanning of the gel (Pharmacia Biotech) using "Total image" 1D GEL ANALYSIS software. DNA marker (Takara, Japan) was run in each gel to confirm the size of PCR product.

Western blotting analysis

To examine the protein expression level of FOXP3 in HCC, whole cell lysate was subjected to SDS-PAGE electrophoresis followed by blotting on a nitrocellulose (NC) membrane. During FOXP3 detection, membranes were probed with goat anti-human FOXP3 polyclonal antibody at 4°C overnight followed by incubation with a secondary horseradish peroxidase-conjugated antibody. The mouse anti-human β -actin monoclonal antibody was used as an internal control (R&D, USA). Chemiluminescent detection was done with the enhanced chemiluminescence detection kit (Anmei, China).

Flow cytometric analysis

To determine the expression levels of FOXP3, hepatoma cell lines were stained for FOXP3 and analyzed by flow cytometry. Cells were washed in PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN₃ before antibody staining followed by fixation with 1% paraformaldehyde.

Fluorescein isothiocyanate (FITC)-conjugated rat anti-human FOXP3 monoclonal antibody was purchased from eBiosciences, USA. A total of 10^5 events were collected using Becton Dickinson FACScaliber (Becton Dickinson, USA). Analysis was performed using the WinMDI 2.8 program (Purdue University Cytometry Laboratories, USA).

Confocal microscopic analysis

For double-label immunofluorescence, formalin-fixed cell line slides were treated in 3% hydrogen peroxide in methanol for 10 min. Following three rinses in PBS, the slides were treated in blocking solution (Zhongshan, China) for 1 h, and then incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-human FOXP3 monoclonal antibody (eBioscience, USA) for another 1 h at room temperature. After rinsed in PBS, the slides were treated with a 1:1000 dilution of diamidino-phenyl-indole (DAPI) (stock solution: 1 mg/mL) (Sigma-Aldrich, USA) for 30 min. DAPI staining was used to visualize nuclei. The slides were mounted with the anti-fade mounting medium. Slides were examined under a Leica TCS-SP laser scanning confocal microscope (Leica, German). All images were collected using a pinhole of 1 Airy.

Immunohistochemistry

Paraffin-embedded resected liver cancer specimens were provided by Xijing Hospital tissue bank. Rat anti-human FOXP3 monoclonal antibody was applied to paraffin-embedded sections after microwave antigen retrieval for 10 min in citrate buffer (pH 6.0). Specimens were treated with 0.3% hydrogen peroxide in methanol for 15 min after incubation with the primary antibody to block endogenous peroxidase activity. The secondary antibody of horseradish peroxidase-labeled goat anti-rat antibody (Zhongshan, China) was incubated for 1 h. These slides were examined systematically using an image analyzer system (Olympus BH-2 microscope, Japan).

Semi-quantitative analysis of IHC

Slides were reviewed under light microscopy by two pathologists separately. Semi-quantitative analysis of FOXP3 staining was assessed as 0, 1+, 2+, and 3+ as previously established^[16,17]. Grade 0 was defined as the complete absence or weak FOXP3 staining in < 1% of the tumor cells; grade 1+ was focal FOXP3 staining in 1%-10% of tumor cells; grade 2+ was positive FOXP3 staining in 11%-50% of tumor cells; and grade 3+ was positive FOXP3 staining in > 50% of tumor cells. A global assessment of the entire tumor was made without selection for the invasive front or areas of active tumor growth. The frequency and semi-quantitative analysis of positive tumors for all regions were calculated for statistical comparisons.

Statistical analysis

Differences in proportions were compared by a Pearson Chi-square test or Fisher's exact test as appropriate. The statistical correlation between the grades of HCC differentiation and the staining level of FOXP3 was analyzed

