



Inflammatory cytokines and alpha-fetoprotein concentrations for predicting survival in patients with hepatocellular carcinoma

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Background: Chronic inflammation is crucial in the evolution of hepatocellular carcinoma (HCC). Circulatory and intratumoral inflammatory factors are always increased in these patients. In this study, we determined the value of pretherapy serum concentrations of several inflammatory cytokines for predicting survival among patients with HCC.

Methods: We retrospectively analyzed data of 85 patients with HCC with known alpha-fetoprotein (AFP) concentrations diagnosed at our institute. Before therapy, serum concentrations of interleukin (IL)-2, -4, -6, -10, interferon gamma, and tumor necrosis factor alpha were measured by flow cytometry. Correlations between these serum factors and clinical parameters were tested. Statistical analysis was made by using non-parametric procedures and survival analysis. The expression of IL-6 and the co-expression of IL-6 and CD11b, marker of myeloid cell in HCC tumor tissues, were evaluated by immunohistochemistry and immunofluorescence, respectively.

Results: The only independent predictor of overall survival (OS) was IL-6 [hazard ratio (HR), 3.338; 95% CI, 1.671 to 6.666; P=0.001]. OS was shorter among patients with high concentrations of both IL-6 (≥ 1.435 pg/mL) and AFP (≥ 400 ng/mL) than it was in those with high concentrations of either IL-6 or AFP; survival was significantly longer in patients with low concentrations of both markers (P<0.001). Immunostaining showed that IL-6 expression was localized to the stroma of the tumor and was co-expressed with CD11b positive cells.

Conclusions: The combination of serum concentrations of IL-6 and AFP more accurately predicts survival in HCC patients.

Keywords: Alpha-fetoprotein (AFP); interleukin 6; inflammatory factor; hepatocellular carcinoma (HCC); prognosis

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Introduction

Worldwide, primary hepatocellular carcinoma (HCC) is the fifth most common malignant tumor, and the second leading cause of cancer mortality in men and the sixth in women (1). The prognosis of patients with resectable HCC remains poor: more than 50% relapse within two years after resection (2). Staging systems, such as the Tumor, Node, Metastases (TNM) system, the Barcelona Clinic Liver Cancer (BCLC) system, and the Cancer of the Liver Italian Program (CLIP) score, are based on clinical characteristics that include tumor factors, general patient condition, and liver function, and are widely used to predict outcomes (3). However, although these characteristics provide adequate information to inform treatment decisions, they do not necessarily reflect the biological behavior of the tumor, and patients with tumors of the same stage may have different outcomes (4). Validated biomarkers would, therefore, be useful in complementing the limited prognostic ability of current staging systems.

Alpha-fetoprotein (AFP) is a specific tumor antigen secreted by hepatoma cells. A subclass of AFP-positive HCCs has been reported to have a unique gene expression signature and worse biological behaviour (5). Higher serum concentrations of AFP suggest more severe tumor invasion and a poorer prognosis (6). These findings indicate that AFP could be a prognostic biomarker because it may directly reflect features of HCC tumor biology. Considering the heterogeneous nature of HCC, complementary markers are needed to provide more general information.

Tumor-associated inflammation is a hallmark of cancer progression. Almost 70–90% of HCC cases occur in the setting of chronic inflammation (7). Cytokines are essential components of the tumor microenvironment and participate in the intricate crosstalk between tumor cells and immune cells (8). Systemic inflammation predicts outcome in patients with surgically resectable HCC and in those patients with more advanced HCC (9). Elevated serum concentrations of various cytokines, including interleukin (IL)-2, IL-6, IL-8, IL-10, and IL-17, have been reported to be associated with prognosis in HCC patients (10–12). However, few cytokines have been independently validated as predictors of outcome.

In this study, we combined immune-related prognostic predictors screened from six tumor-immune-related cytokines with AFP and determined their ability to predict survival in patients with HCC.

Methods

Patients, clinical specimens, and follow-up

Patients were eligible for the study if, between December 2009 and December 2010, they had newly diagnosed HCC based on National Comprehensive Cancer Network guidelines, had no other malignancies or immune-related disease, had complete clinicopathologic and follow-up data until June 2018, a pre-treatment serum sample, and data on AFP concentrations.

