의학박사 학위논문

# 수술적 치료를 받은 간세포암에서 면역관문 조절 인자가 임상경과에 미치는 영향

# The Effects of Immune Checkpoint Modulators on the Clinical Course of Patients with Resectable Hepatocellular Carcinoma

울 산 대 학 교 대 학 원

의 학 과

안 지 현

# The Effects of Immune Checkpoint Modulators on the Clinical Course of Patients with Resectable Hepatocellular Carcinoma

지 도 교 수　이 한 주

이 논문을 의학박사 학위 논문으로 제출함

2017년　12월

울 산 대 학 교 대 학 원

의　　학　　과

안　지　현

안지현의 의학박사학위 논문을 인준함

심사위원 이 한 주 (인)

심사위원 최 문 석 (인)

심사위원 김 강 모 (인)

심사위원 송 기 원 (인)

심사위원 심 주 현 (인)

울 산 대 학 교 대 학 원

2017년 12월

**ABSTRACT**

Immune checkpoint proteins regulating T-cell mediated anti-tumor immunity have been reported to affect clinical outcomes in multiple malignancies. This study aimed to investigate the prognostic effect of histological expression of immune checkpoint proteins in patients with resected hepatocellular carcinoma (HCC). A total of 221 patients with HCC who underwent curative resection were included. Expression of Programmed-cell death ligand-1 in tumor cells (*t*PD-L1) and tumor infiltrating mononuclear cells (TIMCs) (*i*PD-L1), Programmed-cell death-1 in TIMCs (*i*PD-1), and cytotoxic T lymphocyte antigen-4 in TIMCs (*i*CTLA-4) were measured immunohistochemically. Among the 221 patients, histo-positivity for *i*CTLA-4, *i*PD-1, *i*PD-L1, and *t*PD-L1 was 32.1% (n=71), 42.5% (n=94), 35.3% (n=78), and 14.9% (n=33), respectively. Multivariate logistic analyses revealed that male sex and tumor >5cm were variables related to *i*CTLA-4 positivity (odds ratios [ORs] 0.46 and 1.94 respectively; *P*s<0.05). Poor differentiation was related to PD-L1 expression in both tumor cells and TIMCs (ORs 2.88 and 3.46, respectively; *P*s<0.05). Microvascular invasion was significantly associated only with *i*PD-L1, whereas *t*PD-L1 was positively correlated with baseline elevation of serum alpha-fetoprotein (≥200 ng/ml) (ORs 2.24 and 2.45; *P*s<0.05). In time-dependent outcome analyses, expression of immune checkpoint proteins in TIMCs (i.e., *i*CTLA-4, *i*PD-1, and *i*PD-L1) was significantly related to longer overall survival and non-cancer-related survival (all *Ps*<0.05), but not to time-to-recurrence or cancer-specific deaths (all *Ps*>0.05). Concurrent activation of the PD-1:PD-L1 and CTLA-4 pathways predicted improved outcomes in terms of overall survival and non-cancer related survival (P=0.06 and P=0.03, respectively). In conclusion, immune checkpoint proteins upregulated in TIMCs in HCC tissues have

individual and additive effects in prolonging the survival of patients, specifically in terms of survival not related to cancer recurrence.

## Keywords

Liver cancer, prognosis, CTLA-4, PD-L1, PD-1

### List of Abbreviations

CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1. programmed-cell death-1; PD-L1, programmed-cell death ligand-1; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; TIMC, tumor-infiltrating mononuclear cell; OR, odds ratio; CI, confidence intervals; IQR, interquartile range; HR, hazard ratio.

