·Basic Research·

Relationship and clinical significance of TGF-β1 expression with Treg cell infiltration in hepatocellular carcinoma

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[**Abstract**] **Background and Objective:** There are few studies about origins of regulatory T (Treg) cells increased in primary hepatocellular carcinoma (HCC) tissue. Studies showed that Treg cells could be induced by transforming growth factor-β1 (TGF-β1), but the relation between TGF-β1 expression and Treg cell infiltration is unclear in HCC tissue. This study was to investigate the expression of TGF-β1 and correlations with the amount of Treg cells in HCC, and to evaluate their clinical values in predicting the prognosis of HCC. **Methods:** Envision immunohistochemistry was used to detect the expression of TGF-β1 and Foxp3 in 102 specimens of HCC tissue and paired adjacent non-tumor liver tissue. **Results:** Of the 102 specimens of HCC, 41 showed low TGF-β1 expression and 61 (59.8%) showed high expression; of the 102 specimens of adjacent non-tumor tissue, 22 showed low TGF-β1 expression and 80 (78.4%) showed high expression. The high expression rate of TGF-β1 was significantly lower in HCC than in adjacent non-tumor tissues (P = 0.001). Average Foxp3$^+$ cell density in HCC was 2.98 cells/HP, but there was very few or no expression of Foxp3 in adjacent non-tumor liver tissue. Expression of TGF-β1 was positively correlated with expression of Foxp3 in HCC tissues (r = 0.228, P = 0.021). The expression of TGF-β1 was significantly higher in HCC tissues with high preoperative AFP concentration than in those with low preoperative AFP concentration (P = 0.023). TGF-β1 and Foxp3 expression had no correlations with tumor diameter, tumor capsule, liver cirrhosis, and so on. The 5-year survival rate was not different between HCC tissues with high and low TGF-β1 expression (P = 0.790); however, it was significantly lower in HCC tissues with high Treg cell infiltration than in those low infiltration (25% vs. 44%, P = 0.007). Cox multivariate analysis showed that the number of Treg cells and tumor capsule were independent prognostic factors (P = 0.021, P = 0.001). **Conclusions:** Expression of TGF-β1 may relate to the infiltration of Treg cells in HCC tissues, but the relation need to be further investigated. The number of Treg cells in HCC tissues could be used as a potential immunological prognostic indicator for HCC patients after resection.

Key words: Liver neoplasm, regulatory T cells, transforming growth factor-β1

The immune cells in the local immunity microenvironment of tissue play an important role in the occurrence and development of tumor. Our previous studies have found that the level of regulatory (Treg) cells in the peripheral blood and tumor tissue of patients with hepatocellular carcinoma (HCC) was elevated, and appeared to has negative correlation with the prognosis[1,2]. Tumor growth factor-β1 (TGF-β1) is a multifunctional cytokine, which can inhibit the growth of tumor cells[3]. Recent studies have showed that TGF-β1 possessed the biological property inducing the accumulation of local Treg cells in tumor. In vitro study suggested that TGF-β1 could induce $CD4^+CD25^-$ T cells to transform to Treg cells[4]. However, in HCC tissue, the relationship between TGF-β1 and Treg cell infiltration is still unclear. The present study used immunohistostaining to detect the expression of TGF-β1 in HCC and paired adjacent normal liver tissues, analyzed the relationship between TGF-β1 expression and Treg cell infiltration, and investigated the relationship among TGF-β1 expression, Treg cell infiltration, clinicopathologic characteristics and prognosis.

Materials and Methods

Clinical data

The HCC specimens and clinical data of 102 patients with pathologically confirmed primary HCC treated between February

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1999 and November 2001 by radical operation at Sun Yat-sen University Cancer Center were collected. There were 85 men and 17 women, with a median age of 49.5 years old (range: 13–75 years). According to the UICC (2003) staging criteria, 70 patients (68.6%) had stage I disease, 7 (6.9%) had stage II disease, 23 (22.5%) had stage III disease, and 2 (2%) had stage IV disease. None of the patients had received any anti-tumor therapy or immunotherapy before. The patients were followed up till December 30, 2006. The follow-up rate was 100%. The median survival time was 36 months (range: 1–84 months).

