Serum interferon gamma level predicts recurrence in hepatocellular carcinoma patients after curative treatments

I-Cheng Lee¹², Yi-Hsiang Huang¹, Gar-Yang Chau⁶, Teh-Ia Huo¹,⁵, Chien-Wei Su¹², Jaw-Ching Wu²⁶ and Han-Chieh Lin¹

¹ Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
² Institute of Clinical Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan
³ Department of Medicine, National Yang-Ming University Hospital, I-Lan, Taiwan
⁴ Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan
⁵ Institute of Pharmacology, National Yang-Ming University School of Medicine, Taipei, Taiwan
⁶ Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

Host immunity may have important role in the prognosis of hepatocellular carcinoma (HCC). The aim of this study was to evaluate the correlation between circulating immune regulators and clinical outcome in patients with HCC. Sixty-three HCC patients were prospectively enrolled. Serum levels of interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), interferon-gamma (IFN-γ) and interferon-gamma-inducible protein 10 (IP-10) were measured, as well as the prevalence of regulatory T cells (Treg), NK⁺ T cells, invariant natural killer T cells (iNKT), programmed cell death-1 (PD-1⁺)CD8⁺ T cells, T helper 17 cells (Th17), CD69⁺ and CD65R0⁺ T cells in peripheral blood mononuclear cells (PBMC). Correlation between these immune regulators and clinical outcome were analyzed. A low serum IFN-γ level (<50 pg/mL) was significantly associated with tumor stage (BCLC stage B: 61.25% vs. stage A: 25%, p = 0.010) and tumor size (>5 cm: 53.8% vs. <5 cm: 25%, p = 0.047). Recurrence-free survival was evaluated in 48 patients receiving curative treatment of HCC. By multivariate analysis, BCLC stage [hazard ratio (HR) = 32.180, p < 0.001], tumor size (HR = 15.373, p = 0.005), AST (HR = 3.796, p = 0.011) and IFN-γ (HR = 0.354, p = 0.018) levels were independent factors associated with recurrence-free survival. In conclusion, serum IFN-γ level correlates with tumor stage and tumor size in HCC patients. Patients with lower baseline IFN-γ levels have a higher risk of tumor recurrence after curative treatment. IFN-γ may reflect host anti-tumor immunity and may be a potential marker of HCC recurrence after curative treatment.

Hepatocellular carcinoma (HCC) is one of the most common cancers and the third leading cancer death in the world. Its prevalence is high in hepatitis B virus (HBV) and hepatitis C virus (HCV) endemic areas and its incidence is still rising. Currently, surgical resection, local ablation therapy and transarterial chemoembolization (TACE) are the main treatments for HCC. However, recurrence is frequent even after curative treatment and long-term outcome is still unsatisfactory.

Many host and tumor factors are associated with the outcome of HCC. Recent advances in cancer immunology suggest that several immune regulators play certain role in the prognosis of cancer. The host immune system may identify tumor antigens and many host factors are associated with the outcome of HCC.
What's new?

In fighting cancer, the patient’s own immune system can deliver a significant blow to the disease. This study looked at how immune cells circulating in the bloodstream correlate with clinical outcome in liver cancer. They showed that patients with lower levels of IFN-γ prior to treatment have higher risk of recurrence. Serum levels of IFN-γ also correlate with tumor size and stage. It’s possible, then, that measuring a patient’s IFN-γ levels could help predict risk of recurrence following treatment.

and activate particular innate and adaptive immune cells and effector molecules to suppress tumor growth. In contrast, tumors may develop several mechanisms to evade from host immunosurveillance by secretion of immunosuppressive cytokines and up-regulation of negative immune-regulatory cells.

Alterations in populations of several immune cells have been reported in HCC. Recent studies have demonstrated that CD4+CD25+ regulatory T cell (Treg) and IL-17-producing CD4+ T helper cell (Th17) population were increased in tumor-infiltrating lymphocytes and were associated with reduced survival in HCC patients. In animal model, activation of invariant natural killer T cells (iNKT) could suppress liver tumor, which implies iNKT is possessed of antitumor immunity. Programmed cell death 1 (PD-1) and its ligand PD-L1, which correlate with T cell exhaustion in chronic viral hepatitis, have been shown to negatively regulate immune responses. These findings imply that the phenotype of T cells may correlate with outcomes of cancer patients.

