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## Original article

# Clinical significance of circulating IL-10 and fibronectin levels in hepatocellular carcinoma patients with HBV infection

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## ABSTRACT

**Background/Introduction:** Hepatocellular carcinoma (HCC) is the major cause of cancer-related death in Taiwan and is strongly associated hepatitis B virus (HBV) infections. Previous studies observed an imbalanced T-helper (Th)1/Th2 cytokine profile in HCC patients, however, less attention has been paid to the variation of Th2 cytokines, anti-inflammatory cytokines such as IL-4 and IL-10, in HCC patients. Increased expression of Fibronectin, VEGF and TGF- $\beta$ 1 in HCC patients has been observed, the relationship between these factors and other biomarkers remains unknown.

**Purpose:** This study examined the clinical significance of circulating interleukin-10 and fibronectin levels in HBV-infected hepatocellular carcinoma (HCC) patients.

**Methods:** HCC patients were classified according to international tumor-node-metastasis staging system as I ( $n = 8$ ), II ( $n = 24$ ), III ( $n = 20$ ) and IV ( $n = 10$ ). Thirty healthy subjects were included as control group.

**Results:** Compared with the control group, 7 test cytokines [interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, interferon- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ ] were significantly higher in HCC patients ( $p < 0.05$ ). Plasma TNF- $\alpha$  concentration in HCC patients increased from stage to stage ( $p < 0.05$ ), while concentrations of both IL-4 and IL-10 decreased from Stage II to Stage IV ( $p < 0.05$ ). HCC patients also had significantly higher plasma levels of VEGF, TGF- $\beta$ 1 and fibronectin than the control group ( $p < 0.05$ ). Within HCC groups, both vascular endothelial growth factor (VEGF) and fibronectin levels decreased in Stage IV. VEGF, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) or fibronectin were negatively correlated with IL-10, and the correlation coefficients were lower than 0.7. Both VEGF and TGF- $\beta$ 1 were positively correlated with fibronectin, and the correlation coefficients were higher than 0.7.

**Conclusion:** The circulating levels of IL-10 and fibronectin may reflect progression of HCC. Thus, monitoring these biomarkers may benefit HCC progression evaluation.

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## 1. Introduction

Liver cancer, also called hepatocellular carcinoma (HCC), is the most common malignancy in the world [1]. In Taiwan, HCC is

the major cause of cancer-related death [2], and is strongly associated hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections [3,4]. Thus, virus infection is an important variable in clinical pathological investigation of HCC.

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**Table 1 – Mean ± SD baseline characteristics in HCC patients and healthy control group.**

Parameters	HCC				
	Control	I	II	III	IV
	n = 30	n = 8	n = 24	n = 20	n = 10
Body mass index (kg/m <sup>2</sup> )	25.1 ± 2.6	24.5 ± 2.2	23.8 ± 1.7	21.2 ± 2.5	20.3 ± 1.6
Albumin (g/dL)	4.41 ± 0.43	4.17 ± 0.37	3.69 ± 0.51 <sup>§</sup>	3.19 ± 0.67 <sup>§</sup>	3.04 ± 0.73 <sup>§</sup>
Creatinine (mg/dL)	0.74 ± 0.18	1.10 ± 0.29	1.08 ± 0.25	1.23 ± 0.19	1.34 ± 0.26
Uric acid (μmol/L)	210.3 ± 19.4	276.5 ± 22.5 <sup>§</sup>	358.4 ± 40.7 <sup>§</sup>	369.3 ± 52.4 <sup>§</sup>	377.5 ± 34.0 <sup>§</sup>
Bilirubin (mg/dL)	0.49 ± 0.13	0.92 ± 0.21	1.26 ± 0.31	2.29 ± 0.27 <sup>§</sup>	3.31 ± 0.43 <sup>§</sup>
α fetoprotein (ng/L)	25.1 ± 4.2	690.4 ± 170.2 <sup>§</sup>	4872.5 ± 434.7 <sup>§</sup>	7421.0 ± 653.8 <sup>§</sup>	7038.2 ± 559.2 <sup>§</sup>
Associated diseases					
Diabetes	2	1	2	1	0
Renal insufficient	0	0	2	1	1
Hypertension	3	1	0	0	1

<sup>§</sup>Means significantly different from control group, *p* < 0.05.