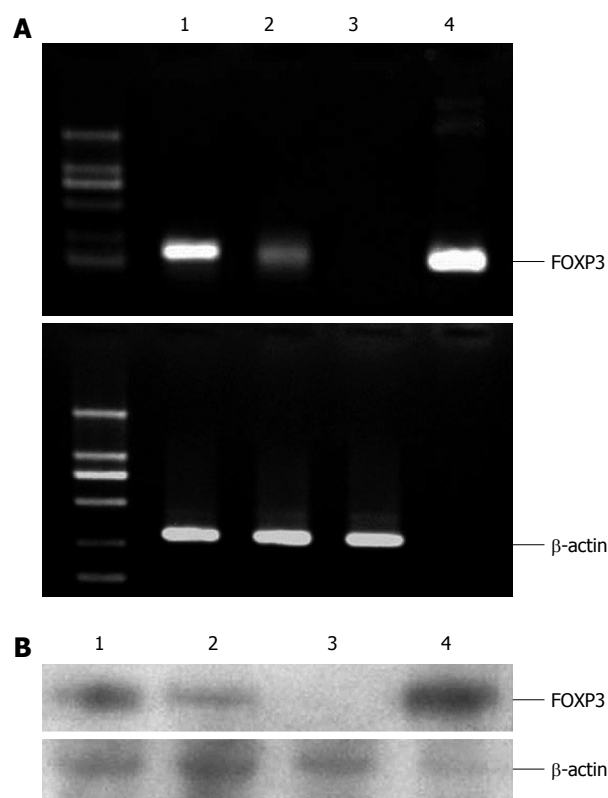


Figure 1 Expression of forkhead box protein 3 in hepatoma cell lines. A: Expression of forkhead box protein 3 (FOXP3) mRNA is different in hepatoma cell lines by reverse-transcription polymerase chain reaction. β -actin was used to verify the integrity of the template cDNA preparations. 1: SMMC-7721, 2: Hepa-G2, 3: Melanocytes, 4: Foxp3-plasmid; B: Expression of FOXP3 protein was different in hepatoma cell lines by Western blotting. Melanocytes served as a negative control. Foxp3 transiently transferred 293 cells were established as a positive control. 1: SMMC-7721, 2: Hepa-G2, 3: Melanocytes, 4: Foxp3/293 cells.

by the Cochran-Mantel-Haenszel test. The dependability between the distribution of cirrhosis or HBV infection and FOXP3 expression was evaluated by *t* test. Differences with a *P* value less than 0.05 were considered to be statistically significant. All analyses were done using SAS statistical software version 9.1 (Cary, USA).

RESULTS

FOXP3 expression in hepatoma cell lines

In RT-PCR analysis, *Foxp3* was found in some hepatoma cell lines. To further validate the changes of *Foxp3*, semi-quantitative RT-PCR analysis from two hepatoma cell lines and melanocytes were conducted. The results revealed a significant overexpression of *Foxp3* in HCC specimens as compared with nonmalignant melanocytes (Figure 1A). The cell lines shown in Figure 1A (SMMC-7721 and Hepa-G2) were uniformly positive for *Foxp3* transcription. But we did not find any *Foxp3* transcription in melanocytes as expected. *Foxp3* full length plasmid was constructed and performed as a positive control.

Furthermore, as shown in Figure 1B, FOXP3 protein expression could be detected in hepatoma cell lines by Western blotting. Because it was the first time to dem-

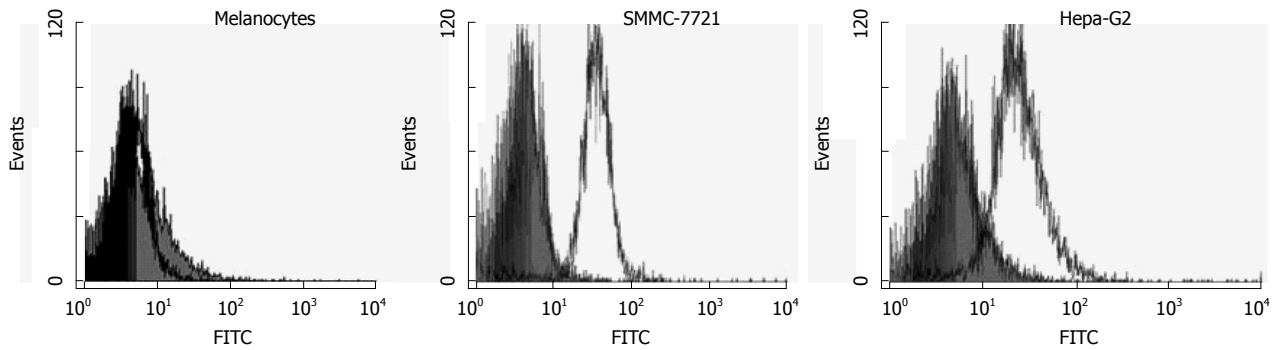


Figure 2 Flow cytometry of forkhead box protein 3 expression in various hepatoma cell lines. Melanocytes served as a negative control. The grey underlaid plot represents staining with the isotype, and the white underlaid plot represents staining with anti-human forkhead box protein 3 antibody. FITC: Fluorescein isothiocyanate.