Several cytokines may also have influence on HCC progression. Interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) are immune-suppressive cytokines and have been shown to inversely correlate with clinical outcome in patients with HCC. Recent studies demonstrate that interferon-gamma (IFN-γ) supplementation can elicit tumor suppressive effects in models of HCC. Interferon gamma-inducible protein 10 (IP-10), also known as CXCL10, is secreted by several types of immune cells in response to IFN-γ. Pretreatment IP-10 levels may predict interferon-based treatment response in patients with chronic hepatitis B and C, but its role in HCC has not been studied.

The correlations between the immune regulatory cells and the prognosis of HCC were mostly demonstrated in animal studies or by analyzing tumor-infiltrating lymphocytes. Whether the populations of these immune markers in peripheral blood, which were easily accessible and practical for clinical application, also have prognostic roles in HCC is unclear. The aim of this study was to evaluate baseline and early predictors of tumor recurrence after curative treatment of HCC by testing these immune markers from peripheral blood.

Material and Methods

Patients and specimens

From January 2009 to August 2011, 63 HCC patients who were Barcelona Clinic Liver Cancer (BCLC) stage A or B diagnosed in Taipei Veterans General Hospital were prospectively enrolled in this study. Of the same period, 20 HBV carriers with alanine aminotransferase (ALT) level <2 × upper limit of normal (including inactive HBV carriers and chronic hepatitis B with mild hepatitis) were also enrolled for comparison. This study was approved by the Institutional Review Board, Taipei Veterans General Hospital, which complied with standards of the Declaration of Helsinki and current ethical guidelines. All patients provided written informed consents for participation of the study.

The diagnosis of HCC was made based mainly on imaging modalities using contrast-enhanced computed tomography (CT), magnetic resonance image (MRI), angiography and/or pathologically by tumor biopsy or alpha-fetoprotein (AFP) >200 ng/mL, which fulfilled the diagnostic criteria of American Association for the Study of Liver Diseases (AASLD) treatment guidelines for HCC. Patients who received curative treatments, including surgery and radiofrequency ablation (RFA), were followed every 3 months with measurement of serum AFP, ultrasonography, CT or MRI. Tumor recurrence was confirmed by contrast-enhanced CT or MRI.

Peripheral blood samples were obtained from patients at baseline before and one month after treatments. The serum was separated for cytokines/chemokine detection. The peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation and frozen for subsequent experiments.

The following clinical features and biochemistry were collected for analysis: age, sex, intrahepatic tumor number, tumor size, Child-Pugh class and serum AFP, ALT, aspartate aminotransferase (AST), albumin, total bilirubin levels, platelet count, prothrombin time [measured by international normalized ratio (INR)], hepatitis B surface antigen (HBsAg) and antihepatitis C antibody (anti-HCV). Serum HBsAg and serum AFP were measured by radio-immunoassay kits (Abbott Laboratories, North Chicago, IL and Serono Diagnosti SA, Coinsin/VD, Switzerland, respectively). Anti-HCV was detected by an enzyme immunoassay kit (Abbott laboratories), while serum albumin, ALT, AST and total bilirubin were measured by systemic multiautoanalyzer (Technicon SMAC, Technicon Instruments, Tarrytown, NY).

Flow cytometric analysis. PBMC from 47 HCC patients and 20 HBV carriers were analyzed. Samples from the same patient were analyzed in parallel. Fluorescein isothiocyanate-conjugated anti-CD3, anti-CD4, anti-CD45RO, anti-CD279
(PD-1), phycoerythrin (PE)-conjugated anti-FoxP3, anti-IL-17A, anti-CD45RA, anti-CD69 and peridinin chlorophyll protein (PerCP)-conjugated anti-CD4, anti-CD8 were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-CD56, anti-Vo24Jx18 and phycoerythrin cyanin 5 (PECy5)-conjugated anti-CD25 were purchased from eBioscience (San Diego, CA). For intracellular IL-17 and FoxP3 staining, PBMC (200 μL) was incubated with phorbol 12-myristate 13-acetate (PMA, 300 ng/mL, Sigma, St. Louis, MO) and ionomycin (1 μg/mL, Sigma-Aldrich) in 800 μL RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) for 4 hr. Monensin (0.4 μM, BD PharMingen) was added during the first hour of incubation. The blood was then lysed with fluorescence-activated cell sorting (FACS) lysing solution (BD PharMingen) and further permeabilized, stained with the corresponding intracellular antibody, fixed and analyzed.