Experimental method

Immunohistochemisty EnVision two-stage method was utilized to detect the expression of Foxp3 and TGF-β1 in HCC and adjacent normal liver tissue. All the fresh specimens were fixed by 10% formalin, drawn within 48 h, embedded by paraffin, sectioned serially to 4 μm in thickness for each piece. Foxp3 mouse anti-human monoclonal antibody was bought from Abcom Company with the concentration of working solution to be 1:50; TGF-β rabbit anti-human monoclonal antibody was bought from Santa Cruz Biotechnology Company, with the concentration of working solution to be 1:500, and PBS was taken to be the substitute for the two primary antibodies as the negative control. EnVision kit was bought from Dako Company. Antigens were repaired by microwaves in sodium citrate damping fluid (pH = 6.0). The other procedures were operated strictly according to manual.

Result assessment

Assessment criteria for positive: brown in Foxp3 staining, located in the nucleus; brown in TGF-β1 staining, located in cytoplasm. $Foxp3^+$ T cell scoring criteria: five various high power fields (×400) counting were selected randomly, and the average value was obtained. TGF-β1 was scored by staining area and intensity. It was scored zero when the area occupied by positive cells was zero; one when the area was less than 10%; two when the area was between 10% and 50%; three when the area was between 51% and 80%; and four when the area was more than 80%. It was scored zero when the staining intensity was negative; one when the intensity was weakly positive; two when the intensity was medium positive; three when the intensity was strong positive. Total score used the method of area multiplied by intensity. Those scored equal to or less than 4 belonged to low expression group, and those more than 4 belonged to high expression group[5].

Statistical analysis

SPSS16.0 software was used for statistical analysis. Measurement data was expressed by mean ± standard deviation, χ^2 test was used for group comparison, Kaplan-Meier and log-rank test analyzing method were utilized for survival analysis, and spearman correlation analysis was used for the correlation analysis between two indexes.

Results

Distribution status of TGF-β1 and Foxp3 in HCC tissue and adjacent normal liver tissue

Foxp3 staining positive cells diffusely distributed in the tumor tissue (Figure 1A). The average density of Treg cells in HCC tissue was 2.98/HP, whereas it was rarely seen in the adjacent normal liver tissue (Figure 1B). The TGF-β1 staining positive cells distributed in lamellar and relatively light staining in HCC tissue (Figure 1C) and adjacent normal liver tissue (Figure 1D). The high expression rate of TGF-β1 was 59.8% consisting of 41 patients with low expression and 61 patients with high expression in HCC tissue. The high expression rate of TGF-β1 was 78.4% consisting of 22 patients with low expression and 80 patients with high expression in adjacent normal liver tissue. The high expression rate of TGF-β1 of the HCC tissue was lower than the adjacent normal liver tissue (P = 0.001).

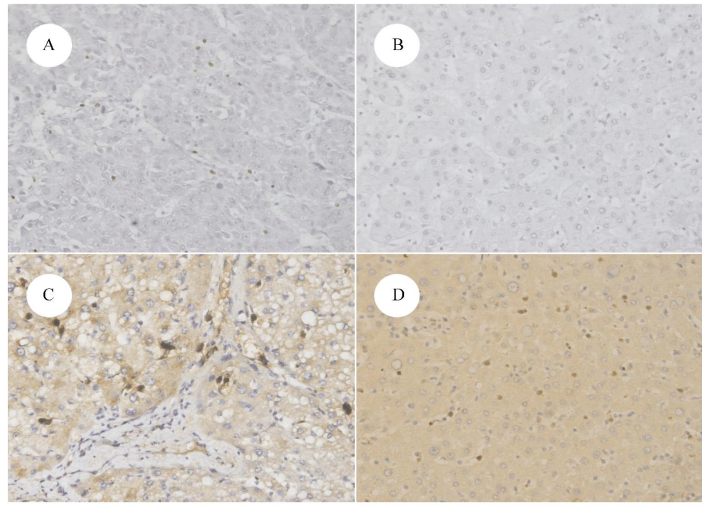

Figure 1 Expression of Foxp3 and TGF-β1 in primary hepatocellular carcinoma and adjacent normal tissues (SP ×400)

A, Foxp3 is expressed in nuclei of hepatocellular carcinoma cells. B, Foxp3 is not expressed in adjacent normal liver tissue. C, TGF-β1 is expressed in cytoplasm of hepatocellular carcinoma cells. D, TGF-β1 is also expressed in cytoplasm in adjacent normal liver tissue.