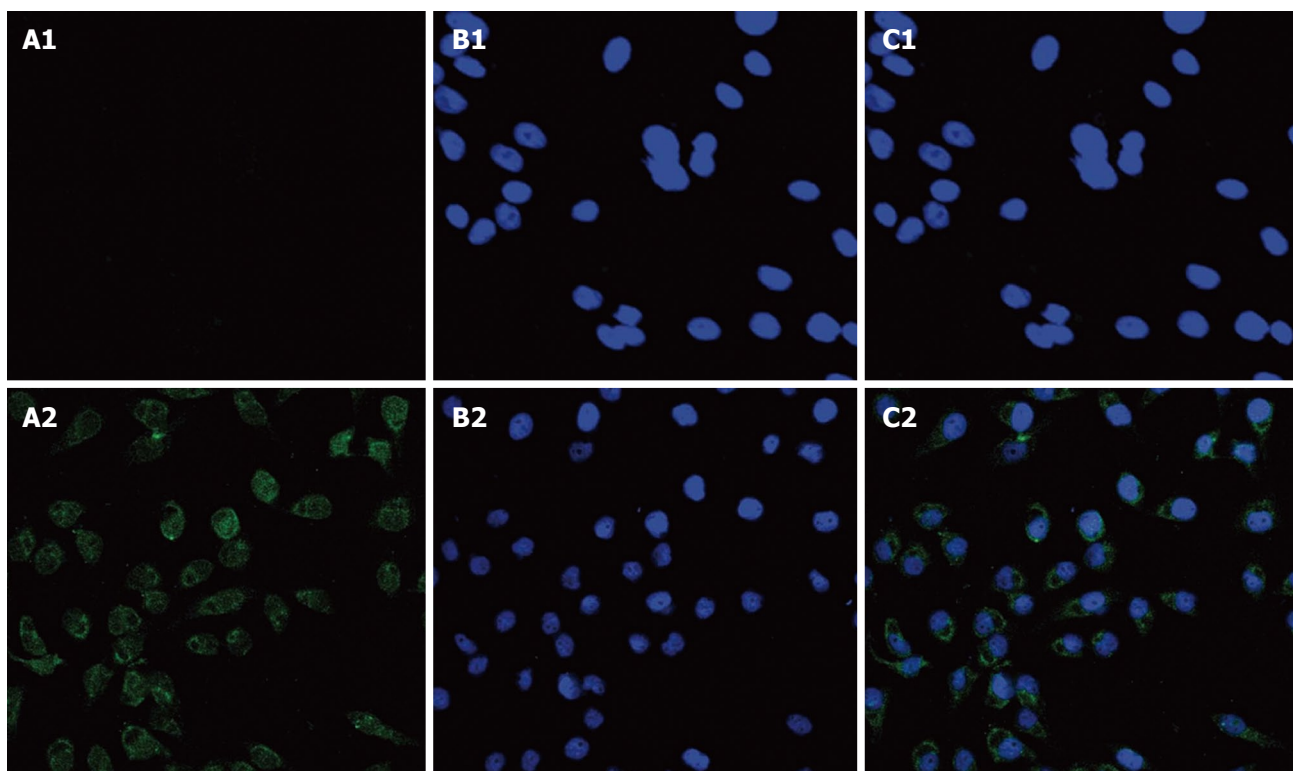


Figure 3 Double-label immunofluorescence analysis of forkhead box protein 3 expression in hepatoma cell line. A: SMMC-7721 cells express forkhead box protein 3 (FOXP3) in both nuclei and perinuclear cytoplasm (imaged with green fluorescent) (A1: Melanocytes FOXP3-FITC, A2: SMMC-7721 FOXP3-FITC); B: Imaged with diamidino-phenyl-indole (DAPI) to identify the nuclei of SMMC-7721 and melanocytes (B1: Melanocytes DAPI, B2: SMMC-7721 DAPI); C: Image superimposed on a differential interference contrast background confirmed colocalization (C1: Melanocytes merge, C2: SMMC-7721 merge).

onstrate FOXP3 expression in liver cells, we confirmed the staining results in Western blotting with two different anti-FOXP3 antibodies (eBioscience and R&D, USA). We found that the intensity of FOXP3 expression varied in different cell lines. In this experiment, *Foxp3* transiently transfected 293 cells were established as a positive control cell line.