The prevalence of CD4+ T cells (CD4+/CD3+ T cells), Th17 (IL17A+/CD4+ T cells), Treg (CD25+ FoxP3+/CD4+ T cells), NK+ T cells (CD56+/CD3+ T cells), iNKT (Vo24Jx18+/CD3+ T cells), PD-1+ CD8+ T cells (PD-1+/CD8+ T cells), CD4+ CD69+ T cells (CD69+/CD4+ T cells), CD8+ CD69+ T cells (CD69+/CD8+ T cells), CD4+ CD45RO+ T cells (CD45RO+/CD4+ T cells) and CD8+ CD45RO+ T cells (CD45RO+/CD8+ T cells) were determined in available PBMC were evaluated. Samples were run on a FACSscan flow cytometer and the data were analyzed by using CellQuest software (Becton Dickinson Immunocytometry System) as previously described.33 The procedure followed the instruction provided by manufactures. The representative FACS data are shown in Supporting Information Figure 1.

Enzyme-linked sorbent assay. The concentrations of IL-10, TGF-β, interferon gamma (IFN-γ) and interleukin-17A (IL-17A) in serum were tested by commercialized human cytokine Enzyme-linked sorbent assay kits (eBioscience, San Diego, CA). Interferon gamma-inducible rotein 10 (IP-10) to IP-10 in serum was tested by Human IP-10 Development Kit (Pepro Tech, Rocky Hill, NJ). The procedure followed the instruction provided by manufactures. The sensitivity was 4.68 pg/mL for IL-10, 125 pg/mL for TGF-β, 7.81 pg/mL for IFN-γ, 31.25 pg/mL for IP-10 and 4 pg/mL for IL17A. Serum IL-17A levels were below the detection limit in all HCC patients and the data were not further presented.

Statistical analysis. Values are expressed as median (ranges) or as mean ± standard deviation when appropriate. The Pearson chi-square analysis or Fisher exact test was used to compare categorical variables, while the Student t test or Mann–Whitney U test was used for continuous variables. Wilcoxon signed ranks test was used to compare serial changes of immune factors. Correlations between the immune factors were tested by Spearman’s test. Survival was estimated by the Kaplan–Meier method and compared by the log-rank test. Analysis of prognostic factors for recurrence-free survival was performed using the Cox proportional hazards model.

Variables that achieved statistical significance (p < 0.05) or those that were close to significance (p < 0.1) by univariate analysis were subsequently included in the multivariate analysis. A two-tailed p < 0.05 was considered statistically significant. The cutoff values for clinical variables were chosen according to clinically significant values, while the cutoff values for immune factors were determined by integers near the mean or median values. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS, Chicago, IL).

Results

The baseline characteristics of 63 HCC patients are shown in Table 1. The mean age was 63.7 ± 11.7 years, and 47 patients (74.6%) were males. About 90% of patients had either chronic HBV (58.7%) or HCV (31.7%) infection. Majority of patients (95.2%) were Child-Pugh class A, and 52.4 and 47.6% of patients were BCLC stage A and B, respectively. Among these patients, 27 (42.9%) received surgical resection, 21 (33.3%) received RFA, 14 (22.2%) received TACE and 1 (1.6%) received best supportive care only. The 48 patients who received curative treatment of HCC, including surgery and RFA, were analyzed for recurrence-free survival. In the median follow-up period of 15.6 months, 28 (58.3%) patients developed tumor recurrence. The estimated 1-year and 2-year recurrence-free survival rate were 58.9 and 43.9%, respectively.