# Index

# Table and figure index

## INTRODUCTION

The immune system plays a dual role in cancer.[1,2] First, it suppresses tumor growth by destroying cancer cells or inhibiting their growth. Second, it promotes tumor progression by selecting tumor cells that are more likely to survive in an immunocompetent host or by establishing conditions within the tumor microenvironment that may facilitate tumor growth.[2,3] Effective antitumor immunity depends on interactions between various T-cell regulatory receptors and ligands including the cytotoxic T lymphocyte antigen-4 (CTLA-4)/B7 and programmed-cell death-1 (PD-1)/its ligand (PD-L1) signaling pathways.[2,4]

These immune checkpoints are known to regulate different stages and signaling processes of the immune response.[3,4] At the initial stage of “priming” of naïve T-cell activation after antigen encounter, CTLA-4:B7 binding blocks stimulatory signals, and stops the development of potentially autoreactive T cells.[5,6] In contrast, a major role of the pathway involving PD-1 and its ligand, PD-L1, is to regulate previously activated T cells at the later “effector” stage of immune response.[7,8] In the tumor microenvironment, antigen-specific T cells induce PD-1 expression on reactive T lymphocytes and upregulate PD-L1 in tumor cells.[8] The subsequent PD-1:PD-L1 interaction results in T-cell exhaustion and immune evasion by tumor cells.[3,7] On the other hand, this interaction can limit collateral tissue damage, as observed for example in response to chronic infection by microorganisms such as hepatitis virus.[3,9-12]

Previous studies have shown that the immune checkpoint proteins CTLA4, PD-1, and PD-L1 can be used as reliable biomarkers for predicting the clinical behavior of many types of tumor.[13-19] These immune molecules are highly expressed in hepatocellular

carcinomas (HCCs) that are recognized histologically as immunogenic tumors.[20-22] In addition, hepatitis B and hepatitis C virus infections, two major causes of HCC, have been shown to interfere with antiviral immunity via the immune checkpoint pathways.[9,10,12,23] The relationships between the biology of HCC and immunomodulatory proteins is unclear and inconsistent across publications.[20-22,24]

Since immunotherapy for HCC is likely to become widespread in future, we aimed to identify different individual or interactive roles of immune checkpoint proteins during the long clinical course after surgical resection. We therefore examined the clinical and pathological factors associated with histological expression of immune checkpoint proteins in a series of patients with operable HCC.

## METHODS

### Study Patients

This clincopathologic study was based on tissue microarrays of paraffin-embedded samples from a cohort of 221 patients undergoing hepatic resection for HCC with curative intent in our center between 2004 and 2011. All included patients with histology-proven HCC had Child-Pugh A or B liver function without extrahepatic metastasis, gross vascular invasion or concomitant cancers. None received neoadjuvant or adjuvant treatment in the perioperative period. The study protocol was approved by the Institutional Review Board of Asan Medical Center.

Of the 221 patients, 165 (74.7%) were male, and the median age was 56 years. The most common cause of chronic liver disease was HBV infection (n=160, 72.4%). The

majority of patients had solitary tumors (89.6%; n=198), which ranged from 0.6 to 18.0 cm in maximum diameter. Microvascular invasion and poor differentiation on resected specimens were observed in 26.7% (n=59) and 64.7% (n=143) of the patients, respectively. Disease stage was classified as 0 (n=6, 2.7%), A (n=199, 90.1%), or B (n=16, 7.2%) according to the Barcelona Clinic Liver Cancer staging system. High preoperative alpha-fetoprotein (AFP) levels (≥200 ng/mL) were detected in 36.7% (n=81) of the patients. The clinical and biological features of the series are summarized in Table 1.

**Table 1. Clinical and pathological features of the 221 patients with hepatocellular carcinoma**

| Variable | N=221 |
|---|---|
| ***Clinical parameter*** | |
| Age, years | 56 (49-63) |
| Male gender | 165 (74.7%) |
| Etiology of chronic liver disease | |
| HBV | 160 (72.4%) |
| HCV | 21 (9.5%) |
| Others | 40 (18.1%) |
| Child-Pugh class A | 219 (99.1%) |
| Liver cirrhosis | 104 (47.1%) |
| BCLC stage | |
| BCLC stage 0 | 6 (2.7%) |
| BCLC stage A | 199 (90.1%) |
| BCLC stage B | 16 (7.2%) |
| Serum AFP, ng/mL | 43.1 (4.8-578.0) |
| Serum AFP ≥200 ng/mL | 81 (36.7%) |
| ***Pathological parameter*** | |
| Maximal tumor diameter, cm | 4.0 (3.0-5.0) |
| Solitary tumor | 198 (89.6%) |