Treatment for HCC patients was determined by our multidisciplinary team based on the BCLC staging system, including surgery, transcatheter arterial chemoembolization, and thermal ablation. Serum samples were collected from patients before treatment and stored at -80°C until processing. Surgically resected tumor specimens were fixed in formalin and embedded in paraffin for routine histologic diagnosis. Patients were followed until June 2018. Serum AFP concentrations were monitored, and liver computed tomography, or magnetic resonance imaging scans were obtained every two months during the first year after surgery and at least every four months after that. Independent physicians performed all examinations without knowledge of the current study. The primary endpoint was overall survival (OS), or the time to death from any cause measured from the date of surgery to the date of death or of last follow-up.

Cytokine measurement

The biomarkers IL-2, IL-4, IL-6, IL-10, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), were measured by a fluorescence-based detection system and flow cytometric analysis. Cytokines were captured on microparticles using a BD Cytometric Bead Array Human Th1/Th2 Cytokine kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. A series of diluted cytokine standards were run in each assay to generate standard curves. The samples were measured using a FACS Canto II (BD Biosciences, San Diego, CA, USA) and analyzed using FCAP array software (BD Biosciences, San Diego, CA, USA).

Immunohistochemistry staining

Formalin-fixed, paraffin-embedded tissue samples were

cut into 5- μ m sections and mounted on glass slides. After the sections were deparaffinized, rehydrated, and washed, endogenous peroxidase was blocked (3% H₂O₂ for 10 min), and microwave antigen retrieval was performed in Tris-EDTA (pH 9.0). Sections were first incubated with blocking serum at room temperature for 30 min and then with mouse anti-IL-6 monoclonal antibody (1:8,000, ab9324; Abcam) at 4 °C overnight. The second incubation was done using goat anti-mouse antibody and visualized with DAB after counterstaining with hematoxylin after serial rinsing.

Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissue samples were cut into 5- μ m sections and mounted on glass slides. After the sections were deparaffinized, rehydrated, and washed, microwave antigen retrieval was performed in Tris-EDTA (pH 9.0). Sections were first incubated with blocking serum at room temperature for 30 min and with mouse anti-IL-6 monoclonal antibody (1:1,000, ab9324; Abcam) combined with rabbit anti-CD11b monoclonal antibody (1:200, ab52478; Abcam) at 4 °C overnight. The second incubation was done using DyLight 488 AffiniPure goat anti-mouse IgG (1:100, E032210; EarthOx) and DyLight 594 AffiniPure goat anti-rabbit IgG (1:100, E032420; EarthOx) after counterstaining with DAPI and serial rinsing with 1 \times PBST.

Statistical methods

Data of cytokine concentrations are presented as means with standard deviations. Cut-off values for defining high and low values of each variable were determined by receiver operating characteristics analysis. Associations of the cytokine concentrations with clinical and pathologic features of the HCC patients were evaluated using Mann-Whitney U test or Kruskal-Wallis tests. Differences between categorical variables were analyzed with Chi-square tests. The correlation was assessed using the nonparametric Spearman's correlation.

Survival curves were analyzed with the Kaplan-Meier method and compared using log-rank tests with the following covariates: age (≤ 50 , > 50 years), sex (female, male), maximum tumor diameter (≤ 50 , > 50 mm), portal vein invasion (yes, no), hepatitis B virus (HBV) genomic DNA copy number ($< 0.01 \times 10^2$, $\geq 0.01 \times 10^2$ IU/mL), hepatitis B (yes, no), BCLC stage (A, B and C) and AFP concentration (≤ 400 , > 400 ng/mL). Concentrations of AFP above 400 ng/mL have been considered to be diagnostic for HCC

in patients with cirrhosis [see the European Association for the Study of the Liver (EASL) guidelines] (13). Patients were divided into four groups based on low and high concentrations of AFP and IL-6 (AFP, ≤ 400 vs. > 400 ng/mL; IL-6, ≤ 1.435 vs. > 1.435 ng/mL). Covariates with P values less than 0.05 were then subsequently analyzed by multivariate time-to-event analysis using Cox proportional hazards regression. Alpha was set at 0.05, and all tests were two-sided. Data were analyzed with GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, LA, USA) and the SPSS statistics package (version 19, SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

Out of 1,179 patients screened, 85 (78 men) met the inclusion criteria and were included in the study (Table 1). The mean age was 50 years. Out of 85 patients, 70 were in BCLC A stage, and 60 had hepatitis B virus (HBV)-related HCC. Median follow-up was 39.5 months (range, 2 to 103 months).