Table 1. Clinical characteristics of the 63 patients with HCC

| Variables                  | HCC patients |
|----------------------------|--------------|
| Age (years)                | 63.7 ± 11.7  |
| Male sex, n (%)            | 47 (74.6)    |
| HBSAg-positive, n (%)      | 37 (58.7)    |
| Anti-HCV-positive, n (%)   | 20 (31.7)    |
| Child-Pugh class A/B, n (%)| 60/3 (95.2/4.8)|
| BCLC stage A/B, n (%)      | 33/30 (52.4/47.6)|
| Intrahepatic tumor, single/multiple, n (%) | 46/17 (73/27) |
| Tumor size (cm)            | 5.52 ± 4.16  |
| Treatment                  |              |
| Surgery, n (%)             | 27 (42.9)    |
| RFA, n (%)                 | 21 (33.3)    |
| TACE, n (%)                | 14 (22.2)    |
| Best supportive care, n (%)| 1 (1.6)      |
| AFP (ng/mL)                | 39.4 (1.7 – 324120) |
| Albumin (g/dL)             | 3.89 ± 0.56  |
| Total bilirubin (mg/dL)    | 0.77 ± 0.33  |
| Prothrombin time (INR)     | 1.05 ± 0.09  |
| ALT (U/L)                  | 58.3 ± 43.3  |
| AST (U/L)                  | 64.8 ± 55.8  |
| Platelet (<10^9/L)         | 174 ± 71     |
The immunologic features of HCC patients and HBV carriers are shown in Supporting Information Table 1. Compared with HBV carriers, HCC patients had significantly lower frequency of Th17. There were also a trend of higher IL-10 levels and higher frequencies of NK$^+$ T cells and PD-1$^+$CD8$^+$ T cells in HCC patients.

Correlation between serum cytokine levels and frequency of immune-regulatory cells. Correlation between serum cytokine levels and frequency of immune-regulatory cells are shown in Figure 1. There were significant correlations among serum levels of IL-10, TGF-β, IFN-γ and IP-10. The immunosuppressive cytokines IL-10 and TGF-β had negative correlation with proinflammatory cytokines IFN-γ and IP-10 ($p < 0.001$). The frequency of Tregs were positively correlated with IL-10, TGF-β levels and negatively correlated with IFN-γ levels. There was also a positive correlation between frequencies of iNKT and PD-1$^+$CD8$^+$ T cells ($R = 0.594, p < 0.001$).

Tumor status and immune regulators. The immunologic features of the patients stratified according to BCLC stage and tumor size are shown in Table 2. A low serum IFN-γ level ($<50$ pg/mL) was significantly associated with tumor stage (BCLC stage B: 61.25% vs. stage A: 25%, $p = 0.010$) and tumor size ($>5$ cm: 53.8% vs. $\leq 5$ cm: 25%, $p = 0.047$). A lower frequency of NK$^+$ T cells ($<2.5%$) was significantly associated with larger tumor size ($p = 0.024$). In contrast, baseline serum levels of IL-10, TGF-β, IP-10 and frequency of Treg, iNKT and PD-1$^+$CD8$^+$ T cells were not associated with BCLC stage or tumor size.

Changes in serum cytokine levels and frequencies of immune-regulatory cells before and after curative treatment. Changes of immune markers were evaluated in 34 of the 48 patients who received curative treatments. Compared with the baseline serum cytokine levels and frequencies of immune cells, there were no significant changes in all measured markers one month after curative treatments (Table 3).

Factors associated with recurrence-free survival in HCC patients. Recurrence-free survival was evaluated in 48 patients who received curative treatment of HCC, including 27 patients receiving surgical resection and 21 patients receiving RFA. BCLC stage, tumor size, serum AST, IL-10, IFN-γ and frequency of Tregs were factors associated with recurrence-free survival by univariate analysis (Table 4). The recurrence-free survival was comparable between patients receiving surgical resection or RFA. Patients with higher serum IFN-γ levels had significantly better recurrence-free survival (Fig. 2). Serum levels of TGF-β, IP-10 and frequency of other immune cells were not associated with tumor recurrence. In multivariate analysis, BCLC stage B ($p < 0.001$), tumor size $>5$ cm ($p = 0.005$), AST $>80$ U/L ($p = 0.011$) and serum IFN-γ $>50$ pg/mL ($p = 0.018$) remained significantly associated with recurrence-free survival (Table 4).