Relation between the expression of TGF-β1 and Foxp3 in the HCC tissue and the clinicopathological characteristics

The relation between the amount of Treg cells in the HCC tissue, the expression of TGF-β1 and the pathological indexes of patients such as age, tumor recurrence, capsule and hepatic cirrhosis extent was analyzed. The results indicated that there was no significant correlation between the amount of Treg cells in the HCC tissue and each clinicopathological index; the expression of TGF-β1 in the HCC tissue was higher in the preoperative high plasma AFP concentration level group than in the preoperative low plasma AFP concentration level group (P =0.023) (Table 1).

Correlation between the expression of TGF-β1 and Treg cell infiltration

Spearman correlation analysis showed that the expression of TGF-β1 in HCC tissue appeared to be positive correlation with

Table 1 Correlations of Foxp3 and TGF-β expression with clinicopathologic characteristics of primary hepatocellular carcinoma

Characteristic	Patient No.	Foxp3 expression (patient No. (%))			TGF-β1 expression (patient No. (%))		
		High	χ^2	*P*	High	χ^2	*P*
Gender			0.196	0.658		2.945	0.086
Male	85	40(47.1)			54(63.5)		
Female	17	9(52.9)			7(41.2)		
Age (years)			0.571	0.450		3.561	0.059
⩽ 60	80	40(50.0)			44(55.0)		
> 60	22	9(40.9			17(77.3)		
AFP (μg/L)			0.827	0.363		5.165	0.023
⩽ 25	33	18(54.5)			25(75.8)		
> 25	69	31(44.9)			36(52.2)		
UICC stage			0.214	0.644		2.075	0.150
I+II	75	35(46.7)			48(64.0)		
III+IV	27	14(51.9)			13(48.1)		
Capsule			0.028	0.867		0.588	0.443
Complete	47	23(48.9)			30(63.8)		
Incomplete	55	26(47.3)			31(56.4)		
Tumor diameter			2.357	0.125		0.903	0.342
⩽ 5 cm	38	22(57.9)			25(65.8)		
> 5 cm	64	27(42.2)			36(56.3)		
Cirrhosis			2.719	0.099		0.028	0.867
No	24	8(33.3)			14(58.3)		
Yes	78	41(52.6)			47(60.3)		
Tumor differentiation			1.312	0.252		0.287	0.592
I+II	58	25(43.1)			36(62.1)		
III+IV	44	24(54.5)			25(56.8)		
Recurrence			0.927	0.336		0.588	0.443
Yes	55	24(43.6)			31(56.4)		
No	47	25(53.2)			30(63.8)		
Tumor thrombus			1.056	0.304		0.330	0.856
Yes	11	5(45.5)			5(45.5)		
No	91	56(61.5)			44(48.4)		

the Treg cells in HCC tissue (r = 0.228, P = 0.021).

Relationship between the Treg cell infiltration in HCC tissue, expression of TGF-β1 and clinical prognosis

Within the period of follow-up, tumor relapse occurred in 55 patients (53.9%) and 40 patients (39.2%) died of tumor recurrence. The five-year survival of patients in high Treg expression group and low Treg expression group was 25% and 44% , respectively. Statistical significance existed in the difference between them (P < 0.05) (Figure 2). There was no statistical significance in the difference between the accumulative survival of patients in high TGF-β1 expression group and low TGF-β1 expression group (P = 0.790).

Cox regression model analysis on the prognostic factors

Multivariate analysis with Cox regression model was adopted to analyze the relationship between the clinical case data including the number of Treg cells, TGF-β1 expression, tumor diameter, tumor capsule, preoperative AFP concentration and cancer embolus and the prognosis. Results showed that the the number of Treg cells in the HCC tissue (P = 0.021) and the tumor capsule (P = 0.001) were the independent risk factors that influencing prognosis (Table 2).

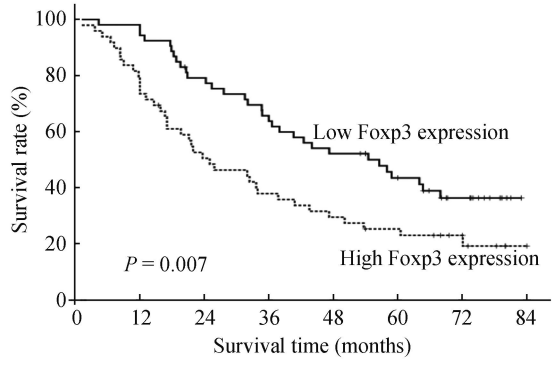

Figure 2 The Kaplan-Meier survival curves of the hepatocellular carcinoma patients with high Foxp3 expression and low Foxp3 expression