An imbalance between T-helper (Th)1 and Th2 cytokines has been observed in HCC patients [5,6]. The increased expression of several proinflammatory cytokines such as interleukin (IL)-6 and Th1 cytokines such as IL-1 in HCC patients has been reported [6,7]. The elevation of these cytokines means HCC deterioration including tumor growth and metastasis [7,8]. So far, less attention has been paid to the variation of Th2 cytokines, anti-inflammatory cytokines such as IL-4 and IL-10, in HCC patients. Since Th2 cytokines possess anti-inflammatory activity, the alteration of these cytokines may also affect HCC progression. Fibronectin is an extra cellular matrix glycoprotein, the expression of which is increased in liver tumor growth [9]; and increased fibronectin has been linked to resistance to therapy [10]. Apparently, fibronectin plays an important role in cancer progression, and is thus hypothesized to be highly associated with HCC progression. It is noted that most information regarding inflammatory stress of HCC is obtained from malignant tumors via surgical process. It may be more practical and feasible if the clinical information associated with inflammation and anti-inflammation, or the so-called Th1/Th2 cytokine profile, of HCC patients could be obtained from circulation via blood sampling.

Vascular endothelial growth factor (VEGF) is an angiogenic factors responsible for tumor angiogenesis: VEGF expression

in tumor tissue is correlated with early metastasis spread and poor prognosis [11,12]. Transforming growth factor-β1 (TGF-β1) is highly expressed in many malignant tumors including HCC [13]. Although increased expression of VEGF and TGF-β1 in HCC patients has been observed, the relationship between these two factors and other biomarkers remains unknown.

Clinically, HCC patients could be classified according to the international tumor-node-metastasis (TNM) staging system [2]. The major purpose of this study was to examine the variation of IL-4, IL-10, fibronectin, VEGF, and TGF-β1 in HBV infected HCC patients classified by TNM staging. These results will enhance the understanding about inflammation variation presented in HCC patients.

## 2. Materials and methods

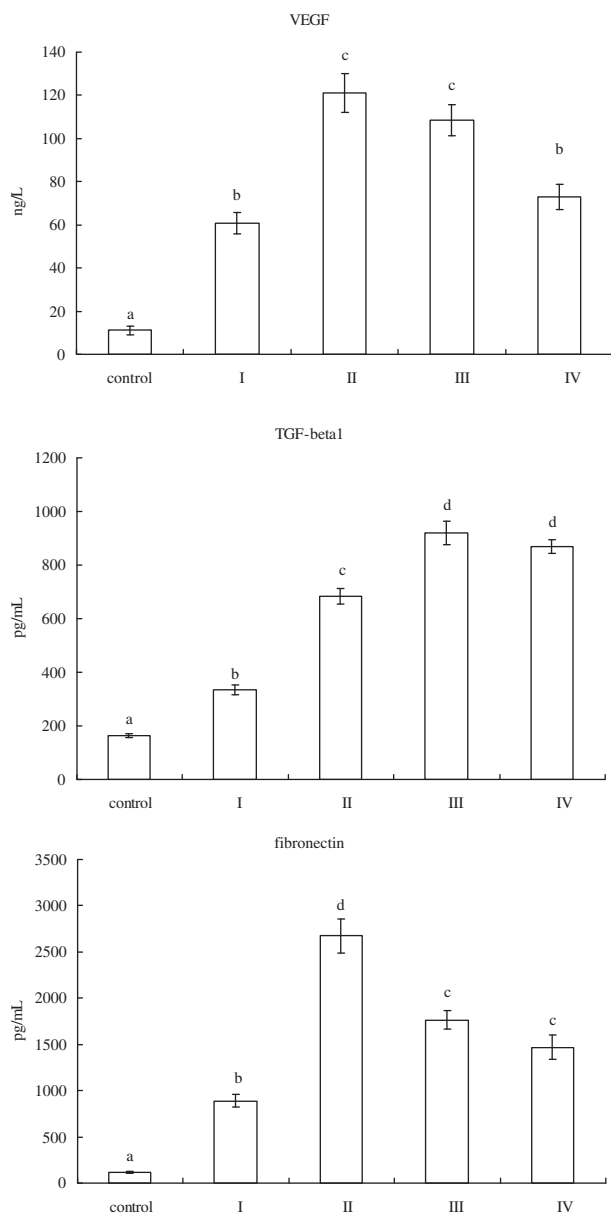
### 2.1. Patients and healthy individuals