Cytokine concentrations and clinical characteristics

Pretreatment serum concentration cut-off values for predicting survival are shown in Table 2. Interleukin-6 concentrations differed significantly by maximum tumor diameters, portal vein invasion, and BCLC stage, while IL-10 concentrations differed significantly by sex (Table 3). Concentrations of IL-6 also differed significantly between patients grouped by maximum tumor diameter analyzed as an ordinal variable with four categories (Figure 1).

Survival analysis

In the univariate analysis, maximum tumor diameter, AFP concentrations, BCLC stage, and IL-6 were significantly related to OS. Covariates with P values less than 0.05 were included in the multivariate analysis. AFP [hazard ratio (HR), 2.282; 95% CI, 1.217 to 4.280; P=0.01], BCLC stage (HR, 3.123; 95% CI, 1.534 to 6.359; P=0.002), and IL-6 concentrations (HR, 3.338; 95% CI, 1.671 to 6.666; P=0.001) independently predicted survival, with cut-off values of 400 ng/mL, BCLC stage A, and 1.435 pg/mL, respectively (Figure 2 and Table 4).

To combine the prognostic scores of IL-6 and AFP, we divided patients into four groups based on low and high

Table 1 Clinical and tumor characteristics of 85 patients with hepatocellular carcinoma

Characteristics	N (%)
Sex	
Male	78 (91.8)
Female	7 (8.2)
Age	
≤50	41 (48.2)
>50	44 (51.8)
Hepatitis B	
Positive	60 (70.6)
Negative	25 (29.4)
Alpha-fetoprotein, ng/mL	
≤400	43 (50.6)
>400	42 (49.4)
Maximum tumor diameter, mm	
30	17 (20.0)
>30 to 50	21 (24.7)
>50 to 100	34 (40.0)
>100	13 (15.3)
Number of tumors	
1	75 (88.2)
≥2	10 (11.8)
Portal vein tumor thrombus	
Absence	76 (89.4)
Presence	9 (10.6)
BCLC stage	
A	70 (82.4)
B	6 (7.1)
C	9 (10.6)
Treatment	
Surgery	62 (72.9)
Transcatheter arterial chemoembolization	17 (20.0)
Thermal ablation	6 (7.1)

BCLC stage, Barcelona Clinic Liver Cancer stage.

Table 2 Pre-treatment serum cytokine concentrations of 85 patients with hepatocellular carcinoma

Cytokine	Serum concentration, mean (SD), pg/mL	Cutoff value ^a , pg/mL
IL-2	0.026 (0.139)	0.360
IL-4	0.057 (0.183)	0.081
IL-6	3.366 (4.036)	1.435
IL-10	2.036 (2.466)	1.271
IFN- γ	0.387 (1.341)	0.296
TNF- α	0.03 (0.176)	0.166

^a, the value maximizing the area under the receiver operating characteristics curve and used to define groups in the Cox proportional hazards analysis.

concentrations of each cytokine. Thus, 24 patients had high values for both cytokines, 19 had low values for both cytokines, 24 had high IL-6 concentrations and low AFP concentrations, and 18 had low IL-6 concentrations and high AFP concentrations. OS differed significantly among the four subgroups (*Figure 3*). Median OS for the whole group was 35.86 months. Median OS for patients with high levels of both cytokines was 9.75 months, which is considerably shorter than that of the other three groups.