Figure 1. Dot plots representing significant correlations between serum cytokine/chemokine levels and frequency of immune cells.
Consistent with previous report, our HCC patients had significantly lower frequencies of NK$^+$ T cells and PD-1$^+$CD8$^+$ T cells in HCC patients. Previous study showed that frequencies of Tregs in HCC patients were higher than in normal controls.12,34 However, our HCC patients had earlier stages and our data did not show significant differences of Tregs population between HBV carriers and HCC patients.

In our study, we observed significant correlations among serum levels of IL-10, TGF-β, IFN-γ and IP-10, which reflects a close interaction between positive and negative immune-regulatory cells to release these proinflammatory and anti-inflammatory cytokines/chemokine in the presence of HCC. Consistent with a recent study, the frequency of Tregs show significant correlations with IL-10, TGF-β and IFN-γ levels, representing the major immune-regulatory role of Tregs in the immunosuppressive environment during HCC progression.34 It is interesting that the population of iNKT positively correlates with the population of PD-1$^+$CD8$^+$ T cells. Prior study has reported that activated iNKT up-regulates the expression of inhibitory costimulatory receptor PD-1 at their cell surface.35 Our observation suggests that iNKT may be counterbalanced by PD-1 expressing CD8$^+$CD45RO$^+$ T cells in HCC patients might represent the altered immune environment from HBV carriers to HCC patients. Previous study showed that frequencies of Tregs in HCC patients were higher than in normal controls.12,34 However, our HCC patients had earlier stages and our data did not show significant differences of Tregs population between HBV carriers and HCC patients.

In our study, we observed significant correlations among serum levels of IL-10, TGF-β, IFN-γ and IP-10, which reflects a close interaction between positive and negative immune-regulatory cells to release these proinflammatory and anti-inflammatory cytokines/chemokine in the presence of HCC. Consistent with a recent study, the frequency of Tregs show significant correlations with IL-10, TGF-β and IFN-γ levels, representing the major immune-regulatory role of Tregs in the immunosuppressive environment during HCC progression.34 It is interesting that the population of iNKT positively correlates with the population of PD-1$^+$CD8$^+$ T cells. Prior study has reported that activated iNKT up-regulates the expression of inhibitory costimulatory receptor PD-1 at their cell surface.35 Our observation suggests that iNKT may be counterbalanced by PD-1 expressing CD8$^+$ T cells in HCC patients.

We demonstrated that lower serum IFN-γ levels correlates with more advanced tumor stage and both lower serum IFN-γ levels and lower frequency NK$^+$ T cells correlates with larger tumor size, suggesting that the host antitumor immunity was higher in early stage. As tumor growth progresses, the immunosuppressive environment counterbalances the antitumor immunity and suppresses effector cells, resulting in decreased secretion of IFN-γ. A recently published study
also showed that immune dysfunction status existed in patients with advanced HCC by augmenting the numbers of Tregs, myeloid-derived suppressor cells, PD-1$^+$ exhausted T cells and diminishing the levels of plasma IFN-γ. The finding can further support our data. Consistent with previous reports, our data showed that IL-10 and TGF-β were not correlated with tumor size or stage. But both cytokines were associated with tumor recurrence in univariate analysis. Serum IL-10 levels have been shown to decline during a period of 6–13 months after surgical resection of HCC. Our observation for serum cytokine levels and immune cell populations did not show significant differences between baseline levels and that one month after curative treatment. The relative earlier measurement time point in our study might result in the insignificant changes in these immune markers. Nevertheless, these cytokine levels or immune cell populations measured within one month after curative treatment may still reflect the baseline host immune environment.