Localization of FOXP3 in hepatoma cell lines

FOXP3 FITC-staining resulted in a shift in the fluorescence of the entire population of hepatoma cell lines compared with the isotype control in the results of flow cytometry. In contrast, melanocytes did not express FOXP3,

as shown in Figure 2. Confocal microscopy was used to examine the distribution of FOXP3 in hepatoma cell lines and melanocytes. In these experiments, the nuclei were stained with DAPI to facilitate analysis. The results showed that SMMC-7721 cells exhibited intense nucleic and less cytoplasmic FOXP3 expression (Figure 3).

Expression and distribution of FOXP3 in HCC tissues

IHC was used to analyze the protein expression and localization of FOXP3 in HCC tissues. FOXP3 staining was done on a set of 29 tissue sections (Table 1) and a tissue array containing 154 cores (Table 2). Those samples were selected from normal and cancerous HCC tissues.

Table 1 Association of forkhead box protein 3 expression with pathologic grades in 21 hepatocellular carcinoma tissue sections by immunohistochemical *n* (%)

Demographic or clinical characteristic	No. of tumor specimens (<i>n</i> = 21)	FOXP3		<i>P</i>	FOXP3 immunohistochemistry intensity score				<i>P</i>
		Positive	Negative		0	1	2	3	
Gender									
Male	20	10 (50)	10 (50)	1.0000 ¹	10 (50)	7 (35)	3 (15)	0 (0)	1.0000 ¹
Female	1	0 (0)	1 (100)		1 (100)	0 (0)	0 (0)	0 (0)	
Age (yr)									
> 60	4	3 (75)	1 (25)	0.3108 ¹	1 (25)	1 (25)	2 (50)	0 (0)	0.1031 ¹
≤ 60	17	7 (41)	10 (59)		10 (59)	6 (35)	1 (6)	0 (0)	
Differentiation grade									
Well	7	4 (57)	3 (43)	1.0000 ¹	3 (43)	3 (43)	1 (14)	0 (0)	1.0000 ¹
Moderate	7	3 (43)	4 (57)		4 (57)	2 (29)	1 (14)	0 (0)	
Poor	7	3 (43)	4 (57)		4 (57)	2 (29)	1 (14)	0 (0)	
Tumor	21	10 (48)	11 (52)	0.0265 ^{1,a}	11 (52)	7 (33)	3 (15)	0 (0)	
Normal	8	0 (0)	8 (100)		8 (100)	0 (0)	0 (0)	0 (0)	

¹Fisher's exact test. ^a*P* < 0.05. FOXP3: Forkhead box protein 3.**Table 2** Association of forkhead box protein 3 expression with pathologic grades in 140 hepatocellular carcinoma tissue arrays by immunohistochemical *n* (%)

Demographic or clinical characteristic	No. of tumor specimens (<i>n</i> = 140)	FOXP3		<i>P</i>	FOXP3 immunohistochemistry intensity score				<i>P</i>
		Positive	Negative		0	1	2	3	
Gender									
Male	119	28 (24)	91 (76)	0.6193 ²	91 (76)	20 (17)	7 (6)	1 (1)	0.4895 ¹
Female	21	6 (28)	15 (72)		15 (72)	3 (14)	3 (14)	0 (0)	
Age (yr)									
> 60	30	9 (30)	21 (70)	0.4103 ²	21 (70)	4 (13)	5 (17)	0 (0)	0.1542 ¹
≤ 60	110	25 (23)	85 (77)		85 (77)	19 (17)	5 (5)	1 (1)	
Differentiation grade									
Well	32	11 (34)	21 (66)	0.2410 ²	21 (66)	8 (25)	2 (6)	1 (3)	0.2887 ¹
Moderate	73	14 (19)	59 (81)		59 (81)	8 (11)	6 (8)	0 (0)	
Poor	35	9 (26)	26 (74)		26 (74)	7 (20)	2 (6)	0 (0)	
Tumor	140	34 (24)	106 (76)	0.0405 ^{1,a}	106 (76)	23 (16)	10 (7)	1 (1)	
Normal	14	0 (0)	14 (100)		14 (100)	0 (0)	0 (0)	0 (0)	

¹Fisher's exact test; ²Pearson χ^2 . ^a*P* < 0.05. FOXP3: Forkhead box protein 3.