Discussion

TGF-β1 is a kind of cytokine with multifunction, and is related

Table 2 Cox regression prognostic analysis for primary hepatocellular carcinoma

Risk factor	B	SE	Wald	P	Exp(B)
Treg	0.614	0.265	5.363	0.021	1.848
Tumor capsule	−1.012	0.274	13.612	0.001	0.363

to cell proliferation, cell differentiation and the formation of extracellular matrix. Its function in tumor is complex, and can stimulate as well as inhibit tumor growth[6]. The expression of TGF-β1 in HCC tissue varied according to different papers. Bedossa *et al*.[7] found that the expression of TGF-β1 in HCC tissue increased and was more than the adjacent normal liver tissue. However, Paik *et al*.[8] found that the expression of TGF-β1 in HCC tissue decreased, and was lower than the adjacent normal liver tissue. The present study was in consistence with the latter one. The reason for the difference mentioned above is still unclear for the present, and it might be concerned with the selected number of cases by researchers and various experimental methods.

Treg is a kind of T lymphocyte subpopulation which has the effect of immune down regulation, inhibiting the proliferation and function of responsive T cells by direct contact or excreting cytokine such as TGF-β1, and taking part in the immune escape of tumor[9]. Removal of Treg cells could improve the immune response of organism, demonstrating promising application prospect. However, it could revive to its former level after removal, which held back the Treg cell-targeted therapy[10]. TGF-β1 is an important cytokine that could induce the generation of Treg cells. Result of animal experiment indicated that TGF-β1 could induce the generation of Treg cells[11]. In vitro experiment suggested that TGF-β1 secreted by renal carcinoma cell line and prostate cancer cell line could induce the spleen $CD4^+CD25^-$ T cell of mice to transform to Treg cells[4]. The present study found that the number of Treg cells in the HCC tissue appeared to be positive correlation with the expression of TGF-β1 (P = 0.021). The Treg cell infiltration was higher in the high TGF-β1 expression group than in the low TGF-β1 expression group, which indicated that the TGF-β1 expression in tumor tissue could increase the Treg cell infiltration in the local tumor, taking part in the immune escape of tumor. What's more, the present study found that the TGF-β1 expression was higher in the adjacent normal liver tissue than in the HCC tissue, while there was little Treg cell accumulation. This might be related to the low TGF-β1 activity in the adjacent normal liver tissue, which failed to induce the local infiltration of Treg cells[12].

When analyzing the prognostic factors of HCC, the present study found that Treg was an independent risk factor influencing prognosis. Clinical study suggested that portal vein tumor thrombus was one of the prognostic risk factors of patients with HCC, while portal vein tumor thrombus failed to enter the final Cox model in the present study, which might be due to the relatively small number of patients with portal vein tumor thrombus in our group. Dispute still exists on the relationship between the expression of TGF-β1 and patients' prognosis at present. Takanami *et al*.[13] detected 88 specimens of lung adenocarcinoma by immunohistochemisty and found that the expression of TGF-β1 appeared to be negative correlation with the clinical prognosis of patients, and those who with high expression had worse clinical prognosis. Hazelbag *et al*.[14] used the method of PCR to detect 108 cervical cancer tissues, and did not find any significant correlation between the expression of TGF-β1 and patients' prognosis. As demonstrated in the report by Ikeguchi *et al*.[15], the expression of TGF-β1 mRNA in the HCC appeared positive correlation with patients' prognosis, and the 5-year survival of patients in the high expression group was higher than that in the low expression group. The differences among the studies mentioned above might be correlated with inconsistent experimental methods used by various researchers. In addition, the studies mentioned above have not further analyzed the active form of TGF-β1. The local active TGF-β1 in gastric cancer tissue was a sensitive index for patients' prognosis, and the active TGF-β1 appeared negative correlation with the prognosis of patients, whereas no significant correlation existed between the total TGF-β1 and patients' prognosis[7,13]. The present study also found that there was no significant correlation between TGF-β1 and the prognosis of patients, which indicated that total TGF-β1 might not be a sensitive index that reflects patients' prognosis.

To sum up, there was significantly more Treg cell infiltration in the HCC tissue than in adjacent normal liver tissue which indicated that the immune function of the organism was in the status of inhibition. The expression of TGF-β1 in the HCC cells appeared positive correlation with the number of Treg cells, which indicated that TGF-β1 was a inducing factor for Treg cell accumulation in HCC tissue. Inhibiting or eliminating TGF-β1 could provide new train of thought for treatment trying to reduce or removal the local infiltrating Treg cells in the HCC tissue.

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