In univariate analysis for recurrence-free survival, BCLC stage, tumor size, serum levels of AST, IL-10, IFN-γ and frequency of Tregs were factors associated with tumor recurrence. Consistent with previous reports, tumor stage and size are still the major determinants of prognosis in HCC. In patients with chronic hepatitis or cirrhosis, an increase in AST/ALT ratio has been shown to associate with liver fibrosis, and elevated AST level has been shown to correlate with poor outcome in HCC. Serum IL-10, an immunosuppressive cytokine with inhibitory effect on cancer immunosurveillance, has also

| Table 4. Univariate and multivariate analyses of factors associated with recurrence-free survival |
|-----------------------------------------------|
| **Univariate** | **Hazard Ratio** | **95% CI** | **P** |
| Age (years) | >60 vs. ≤60 | 1.396 | 0.658–2.961 | 0.384 |
| Sex | female vs. male | 1.686 | 0.779–3.648 | 0.185 |
| Treatment | RFA vs. surgery | 1.159 | 0.558–2.405 | 0.692 |
| BCLC stage | B vs. A | 3.064 | 1.440–6.521 | 0.004 |
| Tumor number | multiple vs. single | 2.323 | 0.991–5.448 | 0.053 |
| Tumor size (cm) | >5 vs. ≤5 | 2.322 | 1.066–5.058 | 0.034 |
| Bilirubin (mg/dL) | >1.2 vs. ≤1.2 | 1.963 | 0.674–5.714 | 0.216 |
| Albumin (g/dL) | >3.8 vs. ≤3.8 | 0.547 | 0.262–1.142 | 0.108 |
| Prothrombin time (INR) | >1.05 vs. ≤1.05 | 1.068 | 0.488–2.342 | 0.869 |
| AFP (ng/mL) | >400 vs. ≤400 | 1.384 | 0.587–3.262 | 0.458 |
| ALT (U/L) | >80 vs. ≤80 | 2.723 | 1.001–5.160 | 0.050 |
| AST (U/L) | >80 vs. ≤80 | 3.669 | 1.404–9.586 | 0.008 |
| Platelet ($\times 10^9$/L) | >150 vs. ≤150 | 0.677 | 0.325–1.409 | 0.297 |
| IL-10 (pg/mL) | >5 vs. ≤5 | 2.521 | 1.187–5.355 | 0.016 |
| TGF-β (pg/mL) | >3500 vs. ≤3500 | 2.060 | 0.976–4.347 | 0.058 |
| IFN-γ (pg/mL) | >50 vs. ≤50 | 0.292 | 0.128–0.664 | 0.003 |
| IP-10 (pg/mL) | >60 vs. ≤60 | 0.469 | 0.220–0.999 | 0.050 |
| Treg (%) | >3 vs. ≤3 | 2.859 | 1.143–7.155 | 0.025 |
| NK$^+$ T cell (%) | >2.5 vs. ≤2.5 | 1.315 | 0.385–4.497 | 0.662 |
| iNKT (% | >2.5 vs. ≤2.5 | 0.770 | 0.298–1.991 | 0.590 |
| PD-1$^+$CD8$^+$ T cell (%) | >15 vs. ≤15 | 0.682 | 0.248–1.874 | 0.458 |

Abbreviations: CI: confidence interval; NA: not adopted; NS: not significant.

Figure 2. Recurrence-free survival of patients with HCC stratified by baseline serum interferon-γ levels.
been reported to predict postoperative recurrence of HCC.\textsuperscript{22} Similarly, increased Tregs in peripheral blood has also been shown to promote disease progression and correlate with poor survival in HCC patients.\textsuperscript{12} IFN-\(\gamma\) is a pleiotropic cytokine with immune-modulatory, antiviral, antiproliferative and anti-tumor effects. In the liver, biologically active IFN-\(\gamma\) can interact with IFN-\(\gamma\) receptor, which initiates subsequent IFN-\(\gamma\) signaling pathways and induce hepatocyte apoptosis or cell cycle arrest.\textsuperscript{27} IFN-\(\gamma\) has direct suppressive effect of tumor growth, inhibition of angiogenesis and effects both on innate and adaptative immune responses against tumor.\textsuperscript{29} IFN-\(\gamma\) also induces autophagy through interferon-regulatory factor-1 signaling pathway in human HCC cells, which contributes to the growth-inhibitory effect of IFN-\(\gamma\).\textsuperscript{37} Based on our results that serum IFN-\(\gamma\) level correlates with tumor status and clinical outcome, serum IFN-\(\gamma\) level may reflect the host antitumor immunity in HCC and may be a potential marker to predict clinical outcome following curative treatment of HCC. Moreover, the estimated 1-year recurrence-free survival rate in our studied patients with IFN-\(\gamma\) \(\leq 50\) or \(>50\) pg/mL were 37 and 85.4\%, respectively. Patients with lower baseline IFN-\(\gamma\) levels have a higher risk of tumor recurrence and should receive more aggressive tumor surveillance after curative treatment.