| | |
|---|---|
| Microvascular invasion | 59 (26.7%) |
| Edmondson-Steiner grade | |
| I or II | 78 (35.3%) |
| III or IV | 143 (64.7%) |
| ***Immuno-histochemical parameter*** | |
| CTLA-4 expression on TIMC (*i*CTLA-4) | 71 (32.1%) |
| PD-1 expression on TIMC (*i*PD–1) | 94 (42.5%) |
| PD-L1 expression on TIMC (*i*PD–L1) | 78 (35.3%) |
| PD-L1 expression on tumor cells (*t*PD–L1) | 33 (14.9%) |

Data are presented as number (%) or median (interquartile range).

HBV, hepatitis B virus; HCV, hepatitis C virus; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; PD-L1, programmed cell-death ligand-1; TIMC, tumor-infiltrating mononuclear cells; PD-1, programmed cell death-1; CTLA-4, Cytotoxic T lymphocyte antigen-4.

## Immunohistochemical Staining and Evaluation

Serial 4-μm thick sections of formalin-fixed paraffin-embedded samples of HCCs and adjacent tissue were used for immunohistochemical staining. All the slides were processed on an automated immunostaining device (Ventana Medical System, Tucson, AZ), with an OptiView DAB IHC Detection Kit (Ventana Medical System) according to the manufacturer's instructions. The following primary antibodies were used: anti-PD-L1 (rabbit monoclonal E1L3N, 1/100; Cell Signaling, Danvers, MA), anti-PD-1 (mouse monoclonal ab52587, 1/100; Abcam, Cambridge, UK), and anti-CTLA-4 (Mouse monoclonal ab134090, 1/500; Abcam, Cambridge, UK). All immunostaining was independently reviewed by two pathologists (EY, HK.) specialized in liver diseases, who was blinded to clinical outcomes.

PD-L1 expression was assessed in both tumor cells (*t*PD-L1) and intratumoral

inflammatory cells, identified as T cells, macrophages and dendritic cells that infiltrated tumor cell nests (*i*PD-L1). The percentages of cells with surface PD-L1 were scored, and cases with ≥1% tumor cell expression were considered positive.[13,15,25] PD-1 expression was only observed in intratumoral lymphocytes, and ≥1% positive cells were regarded as positive.[21,26] Samples with any expression of CTLA-4 in tumor-infiltrating mononuclear cells and tumor cells were classified as positive.[27,28] Representative images of positive staining of each molecule were shown in Fig. 1.