This study protocol was approved by Ethical Committee of the Medicine Faculty at Chung Shan Medical University. Sixty-two patients with HBV infection and cytologically or histologically confirmed liver cancer at Chung Shan Medical University Hospital between May 2005 and October 2006 were included in

**Table 2 – Mean ± SD plasma concentrations of two proinflammatory cytokines (IL-6, TNF-α), three Th1 cytokine (IL-1, IL-2, IFN-γ), and two Th2 cytokines (IL-4, IL-10) in healthy control group and HCC patients at different TNM stage.<sup>1</sup>**

	Control	HCC			
		I	II	III	IV
IL-6, pg/mL	19.2 ± 3.6 <sup>a</sup>	135.3 ± 10.1 <sup>b</sup>	240.8 ± 21.5 <sup>c</sup>	332.7 ± 29.2 <sup>d</sup>	316.4 ± 32.5 <sup>d</sup>
TNF-α, pg/mL	23.8 ± 4.5 <sup>a</sup>	165.7 ± 16.3 <sup>b</sup>	257.6 ± 25.4 <sup>c</sup>	370.5 ± 34.8 <sup>d</sup>	454.7 ± 37.6 <sup>e</sup>
IL-1, pg/mL	19.4 ± 6.2 <sup>a</sup>	89.6 ± 10.5 <sup>b</sup>	180.5 ± 23.4 <sup>c</sup>	223.7 ± 28.6 <sup>c</sup>	245.7 ± 23.4 <sup>c</sup>
IL-2, pg/mL	21.3 ± 6.7 <sup>a</sup>	76.8 ± 13.2 <sup>b</sup>	151.7 ± 20.6 <sup>c</sup>	198.6 ± 23.8 <sup>c</sup>	160.5 ± 15.7 <sup>c</sup>
IFN-γ, pg/mL	18.0 ± 4.8 <sup>a</sup>	84.0 ± 9.3 <sup>b</sup>	177.4 ± 14.9 <sup>c</sup>	238.0 ± 22.5 <sup>d</sup>	263.2 ± 27.8 <sup>d</sup>
IL-4, pg/mL	18.7 ± 5.4 <sup>a</sup>	134.9 ± 18.3 <sup>c</sup>	156.3 ± 21.7 <sup>c</sup>	97.1 ± 9.2 <sup>b</sup>	80.6 ± 9.4 <sup>b</sup>
IL-10, pg/mL	20.5 ± 8.5 <sup>a</sup>	140.5 ± 15.7 <sup>c</sup>	245.2 ± 17.4 <sup>d</sup>	166.8 ± 11.6 <sup>c</sup>	71.3 ± 8.5 <sup>b</sup>

<sup>a–e</sup>Means in a row without a common letter differ, *p* < 0.05.



**Fig. 1** – Mean  $\pm$  SD plasma level of vascular endothelial growth factor (VEGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and fibronectin in healthy control group and HCC patients at different TNM stage. <sup>a-d</sup>Means among bars without a common letter differ,  $p < 0.05$ .