Correlation analysis

Correlation analysis were performed in IL-6 and AFP concentrations in serum samples of 85 HCC patients, and *IL-6* and *AFP* mRNA in HCC tumour tissues from The Cancer Genome Atlas (TCGA) database. Concentrations of IL-6 and AFP in serum samples were not correlated ($r=0.095$, $P=0.699$; *Figure 4*)—a fact confirmed by RNA-sequencing data for HCC tumour tissue samples from TCGA database (14) ($r=-0.067$, $P=0.197$; *Figure 4*).

Immunohistochemical expression of IL-6 and CD11b in tumor tissue

We confirmed the expression pattern of IL-6 in tumor tissues with immunohistochemical staining, which showed that IL-6 was localized predominantly in the tumor stroma, i.e., tumor infiltrating cells. Furthermore, in immunofluorescence staining, IL-6 and CD11b were co-expressed, suggesting that the myeloid cells, not tumor cells, were the primary source of IL-6 (*Figure 5*).

Table 3 Number and percentage of patients by cytokine concentrations and clinical features in 85 patients with hepatocellular carcinoma

Characteristics	IL-2 (<0.36 pg/mL)		IL-4 (<0.08 pg/mL)		IL-6 (<1.435 pg/mL)		IL-10 (<1.27 pg/mL)		TNF- α (<0.16 pg/mL)		INF- γ (<0.29 pg/mL)	
	n (%)	P	n (%)	P	n (%)	P	n (%)	P	n (%)	P	n (%)	P
Age (years)												
≤50 (n=41)	40 (97.6)	0.808	37 (90.2)	0.806	20 (48.8)		15 (36.6)		40 (97.7)	0.869	37 (90.2)	0.772
>50 (n=44)	43 (97.7)		38 (86.4)		17 (38.6)	0.479	20 (45.5)	0.366	42 (95.5)		37 (84.1)	
Sex												
Female (n=7)	7 (100.0)	1	7 (100.0)	0.344	3 (42.9)	0.159	7 (100.0)	0.001	7 (100.0)	1	7 (100.0)	0.655
Male (n=78)	76 (97.4)		68 (87.2)		34 (43.6)		28 (35.9)		75 (96.2)		67 (85.9)	
Maximum tumor diameter (mm)												
≤50 (n=38)	37 (97.4)	0.812	34 (89.5)	0.984	23 (60.5)	0.006	16 (42.1)	0.747	36 (94.7)	0.361	33 (86.8)	0.83
>50 (n=47)	46 (97.9)		41 (87.2)		14 (29.8)		19 (40.4)		46 (97.9)		41 (87.2)	
Portal vein invasion												
No (n=76)	74 (97.4)	0.366	69 (90.8)	0.256	35 (46.1)	0.029	32 (42.1)	0.123	73 (96.1)	1	68 (89.5)	0.231
Yes (n=9)	9 (100.0)		6 (66.7)		2 (22.2)		3 (33.3)		9 (100.0)		6 (66.7)	
HBV DNA copy number ($\times 10^2$ IU/mL)												
≤0.01 (n=32)	32 (100.0)	0.534	28 (87.5)	0.509	12 (37.5)	0.343	15 (46.9)	0.335	31 (96.9)	1	27 (84.4)	0.827
>0.01 (n=53)	51 (96.2)		47 (88.7)		25 (47.2)		20 (37.7)		51 (96.2)		47 (88.7)	
Hepatitis-B												
No (n=25)	25 (100.0)	0.789	22 (88.0)	0.668	10 (40.0)	0.458	13 (52.0)	0.427	24 (96.0)	1	21 (84.0)	0.62
Yes (n=60)	58 (96.7)		53 (88.3)		27 (45.0)		22 (36.7)		58 (96.7)		53 (88.3)	
AFP (ng/mL)												
≤400 (n=43)	42 (97.7)	1	38 (88.4)	0.748	19 (44.2)	0.728	15 (34.9)	0.998	41 (95.3)	0.619	36 (83.7)	0.671
>400 (n=42)	41 (97.6)		37 (88.1)		18 (42.9)		20 (47.6)		41 (97.6)		38 (90.5)	
BCLC stage												
A (n=70)	68 (97.1)	1	63 (90.0)	0.516	34 (48.6)	0.004	30 (42.9)	0.062	67 (95.7)	0.633	62 (88.6)	0.366
B and C (n=15)	15 (100.0)		12 (80.0)		3 (20.0)		5 (33.3)		15 (100.0)		12 (80.0)	

HBV, hepatitis B virus; AFP, alpha-fetoprotein; BCLC stage, Barcelona Clinic Liver Cancer.