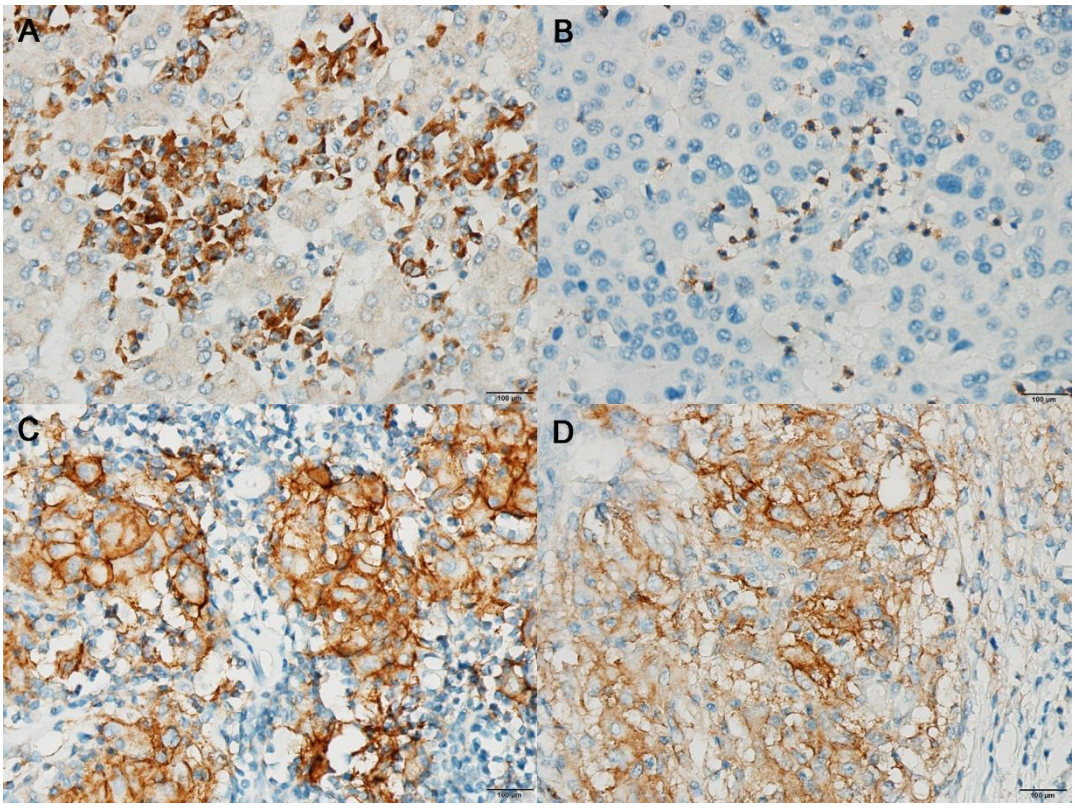


**FIG. 1. Presence of immune checkpoint proteins in formalin-fixed paraffin-embedded samples. Positive staining of each molecule is shown as follows (X400): (A) CTLA-4 in tumor-infiltrating mononuclear cells (TIMCs), (B) PD-1 in TIMCs, (C) PD-L1 in TIMCs, and (D) PD-L1 in tumor cells.**

## Statistical Analysis

Continuous variables and proportions were compared using the Mann-Whitney, chi-square, and Fisher's exact tests, as appropriate. Probabilities of overall survival, cancer-specific survival, non-cancer related survival, and time-to-tumor recurrence were estimated using the Kaplan–Meier method and compared using Cox proportional hazards regression models according to the expression of individual and combinations of immune checkpoints. Death due to HCC progression was considered cancer-specific survival. Hazard ratios were adjusted for age, gender, etiology of liver disease, presence of cirrhosis, Child-Pugh class, serum AFP, tumor stage at diagnosis, tumor number, tumor size, presence of vascular invasion, and histologic differentiation. A backward elimination approach involving candidate variables with P-values ≤0.10 in the univariate analysis was used in the multivariable analysis. The associations between clinical and pathological variables and immune checkpoint expression were analyzed by the logistic regression method. Correlations between pairs of immune checkpoints were evaluated by Pearson's coefficient method. Two-tailed values of $P<0.05$ were considered statistically significant. Statistical analyses were performed with SPSS 22.0 (SPSS Inc., Chicago, IL).

# RESULTS

## Expression profiles and Inter-relationships of Immune Checkpoint Proteins

The immunohistochemical features of the specimens are summarized in Table 1. *i*PD-

1, *i*PD-L1, and *i*CTLA-4 were expressed in 42.5% (n=94), 35.3% (n=78), and 32.1% (n=71) of the tumor-infiltrating mononuclear cells (TIMCs), respectively, in the entire 221 samples; and *t*PD-L1 was positive in 14.9% (n=33) of the tumor cells. No CTLA-4-positive neoplastic cells were detected in any sample.

Univariate and subsequent multivariate logistic regressions showed that poorly differentiated histology was the only variable independently associated with the expression of both *i*PD-L1 and *t*PD-L1 (adjusted odds ratio [OR] 2.88, 95% confidence intervals [CI] 1.31-6.32 *P*=0.008, and OR 3.46, 95% CI 1.11-10.84, *P*=0.033, respectively; Table 2). Microvascular invasion was only significantly related to *i*PD-L1 expression (OR 2.24, 95% CI 1.03-4.86, *P*=0.041). *i*CTLA-4 was associated with male sex (OR 0.46, 95% CI 0.24-0.87, *P*=0.017), and *i*PD-1 positivity was not significantly related to clinical or other pathological parameters (Table 2).