Pathologists evaluated the expression level of FOXP3 in different HCC tissue samples according to the percentage of FOXP3 staining (Figure 4). Ten of 21 (48%) HCC tissue sections displayed FOXP3 staining. Interestingly, we noticed that FOXP3 was mainly localized in the nucleus of well differentiated HCC tissues and cytoplasm of moderately and poorly differentiated HCC tissues in tissue sections and array. To confirm the validity of the observation, we used two different anti-human FOXP3 antibodies (eBioscience and R&D, USA) to repeat the experiments. Both antibodies gave similar patterns about FOXP3 localization in HCC tissues.

Occurrence of FOXP3 is not associated with differentiation

The expression of FOXP3 at each histopathological grade were examined and recorded in all the patients. It is interesting that many cancerous patients were found to be FOXP3 positive, whereas the intensity of FOXP3 staining was low. The proportion of FOXP3 staining

cells had no differences among the histopathological grades in HCC tissue array. In this experiment, 11 of 32 grade I (34%), 14 of 73 grade II (19%), and 9 of 35 grade III (26%) carcinoma cores (Figure 5) were positive in FOXP3 staining (*P* = 0.2410, Pearson χ^2 test; Table 2). This result implicated that FOXP3 staining might be a useful prognostic factor, but not a clue in differentiation of HCC patients. We also found that normal liver tissues were devoid of FOXP3 expression (Figures 4 and 5). In addition, there was no relationship between FOXP3 expression and gender or age, no matter in tissue section or array (Tables 1 and 2).

Relationship between HCC risk factors and FOXP3 expression in tumor tissues

HCC is a heterogeneous tumor. Cirrhosis, HBV and HCV infections represent the major known risk factors for HCC. Those factors could cooperate or act alone to promote the incidence of HCC depending on the different pathways and molecules.

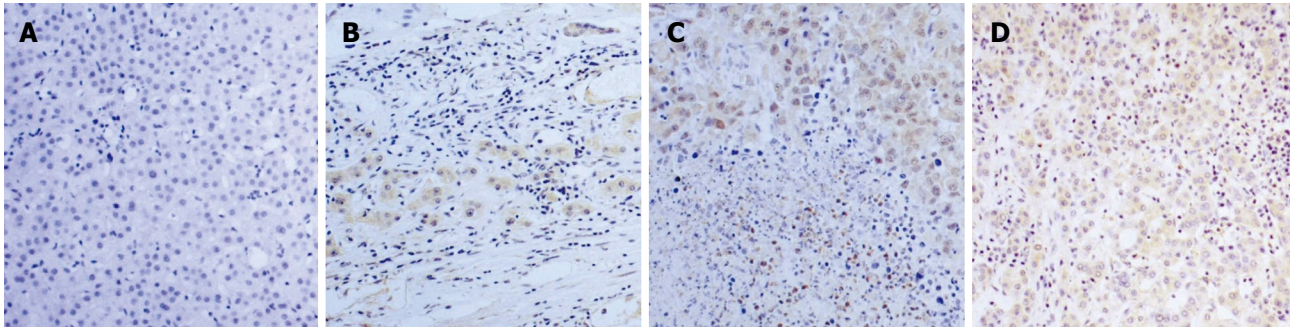


Figure 4 Hepatocellular carcinoma immunohistochemical score ($\times 100$). Forkhead box protein 3 (FOXP3) expression in hepatocellular carcinoma (HCC) by immunohistochemical (IHC). Images represent HCC tissues with IHC scores of 0 (A), 1 (B), 2 (C) and 3 (D). In IHC score 0, many FOXP3-positive lymphocytes are tumor-infiltrating Tregs.