IP-10, a chemokine secreted from cells stimulated with IFN-\(\gamma\), is a chemoattractant for activated T cells and may be a predictor of virological response in treatment of chronic hepatitis B and C.\textsuperscript{28,29} In this study, we showed significant correlation between serum IP-10 and IFN-\(\gamma\) levels, but IP-10 was not associated with tumor status or prognosis. Consistent with the tumor suppressive function of NKT cells in animal model,\textsuperscript{16} our HCC patients with higher frequencies of NK\(^+\) T cells had smaller tumor size, but peripheral NKT cells were not associated with tumor recurrence. Previous studies showed that frequencies of PD-1 expression could predict postoperative recurrence in patients with HCC.\textsuperscript{20} In our study, PD-1 expression was not associated with tumor recurrence. However, our enrolled patients had earlier tumor stages, which might contribute to less significant results in our study. The predictive value of TGF-\(\beta\) in the prognosis of HCC was not shown in our patients with early stage HCC as well as in previous study with unresectable HCC.\textsuperscript{25}

In 26 patients with available samples, we further tested the population of Th17 and T cells expressing CD69 and CD45RO. CD69 is an early activation marker of T cell, and CD4\(^+\)CD69\(^+\) T cells may be associated with HCC progression.\textsuperscript{38} CD45RO\(^+\) T cells, which represent subsets of T cells with effector and memory functions, have been shown to correlate with prognosis in patients with colon cancer.\textsuperscript{39} As shown in Supporting Information Table 2, a higher frequency of CD45RO\(^+\)CD4\(^+\) T cells correlated with more advanced tumor stage and larger tumor size. Recent studies have shown that different subsets of CD4 T cells, such as CD4\(^+\) cytotoxic T cells, Th17 and Tregs, may have different impact on the outcome of HCC.\textsuperscript{12,14,40} Due to small sample size, further study to delineate the subset of CD45RO\(^+\) CD4 T cells is warranted.

There was no significant change of populations of Th17, CD69\(^+\) and CD45RO\(^+\) T cells before and one month after curative treatment (Supporting Information Table 3). Higher frequencies of intratumoral and peritumoral Th17 cells have been shown to correlate with poor survival in patients with HCC.\textsuperscript{14} However, our data did not show this correlation by testing peripheral Th17 cells, and neither CD69\(^+\) nor CD45RO\(^+\) subsets of peripheral CD4 or CD8 T cells correlated with tumor recurrence in HCC patients (Supporting Information Table 4). The negative results might be due to the difference between peripheral blood and the tumor microenvironment.

Depletion of immunosuppressive cell subsets can potentially restore T-cell function in patients with advanced HCC.\textsuperscript{34} Our data provide the rationale to further investigate the mechanisms by which IFN-\(\gamma\) suppresses HCC growth and whether modulating IFN-\(\gamma\)-mediated immune response have clinical benefit for HCC patients. Previous reports have suggested the potential effect of IFN-\(\gamma\)-therapy for inoperable HCC.\textsuperscript{41,42} The survival benefit of adjuvant IFN-\(\gamma\)-therapy as well as depletion of suppressor cell populations for HCC after curative treatment warrants future research.