**Table 2. Independent clinico-pathological features related to expression of immune checkpoint proteins**

| Variable | ***i*CTLA-4** | | | ***i*PD-1** | | | ***i*PD-L1** | | | ***t*PD-L1** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI | *P* | OR | 95% CI | *P* | OR | 95% CI | *P* | OR | 95% CI | *P* |
| Male sex | 0.46 | 0.24-0.87 | 0.017 | - | - | - | - | - | - | - | - | - |
| HBV infection | - | - | - | 2.20 | 1.00-4.84 | 0.051 | 1.61 | 0.72-3.64 | 0.249 | 1.67 | 0.55-5.05 | 0.362 |
| AFP ≥200 ng/mL | 0.91 | 0.50-1.68 | 0.767 | - | - | - | 1.45 | 0.68-3.08 | 0.337 | 2.45 | 1.07-5.62 | 0.034 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tumor size >5cm | 1.94 | 1.01-3.69 | 0.045 | - | - | - | - | - | - | - | - | - |
| Vascular invasion | - | - | - | - | - | - | 2.24 | 1.03-4.86 | 0.041 | 1.43 | 0.56-3.61 | 0.456 |
| Poor differentiation | - | - | - | 1.05 | 0.51-2.16 | 0.899 | 2.88 | 1.31-6.32 | 0.008 | 3.46 | 1.11-10.84 | 0.033 |

Variables including age, presence of liver cirrhosis, Child—Pugh class, and number of tumors were not significantly related to expression of any immune checkpoint molecules in the univariate analysis (all $P \geq 0.10$).

*i*CTLA-4, Cytotoxic T lymphocyte antigen-4 in tumor-infiltrating mononuclear cells; *i*PD-1, programmed cell death-1 in tumor-infiltrating mononuclear cells; *i*PD-L1, programmed cell-death ligand-1 in tumor-infiltrating mononuclear cells; *t*PD-L1, programmed cell-death ligand-1 in tumor cells; OR, odds ratio; CI, confidence interval; HBV, hepatitis B virus; AFP, alpha-fetoprotein.

In terms of the relationships between different immune checkpoints, Pearson's correlation analyses revealed that the expression of all three molecules in TIMCs and tumor cells was significantly correlated, with coefficients ranging from 0.282 to 0.437 (all *Ps*<0.001: Table 3).

**Table 3. Interrelationships of the individual immune checkpoint proteins in the different cell types**

| **Variable** | ***i*CTLA-4** | | ***i*PD-1** | | ***i*PD-L1** | | ***t*PD-L1** | |
|---|---|---|---|---|---|---|---|---|
| | *r* | *P* | *r* | *P* | *r* | *P* | *r* | *P* |
| ***i*CTLA-4** | - | - | 0.427 | <0.001 | 0.303 | <0.001 | 0.283 | <0.001 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***i*PD-1** | 0.427 | <0.001 | - | - | 0.437 | <0.001 | 0.282 | <0.001 |
| ***i*PD-L1** | 0.303 | <0.001 | 0.437 | <0.001 | - | - | 0.567 | <0.001 |
| ***t*PD-L1** | 0.283 | <0.001 | 0.282 | <0.001 | 0.567 | <0.001 | - | - |

*i*CTLA-4, Cytotoxic T lymphocyte antigen-4 in tumor-infiltrating mononuclear cells; *i*PD-1, programmed cell death-1 in tumor-infiltrating mononuclear cells; *i*PD-L1, programmed cell-death ligand-1 in tumor-infiltrating mononuclear cells; *t*PD-L1, programmed cell-death ligand-1 in tumor cells.