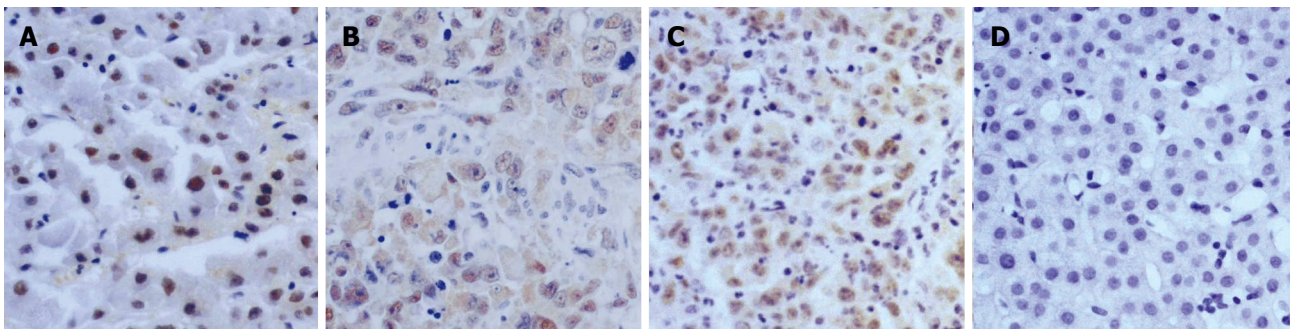


Figure 5 Hepatocellular carcinoma immunohistochemical staining ($\times 200$). Immunohistochemical (IHC) staining of paraffin embedded hepatocellular carcinoma (HCC) tissues revealed different levels of forkhead box protein 3 (FOXP3) expression in HCC cells (PCH101 antibody, eBioscience). A: Well-differentiated HCC (nuclear FOXP3 staining); B: Moderately-differentiated HCC (cytoplasmic FOXP3 staining); C: Poorly-differentiated HCC (cytoplasmic FOXP3 staining); D: Normal liver tissue (nuclear FOXP3 staining in Tregs).

Table 3 Relationship between hepatitis B virus infection (or cirrhosis) distribution and forkhead box protein 3 expression in 21 hepatocellular carcinoma samples

		FOXP3 expression		P
		-	+	
HBV infection	-	2	1	0.021 ^a
	+	9	9	
Cirrhosis	-	1	3	0.092
	+	10	7	

FOXP3: Forkhead box protein 3; HBV: Hepatitis B virus. ^a $P < 0.05$.

We detected the circulating HBV markers and liver cirrhosis levels to analyze the relationship between the expression of FOXP3 and HBV/cirrhosis to evaluate the potential clinical characteristics of FOXP3.

As shown in Table 3, among the HCC patients, 42.9% HBV infection and 33.3% cirrhosis of the section samples were FOXP3 positive. Statistical results revealed that FOXP3 expression coincided with the occurrence of cirrhosis ($P = 0.092$), while it is highly significant with HBV infection ($P = 0.021$) in HCC patients.

DISCUSSION

Although the factors and molecular events associated with

the progression of HCC are complex and not well established, FOXP3 has been shown to play an important role in Tregs in HCC invasion^[1,5,11]. In this study, we assessed the expression and subcellular localization of FOXP3 in hepatoma cell lines and HCC tissues to identify the fact that FOXP3 is expressed by tumor cells. We also identified some clinical characteristics of FOXP3 in HCC.

Few studies assessing FOXP3 expression in tumor tissues and cell lines have been reported. Hinz *et al.*^[12] described for the first time the expression and function of FOXP3 in pancreatic ductal adenocarcinoma cells and tissues. They detected FOXP3 expression in tumor cells of 24/39 patients with pancreatic carcinoma. Although they were unable to find a correlation between FOXP3 expression and tumor stage or survival rate, their findings indicate that pancreatic carcinoma cells share growth suppressive effects with Tregs depending on the function of FOXP3. Their results also suggest that FOXP3 may promote tumor cells to mimic characters of Tregs to represent an immune evasion function in microenvironment. Ebert *et al.*^[13] and Karanikas *et al.*^[14] reported that FOXP3 transcription factor was expressed by melanoma cells and lots of tumor cells. These evidences suggest that FOXP3 is related to tumor escape and can be used as a potential tumor antigen.

In agreement with their findings, *Foxp3* transcription and protein expression were found in hepatoma cell lines

(SMMC-7721 and Hepa-G2) in this study. After Confocal analysis, we noticed that nuclear and less cytoplasm FOXP3 expression was more prevalent in hepatoma cell lines. To our knowledge, this study provides the first evidence of nuclear and cytoplasm localization of FOXP3 in hepatoma cell lines. In addition, we found that FOXP3 was mainly expressed in the nucleus in well differentiated HCC tissues and cytoplasm in moderately and poorly differentiated HCC tissues. It implicates that the factors (such as cytokines, immune cells, ligand and receptor of tumor cells) coming from microenvironment and differentiation of tumor might induce a change in the subcellular localization of FOXP3 between cytoplasmic and nuclear expression patterns, which would result from post-translational modifications^[18]. Therefore, the heterogeneous subcellular localization of FOXP3 in hepatoma cell lines and HCC tissues may reflect the different post-translationally modified forms of FOXP3. In particular, previous reports revealed the interaction of FOXP3 with nuclear factor of activated T cells (NFAT, a transcription factor) in Tregs^[19]. It revealed that FOXP3 plays a key role in the formation of nuclear complexes that are important to regulate the transcription of functional genes^[20].