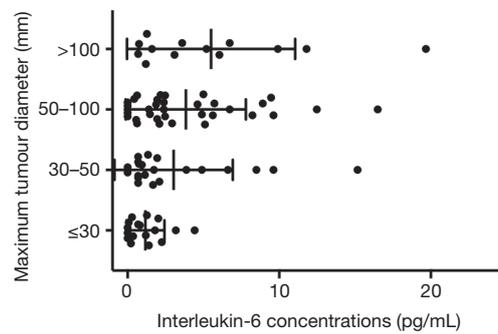


Figure 1 Serum IL-6 concentrations in patients with hepatocellular carcinoma, by maximum tumor diameter. Using Kruskal-Wallis test, serum concentrations of IL-6 differed significantly among the four groups (overall group differences significant at $P=0.012$); Using Mann-Whitney U test, IL-6 in the tumors between 50–100 mm group and >100 mm group were significantly higher than in the tumors ≤ 30 mm group ($P=0.01$, $P=0.004$).

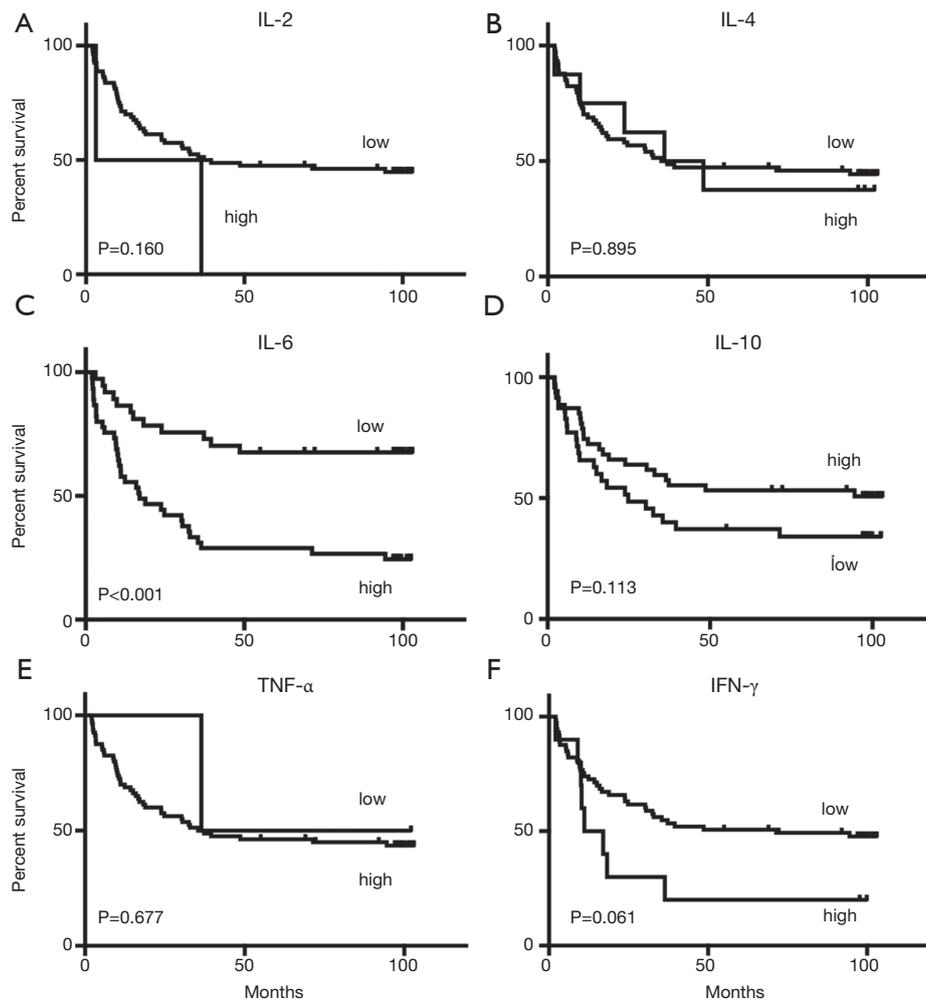


Figure 2 Overall survival in patients with hepatocellular carcinoma. (A) IL-2, (B) IL-4, (C) IL-6, (D) IL-10, (E) TNF- α , and (F) IFN- γ . Kaplan-Meier curves indicating the relationship between months to death after initial treatment were grouped according to cut-off values determined by ROC analysis. ROC, receiver operating characteristic curve.

Table 4 Univariate and multivariate analysis of the clinical characteristics predicting overall survival in 85 patients with hepatocellular carcinoma

Characteristic	Patients (%)	Overall survival		
		Univariate analysis	Multivariate analysis	
			P	HR (95% CI)
Age ≤50 years	51	0.619	–	–
Sex, male	91	0.115	–	–
Maximum tumor diameter ≤50 mm	46	0.002	1.321 (0.674–2.590)	0.417
Portal vein invasion	22	0.228	–	–
Hepatitis-B	94	0.136	–	–
HBV DNA copy <0.01×10 ² IU/mL	32	0.18	–	–
AFP concentration ≤400 ng/mL	45	0.003	2.282 (1.217–4.280)	0.01
BCLC stage A	75	0.001	3.123 (1.534–6.359)	0.002
IL-2 <0.36 pg/mL	97	0.160	–	–
IL-4 <0.08 pg/mL	88	0.895	–	–
IL-6 <1.40 pg/mL	40	0.001	3.338 (1.671–6.666)	0.001