this study. Chronic HBV infection was confirmed by the presence of serum hepatitis B virus surface antigen (HBsAg), hepatitis B virus extracellular antigen (HBeAg) and HBV DNA. HBsAg and HBeAg were measured by radioimmunoassay (Abbott Laboratories, Chicago, IL, USA) and electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, USA), respectively. Patients infected with HCV, and those with habitual alcohol intake, any other liver diseases (alcohol-, drug-, or obesity-induced liver disease, autoimmune hepatitis, hemochromatosis,  $\alpha$ -1 anti-trypsin deficiency, Wilson disease or cirrhosis) were excluded. Patients with serum creatinine  $>15$  mg/L, absolute neutrophil count  $<1 \times 10^9/L$ , platelet count  $<50 \times 10^9/L$  or hemoglobin  $<100$  g/L were also excluded. These patients were aged 37 to 80 years (mean 64.1 years), were taking no therapy, and were newly diagnosed. HCC patients were classified according to the TNM staging system. The clinicopathological characteristics of these 62 HCC patients are shown in Table 1. Thirty healthy control participants (17 male, age 47–82 years, mean 61.3 years) were also included for comparison.

## 2.2. Blood sampling and biochemical measurements

Informed consent for study participation was obtained from 62 HCC patients and 30 healthy control subjects. A 15 mL peripheral blood sample was drawn from each participant after an overnight fasting. Plasma was separated from erythrocyte immediately after blood collection. Plasma levels of IL-1, IL-2, IL-4, IL-6, IL-10, interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA, USA). Samples were run in duplicate, and according to the manufacturer's instructions. The sensitivity of assay with the lower limit was 5 nmol/L for IL-1, IL-2, IL-4, IL-6, IL-10 and 10 nmol/L for IFN- $\gamma$  and TNF- $\alpha$ . Plasma TGF- $\beta$ 1 and VEGF levels were measured by commercial ELISA kit (Quantikine Human VEGF, R&D System, Minneapolis, MN, USA). The sensitivity of assay with the lower limit was 5.0 ng/L, the intra-assay and interassay variabilities were 6.7% to 5.1% and 8.8% to 6.2%, respectively.

## 2.3. Statistical analysis

Each measurement was analyzed from 62 liver cancer patients and 30 healthy controls. All data presented in this study are mean  $\pm$  SD. Data were subjected to analysis of variance (ANOVA) and differences with  $p < 0.05$  were considered to be significant. Correlations between two variables

**Table 3** – Correlation coefficients among IL-1, IL-2, IL-4, IL-10, VEGF, fibronectin, and TGF- $\beta$ 1 in 62 HCC patients.

	IL-1	IL-2	IL-4	IL-10	VEGF	fibronectin	TGF- $\beta$ 1
IL-1	1.000	0.483	-0.464	-0.545	0.570	0.528	0.493
IL-2		1.000	-0.425	-0.624	0.584	0.605	0.414
IL-4			1.000	0.672	-0.636	-0.715*	-0.702*
IL-10				1.000	-0.749*	-0.827*	-0.811*
VEGF					1.000	0.784	0.773*
Fibronectin						1.000	0.841*
TGF- $\beta$ 1							1.000

\*Means  $p < -0.05$ .

were calculated by simple regression analysis (Minitab Inc., State College, Philadelphia, USA).

### 3. Results

As shown in Table 1, HCC patients had lower albumin, higher uric acid, and higher  $\alpha$  fetoprotein concentrations in plasma than the control group ( $p < 0.05$ ). Bilirubin level in HCC patients at Stages III and IV was significantly increased ( $p < 0.05$ ). Plasma levels of cytokines in HCC patients are shown in Table 2. Compared with the control group, the concentrations of 7 test cytokines were significantly higher in HCC patients ( $p < 0.05$ ). Plasma TNF- $\alpha$  concentration in HCC patients increased from stage to stage ( $p < 0.05$ ). Both IL-4 and IL-10 levels were decreased from Stage II to Stage IV ( $p < 0.05$ ). Plasma levels of VEGF, TGF- $\beta$ 1 and fibronectin from healthy control group and HCC patients at different TNM stage are presented in Fig. 1. HCC patients had significantly higher plasma concentrations of VEGF, TGF- $\beta$ 1 and fibronectin than the control group ( $p < 0.05$ ). Within HCC groups, both VEGF and fibronectin concentrations decreased in Stage IV. The relationships among test factors in HCC patients is shown in Table 3. VEGF, TGF- $\beta$ 1, and fibronectin were negatively correlated with IL-10, with a correlation coefficient lower than  $-0.7$ . Both VEGF and TGF- $\beta$ 1 were positively correlated with fibronectin, and the correlation coefficient was higher than 0.7.