In this study, we also found some clinical characteristics of FOXP3 in HCC. The statistical results revealed that HBV infection had different distribution patterns compared with the expression of FOXP3 in HCC. In our previous study, FOXP3 was not found in the hepatitis and normal liver cells. Therefore, we may draw a conclusion that FOXP3 of tumor cells has little relationship with the inflammation induced by HBV infection alone. But because of the limited number of patients, this still needs further studies to validate. On the other hand, 70% of FOXP3 positive HCC cases were companied with liver cirrhosis. This indicates that the pathogenesis and molecules of cirrhosis may have some effects on the regulation of FOXP3 expression. FOXP3 may be involved in the progression of cirrhosis-induced HCC.

On the contrary, Zou *et al.*^[21,22] reported that functional somatic mutations and down-regulation of the *Foxp3* gene were commonly found in human breast cancer tissues. The expression of FOXP3 was correlated with HER-2/Erbb2 and SKP-2 overexpression. So the function and mechanism of FOXP3 may be different in various tumor cells, which still remains to be elucidated.

In conclusion, this is the first report of FOXP3 staining in hepatoma cell lines and HCC tissues, creating a new focus that FOXP3 is widely expressed in tumor cells and tissues. As the molecular mechanisms of FOXP3 still remain unclear, the real function of FOXP3 in different tumors need to be further studied so as to understand the critical molecular events associated with tumor progression.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. It is highly resistant to many conventional treatments and has a poor prognosis. forkhead box protein 3 (FOXP3) is considered to play an important role in naturally occurring regulatory T cells (Tregs). Recent reports have implicated that FOXP3 might have different effects on tumor cells. But the expression and mechanism of FOXP3 in HCC cells remain unclear.

Research frontiers

Few reports showed the expression of FOXP3 in pancreatic carcinoma cells, melanoma cells and other tumor cells. The reports found that FOXP3 expression was related to the regulation of several cytokines, such as IL-10 and TGF- β 2. FOXP3 might mediate the immune inhibiting efficacy of tumor cells to escape immune destruction. The authors assumed that FOXP3 may also be functional in HCC.

Innovations and breakthroughs

Interestingly, the authors found that hepatoma cell lines expressed FOXP3 mainly in nuclear and cytoplasm. But FOXP3 was almost expressed in nuclear in well differentiated HCC tissues and cytoplasm in moderately and poorly differentiated HCC tissues. It suggests that the microenvironment and differentiation of tumor might induce a change in the subcellular localization of FOXP3 between cytoplasmic and nuclear expression patterns. Therefore, the heterogeneous subcellular localization of FOXP3 in hepatoma cell lines and tissues may reflect the different post-translationally modified forms of FOXP3. The authors also found that the distribution of FOXP3 was similar to that of the cirrhosis, but not to that of HBV infection in HCC. Although the function of FOXP3 in HCC is not clear, the result suggests that FOXP3 may be involved in the cirrhosis-induced HCC. Altogether, those findings implicate that FOXP3 staining seems to be associated with a high risk in HCC.

Applications

This study is creating a new focus that FOXP3 is widely expressed in HCC cells and tissues. However, the real function of FOXP3 in HCC needs to be further studied so as to understand the critical molecular events in HCC progression.

Peer review

The manuscript by Wang *et al* provides evidences supporting FOXP3 expression both in human hepatoma cells and in HCC tumors. Since this is the first time that expression of FOXP3 has been reported in liver cancer, the information is of interest.