IL-10 <1.27 pg/mL	45	0.113	–	–
TNF-α <0.16 pg/mL	97	0.677	–	–
IFN-γ <0.29 pg/mL	85	0.061	–	–

HBV, hepatitis B virus; AFP, alpha-fetoprotein; BCLC stage, Barcelona Clinic Liver Cancer.

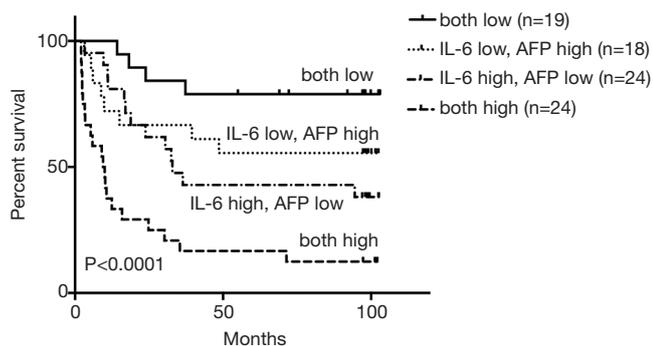


Figure 3 Overall survival in 85 patients with hepatocellular carcinoma, by IL-6 and AFP concentrations. Patients were divided into four groups based on low and high concentrations of each cytokine (AFP, ≤400 vs. > 400 ng/mL; IL-6, <1.435 vs. ≥1.435 ng/mL). Among the four groups, patients with high serum concentrations of both cytokines had the worst survival ($P<0.001$). AFP, alpha-fetoprotein.

Discussion

Chronic inflammation is a crucial factor in the development of HCC; the process has been referred to as the “inflammation-

fibrosis–cancer axis” (15), which is characterized by the continuous expression of cytokines and recruitment of immune cells into the liver (16). Increased circulatory or intratumoral cytokine concentrations have been reported to be associated with different outcomes in HCC (10,17–19).

In the current study, we measured serum concentrations of six cytokines in 85 patients with HCC. Among them, lower IL-10 and higher IL-6 levels were associated with poorer survival. The tumor-related implications of IL-10 are complex, and both enhancing and inhibitory effects have been observed after IL-10 treatment in different *in vivo* experimental models (20). We found that lower circulating concentrations of IL-10 predicted, without significance, poorer survival in our patients, but it was not an independent predictor.