### 4. Discussion

The increased expression of several inflammatory cytokines such as IL-6 and TNF- $\alpha$  in HBV related liver cancer development has been reported previously [14]. Our present study further found that the release of Th1 and Th2 cytokines including IL-1, IL-2, IL-4, and IL-10 in circulation was markedly increased in HCC patients, which supported that inflammation and imbalance between Th1 and Th2 cytokines were involved in HBV-associated HCC deterioration.

It is known that IL-4 and IL-10 are anti-inflammatory immunomodulatory cytokines because they can induce expression of the IL-1R antagonist, and down-regulate the production of proinflammatory cytokines from human monocytes [15]. Moreover, IL-4 has a direct inhibitory effect on the development of human Th1 cells, and IL-10 is able to prevent Th1 effector function by reducing long-lasting T cell responsiveness [16,17]. Thus, the observed IL-4 and IL-10 increase in HCC patients at early stages (I+II) in our present study implied that the host self-defense system tended to suppress the inflammation reaction or to maintain cytokine balance. However, the overwhelming inflammation occurring in the late stages of liver cancer lowered IL-4 and IL-10 production, which suggested that the host's self-protection capability was diminished. Furthermore, the reduced IL-4 and IL-10 expression might indirectly favor expression of Th1 cytokines, which in turn exacerbated imbalance between Th1 and Th2 cytokines. It has been indicated that IL-4 is a potent inhibitor of hepatocyte growth factor, and may retard invasion and metastasis of carcinoma cells [18]. Thus, the variation of circulating IL-4 and IL-10 levels could be

considered as predictors for evaluating host self-defense capability, liver immune function, and/or cancer progression.

Tumor angiogenesis is essential for solid tumorigenesis, growth, invasion and metastasis [19]. The elevation of circulating VEGF level indicated a promotion of tumor angiogenesis because VEGF benefited cancer cells spreading into normal liver parenchyma [19,20]. TGF- $\beta$ 1 could stimulate the metastatic capacity of tumor cells, and thus has been considered as a predictor for poor survival in HCC patients [21,22]. In the present study, TGF- $\beta$ 1 and VEGF levels in HCC patients were elevated, which supported that both were indicators to reflect HCC progression. Fibronectin could activate focal adhesion kinase, increase matrix metalloproteinase expression and promote cancer cell invasion and/or migration [23,24]. We found that circulating VEGF and fibronectin levels were dramatically reduced in patients at Stage IV. Although the mechanism remains unknown, it is possible that patients at the final cancer stage lost their capability to synthesize these molecules because of liver malfunctions. Further large scale clinical study is necessary to confirm the role of VEGF and fibronectin in HCC deterioration. In addition, we noted that VEGF, TGF- $\beta$ 1 and fibronectin levels in circulation were negatively correlated with IL-10 and IL-4. These relationships imply that the increased production of VEGF, TGF- $\beta$ 1, and fibronectin impaired the host's anti-inflammatory protection, and enhance inflammatory reactions. These findings indicate the clinical significance of these biomarkers in HCC progression. Plasma concentrations of IL-4, IL-10, VEGF, TGF- $\beta$ 1, and fibronectin are not routinely measured for HCC patients, at least in Taiwan. Clinical physicians and researchers should consider measuring these factors to assist clinical evaluation for HCC patients.

In conclusion, this clinical study provided several novel findings regarding the variation in the circulating levels of IL-4, IL-10, VEGF, TGF- $\beta$ 1 and fibronectin in HBV-infected HCC patients at different stages. The reduction of circulating IL-4 and IL-10 levels implies that patients lose their self-defense capability. Fibronectin profile might reflect HCC deterioration. Thus, monitoring these molecules in HCC patients might benefit diagnosis and/or prediction.

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