REFERENCES

- 1 **Bosch FX**, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127**: S5-S16
- 2 **Tsai WL**, Chung RT. Viral hepatocarcinogenesis. *Oncogene* 2010; **29**: 2309-2324
- 3 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50
- 4 **Korangy F**, Ormandy LA, Bleck JS, Klempnauer J, Wilkens L, Manns MP, Greten TF. Spontaneous tumor-specific humoral and cellular immune responses to NY-ESO-1 in hepatocellular carcinoma. *Clin Cancer Res* 2004; **10**: 4332-4341
- 5 **Ormandy LA**, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. *Cancer Res* 2005; **65**: 2457-2464
- 6 **Hori S**, Sakaguchi S. Foxp3: a critical regulator of the development and function of regulatory T cells. *Microbes Infect* 2004; **6**: 745-751
- 7 **Tanswell P**, Garin-Chesa P, Rettig WJ, Welt S, Divgi CR, Casper ES, Finn RD, Larson SM, Old LJ, Scott AM. Population pharmacokinetics of antifibroblast activation protein

- monoclonal antibody F19 in cancer patients. *Br J Clin Pharmacol* 2001; **51**: 177-180
- 8 **Scott AM**, Wiseman G, Welt S, Adjei A, Lee FT, Hopkins W, Divgi CR, Hanson LH, Mitchell P, Gansen DN, Larson SM, Ingle JN, Hoffman EW, Tanswell P, Ritter G, Cohen LS, Bette P, Arvay L, Amelsberg A, Vlock D, Rettig WJ, Old LJ. A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. *Clin Cancer Res* 2003; **9**: 1639-1647
 - 9 **Yagi H**, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 2004; **16**: 1643-1656
 - 10 **Allan SE**, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, Ziegler SF, Roncarolo MG, Levings MK. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* 2005; **115**: 3276-3284
 - 11 **Kobayashi N**, Hiraoka N, Yamagami W, Ojima H, Kanai Y, Kosuge T, Nakajima A, Hirohashi S. FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis. *Clin Cancer Res* 2007; **13**: 902-911
 - 12 **Hinz S**, Pagerols-Raluy L, Oberg HH, Ammerpohl O, Grüssel S, Sipos B, Grützmann R, Pilarsky C, Ungefroren H, Saeger HD, Klöppel G, Kabelitz D, Kalthoff H. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Res* 2007; **67**: 8344-8350
 - 13 **Ebert LM**, Tan BS, Browning J, Svobodova S, Russell SE, Kirkpatrick N, Gedye C, Moss D, Ng SP, MacGregor D, Davis ID, Cebon J, Chen W. The regulatory T cell-associated transcription factor FoxP3 is expressed by tumor cells. *Cancer Res* 2008; **68**: 3001-3009
 - 14 **Karanikas V**, Speletas M, Zamanakou M, Kalala F, Loules G, Kerenidi T, Barda AK, Gourgoulialis KI, Germeis AE. Foxp3 expression in human cancer cells. *J Transl Med* 2008; **6**: 19
 - 15 **Kuniholm MH**, Lesi OA, Mendy M, Akano AO, Sam O, Hall AJ, Whittle H, Bah E, Goedert JJ, Hainaut P, Kirk GD. Aflatoxin exposure and viral hepatitis in the etiology of liver cirrhosis in the Gambia, West Africa. *Environ Health Perspect* 2008; **116**: 1553-1557
 - 16 **Iwasa S**, Jin X, Okada K, Mitsumata M, Ooi A. Increased expression of seprase, a membrane-type serine protease, is associated with lymph node metastasis in human colorectal cancer. *Cancer Lett* 2003; **199**: 91-98
 - 17 **Ariga N**, Sato E, Ohuchi N, Nagura H, Ohtani H. Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast. *Int J Cancer* 2001; **95**: 67-72
 - 18 **Chen C**, Rowell EA, Thomas RM, Hancock WW, Wells AD. Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *J Biol Chem* 2006; **281**: 36828-36834
 - 19 **Wu Y**, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006; **126**: 375-387
 - 20 **Marson A**, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, Levine SS, Fraenkel E, von Boehmer H, Young RA. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 2007; **445**: 931-935
 - 21 **Zuo T**, Wang L, Morrison C, Chang X, Zhang H, Li W, Liu Y, Wang Y, Liu X, Chan MW, Liu JQ, Love R, Liu CG, Godfrey V, Shen R, Huang TH, Yang T, Park BK, Wang CY, Zheng P, Liu Y. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell* 2007; **129**: 1275-1286
 - 22 **Zuo T**, Liu R, Zhang H, Chang X, Liu Y, Wang L, Zheng P, Liu Y. FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. *J Clin Invest* 2007; **117**: 3765-3773

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