The activity of chronic HBV or HCV infection, which comprised 90\% of our studied population, may interfere with cytokine levels and frequency of immune regulatory cells, since these immune regulators all participate in the course of chronic HBV or HCV infection. However, this is inevitable in the study of HCC patients. Another limitation of this study is the lack of tumor tissues for analysis. A significant proportion of patients in this study received RFA as curative treatment for HCC, in whom tumor specimens were not available. However, peripheral blood samples are easily accessible and our results are practical for clinical application.

In conclusion, serum IFN-\(\gamma\) level has an inverse correlation with tumor stage and tumor size in BCLC stage A and B HCC patients. Patients with lower baseline IFN-\(\gamma\) levels have a higher risk of tumor recurrence after curative treatment. IFN-\(\gamma\) may reflect host anti-tumor immunity and may be a potential marker of HCC recurrence after curative treatment.

**REFERENCES**

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011;61: 69–90.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.
3. El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011;365: 1118–27.
4. Huang YH, Chen CH, Chang TT, et al. The role of transcatheter arterial embolization in patients with resectable hepatocellular carcinoma: a nation-wide, multicenter study. Liver Int 2004;24: 419–24.
5. Huang YH, Chen CH, Chang TT, et al. Evaluation of predictive value of CLIP, Okuda, TNM and JIS staging systems for hepatocellular
12. Fu J, Xu D, Liu Z, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 2007;133:2328–39.

13. Gao Q, Qiu SJ, Fan J, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol 2007;25:2586–93.

14. Zhang JP, Yan J, Xu J, et al. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. J Hepatol 2009;50:980–9.

15. Wu J, Du J, Liu L, et al. Elevated pretherapy serum IL17 in primary hepatocellular carcinoma patients correlates to increased risk of early recurrence after curative hepatectomy. PLoS One 2012;7:e50035.

16. Tatsuki T, Takehara T, Yamaguchi S, et al. Intrahepatic delivery of alpha-galactosylceramide-pulsed dendritic cells suppresses liver tumor. Hepatology 2007;45:22–30.

17. Wherry EJ. T cell exhaustion. Nat Immunol 2011;12:492–9.

18. Watanabe T, Bertolotti A, Tanoto TA, PD-L1/PD-L1 pathway and T-cell exhaustion in chronic hepatitis virus infection. J Viral Hepat 2010;17:453–8.

19. Gao Q, Wang XY, Qiu SJ, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 2009;15:971–9.

20. Shi F, Shi M, Zeng Z, et al. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. Int J Cancer 2011;128:887–96.

21. Budhu A, Wang XY. The role of cytokines in hepatocellular carcinoma. J Leukoc Biol 2006;80:1197–213.

22. Chau GY, Wu CW, Lui WY, et al. Serum interleukin-10 but not interleukin-6 is related to clinical outcome in patients with resectable hepatocellular carcinoma. Ann Surg 2000;231:552–8.

23. Blobe GC, Schiennmann WP, Locnh HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342:1350–8.

24. Achyut BR, Yang L. Transforming growth factor-beta in the gastrointestinal and hepatic tumor microenvironment. Gastroenterology 2011;141:1167–78.

25. Okumoto K, Hattori E, Tamura K, et al. Possible contribution of circulating transforming growth factor-beta to immunity and prognosis in unresectable hepatocellular carcinoma. Liver Int 2004;24:21–8.

26. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoeediting. Cytokine Growth Factor Rev 2002;13:95–109.

27. Horras CJ, Lamb CL, Mitchell KA. Regulation of hepatocyte fate by interferon-gamma. Cytokine Growth Factor Rev 2011;22:35–43.

28. Sonneveld MJ, Arends P, Boonstra A, et al. Serum levels of interferon-gamma-inducible protein-10 and response to peginterferon therapy in HBeAg-positive chronic hepatitis B. J Hepatol 2013;58:989–903.

29. Lagging M, Romero AI, Westin J, et al. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. Hepatology 2006;44:1617–25.

30. Lee IC, Lin CH, Huang YH, et al. IL28B polymorphism correlates with active hepatitis in patients with HBeAg-negative chronic hepatitis B. PLoS One 2013;8:e58071.

31. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–36.

32. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020–2.