## Survival and Recurrence Analyses as a function of Expression of Immune Checkpoint Proteins

During a median 7.09 years of follow-up (interquartile range [IQR], 5.52-8.93 years), 71 patients (32.1%) died, and 49.3% of the deaths (n=35) were related to HCC progression. In Kaplan-Meier models, 5-year overall survival rates in patients with and without *i*CTLA-4, *i*PD-1, *i*PD-L1, and *t*PD-L1 expression were 85.9% and 75.3% (*P*=0.076); 85.1% and 74.1% (P=0.010); 84.6% and 75.5% (*P*=0.022); and 75.8% and 79.3% (*P*=0.511) (Fig. 2). After adjustment of confounding covariates with *P*-values of <0.10 in the univariate analysis, the individual prognostic values of *i*CTLA-4, *i*PD-1, and *i*PD-L1 positivity for overall survival remained significant (adjusted hazard ratio [HR] 0.49, 95% CI 0.28–0.87, *P*=0.014, HR 0.53, 95% CI 0.32–0.88, *P*=0.015; and HR 0.52 95% CI 0.29-0.91, *P*=0.023, respectively, Table 4).

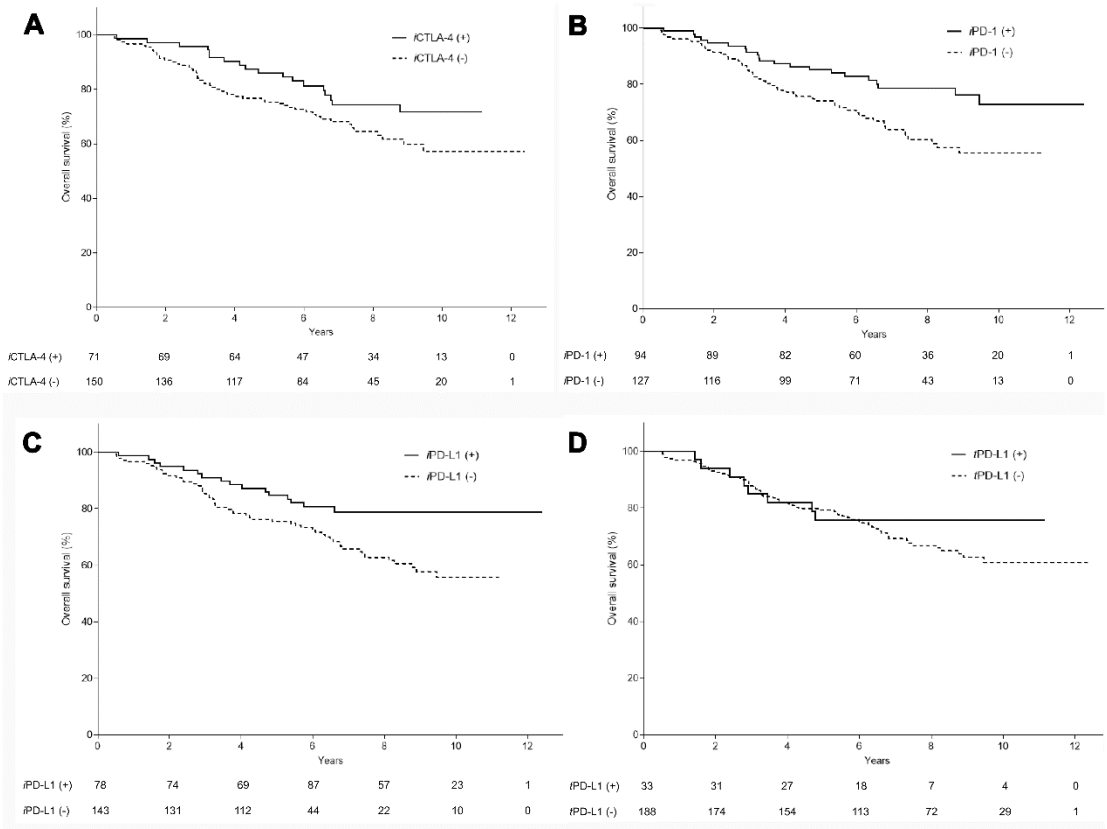


**FIG. 2. Associations between presence of immune checkpoint proteins and overall survival. Enhanced expression of Immune checkpoint molecules in tumor-infiltrating mononuclear cells ([A] *i*CTLA4, [B] *i*PD-1, and [C] *i*PD-L1) was significantly associated with longer survival of HCC patients, whereas tumoral PD-L1 ([D] *t*PD-L1) had no prognostic significance.**