Serum IL-6 concentrations are a promising diagnostic marker for HCC (21), and the function of the IL-6 signaling pathway in promoting hepatocarcinogenesis has received increasing attention (22). However, the prognostic value of IL-6 has not been extensively investigated.

Interleukin-6 is an inflammatory cytokine involved in cell proliferation and the acute-phase reaction (21). As a growth-promoting cytokine, IL-6 provides hepatoprotection

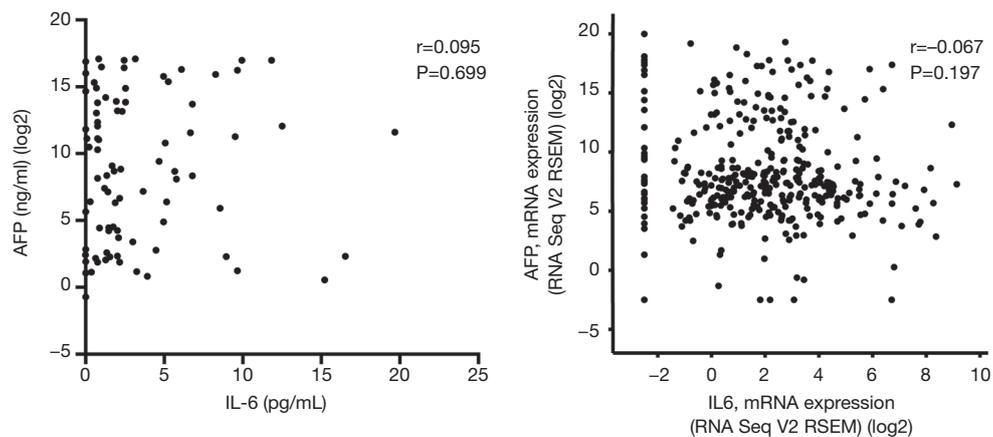


Figure 4 Co-expression analysis of IL-6 and AFP expression in patients with hepatocellular carcinoma. (A) Interleukin-6 and AFP concentrations were not correlated in serum samples of 85 HCC patients ($r=0.095$; $P=0.699$). (B) Co-expression analysis shows no correlation between IL-6 and AFP mRNA in tumour tissue samples in the TCGA HCC dataset ($r=-0.067$; $P=0.197$). Levels of target mRNAs were measured using RNA-sequencing in whole tissue section samples and obtained through the cBioportal analysis platform. AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

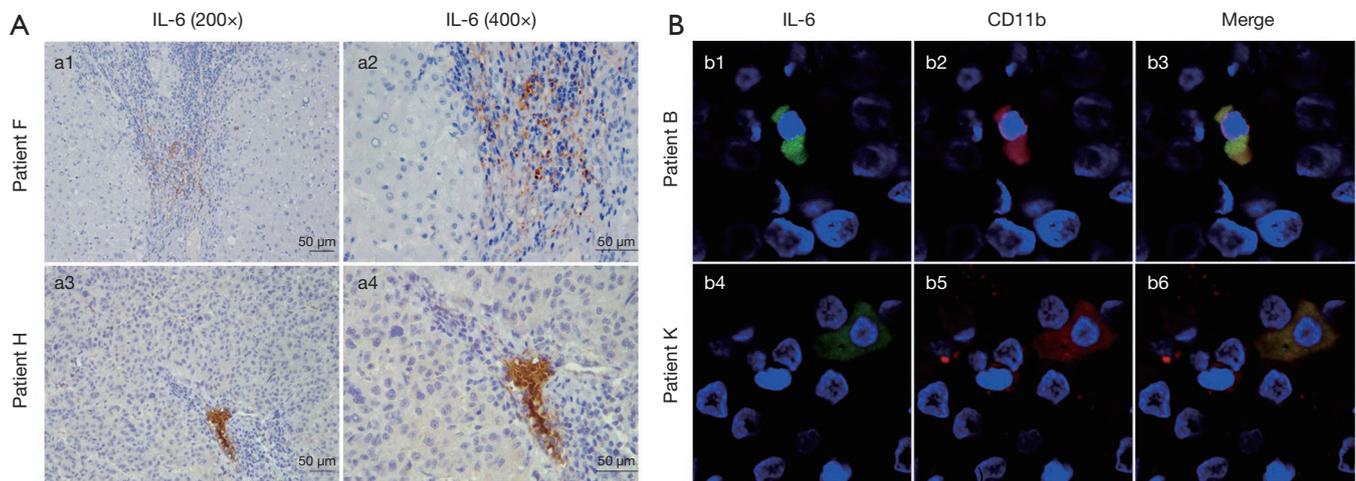


Figure 5 The expression and localization of Interleukin 6 (IL-6) and the cluster of differentiation molecule 11b in hepatocellular carcinoma tumor tissue. (A) (a1,a2) IL-6 (brown chromogen) immunohistochemical staining from tumor samples of patient F (200 \times and 400 \times magnification); (a3,a4) IL-6 immunohistochemical staining from tumor samples of patient H (200 \times and 400 \times magnification). (B) (b1,b2) Immunofluorescence staining for IL-6 cells (green) and CD11b (red) in tumor sections of patient B with hepatocellular carcinoma (400 \times magnification); (b4,b5) Immunofluorescence staining for IL-6 cells (green) and CD11b (red) in tumor sections of patient K with hepatocellular carcinoma (400 \times magnification); (b3,b6) Merge of IL-6 and CD11b in tumor sections of two patients.

by stimulating liver regeneration in liver-injury models (23). However, some evidence indicates that the IL-6 signaling pathway is also important in tumor formation. Serum concentrations of IL-6 have been reported to be elevated in patients with pancreatic ductal adenocarcinoma (24), myeloma (25), non-Hodgkin's lymphoma (26), and

HCC (10). Interleukin-6 signaling occurs through two distinct pathways, termed "classic signaling" and "IL-6 trans-signalling." Mice with impaired IL-6 trans-signaling were protected from the development of HCC, indicating a crucial function for this signaling in hepatocellular carcinogenesis (22). Also, IL-6 promotes the

metastatic potential of HCC by up-regulating osteopontin expression (27). We also found that IL-6 concentrations were associated with portal vein invasion and BCLC stage, thus suggesting the tumorigenic character of IL-6. Our results indicate that IL-6 levels are useful predictors in HCC, a finding consistent with those of Jang *et al.* (28).

In our study, the expression pattern of IL-6 in HCC tumor tissues localized to tumor stroma, i.e., tumor infiltrating cells. Immunofluorescence staining indicated co-localization of IL-6 and CD11b⁺ cells and revealed that myeloid cells were one of the sources of IL-6. Hence, the circulating expression of IL-6 may reflect the infiltration of tumor-related immune cells, as well as immune status, in HCC.

AFP is secreted by HCC cells and is associated with the malignant degree of HCC (6). Because IL-6 and AFP are produced independently in HCC and their concentrations are not correlated, these two cytokines might be complementary markers for HCC and thus combining them may have prognostic value. When we divided patients into four subgroups according to their serum concentrations of both IL-6 and AFP, the patients with high serum concentrations of both cytokines had the worst survival ($P < 0.001$). Further, HCC is a highly malignant tumor with a poor prognosis, and different outcomes have been reported, even among patients with early-stage disease, who are generally thought to have a better prognosis (29,30). Of our 85 patients, 70 (82.4%) had BCLC stage A cancer, indicating that even patients with early-stage HCC who also have high serum concentrations of both IL-6 and AFP may have a poorer prognosis.

The main limitation of our study was the small sample size. In addition, according to the restriction of our detection method, values below the detection limit could not be analyzed and were considered as zero.

Conclusions

We believe our data support the conclusion that the concentration of serum IL-6 is an independent prognostic factor for survival in patients with HCC and that its predictive accuracy can be improved by combining it with the AFP concentration. Patients with high concentrations of both IL-6 and AFP may have the most unsatisfactory outcomes.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.08.09>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Committee of Sun Yat-sen University Cancer Centre in Guangzhou, China (YB2015-018-01). All procedures were performed after obtaining informed consent in accordance with the approved protocol. Written informed consent to the procedure was obtained from patients and their family.

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