**Table 4. Associations between expression of immune checkpoint proteins and time-dependent outcomes in 221 patients with hepatocellular carcinoma**

| Variable | Univariate analysis | | | Multivariate analysis* | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| ***Overall survival*** | | | | | | |
| *i*CTLA-4 | 0.62 | 0.36-1.06 | 0.078 | 0.49 | 0.28-0.87 | 0.014 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *i*PD-1 | 0.52 | 0.31-0.86 | 0.011 | 0.53 | 0.32-0.88 | 0.015 |
| *i*PD-L1 | 0.53 | 0.30-0.92 | 0.024 | 0.52 | 0.29-0.91 | 0.023 |
| *t*PD-L1 | 0.78 | 0.37-1.63 | 0.512 | - | - | - |
| ***Cancer-specific survival*** | | | | | | |
| *i*CTLA-4 | 0.80 | 0.39-1.62 | 0.530 | - | - | - |
| *i*PD-1 | 0.78 | 0.40-1.52 | 0.460 | - | - | - |
| *i*PD-L1 | 0.70 | 0.34-1.46 | 0.340 | - | - | - |
| *t*PD-L1 | 0.78 | 0.28-2.20 | 0.636 | - | - | - |
| ***Non-cancer-related survival*** | | | | | | |
| *i*CTLA-4 | 0.46 | 0.20-1.05 | 0.065 | 0.39 | 0.17-0.90 | 0.028 |
| *i*PD-1 | 0.31 | 0.14-0.71 | 0.006 | 0.35 | 0.15-0.83 | 0.016 |
| *i*PD-L1 | 0.37 | 0.15-0.90 | 0.028 | 0.35 | 0.14-0.87 | 0.024 |
| *t*PD-L1 | 0.79 | 0.28-2.23 | 0.650 | - | - | - |
| ***Time-to-recurrence*** | | | | | | |
| *i*CTLA-4 | 0.71 | 0.46-1.08 | 0.115 | - | - | - |
| *i*PD-1 | 0.72 | 0.49-1.08 | 0.115 | - | - | - |
| *i*PD-L1 | 0.74 | 0.48-1.12 | 0.156 | - | - | - |
| *t*PD-L1 | 0.70 | 0.38-1.27 | 0.237 | - | - | - |

Hazard ratios and confidence intervals were obtained using Cox proportional hazard models adjusted for age, sex, hepatitis B virus infection, presence of liver cirrhosis, Child-Pugh class, serum alpha-fetoprotein, tumor size, tumor number, presence of vascular invasion, and poor differentiation.

HR, hazard ratio; CI, confidence interval; iCTLA-4, Cytotoxic T lymphocyte antigen-4 in tumor-infiltrating mononuclear cells; *i*PD-1, programmed cell death-1 in tumor-infiltrating mononuclear cells; *i*PD-L1, programmed cell-death ligand-1 in tumor-infiltrating mononuclear cells; *t*PD-L1, programmed cell-death ligand-1 in tumor cells.

The adjusted confounding covariates were age, sex, cirrhosis, HBV infection, liver cirrhosis, Child-Pugh class, serum AFP, size and number of tumors, microvascular invasion, and poor differentiation. In subsequent survival analyses by specific cause of death, histologic upregulation of any of the three proteins did not influence cancer-related survival (all *P*s>0.05; Table 4 and Fig. 3). However, positive expression of

*i*CTLA-4, *i*PD-1, or *i*PD-L1 was independently associated with reduced mortality from causes other than HCC (HR 0.39, 95% CI 0.17–0.90, *P*=0.028, HR 0.35, 95% CI 0.15–0.83, *P*=0.016; and HR 0.35 95% CI 0.14-0.87, *P*=0.024, respectively, Table 4 and Fig. 4).

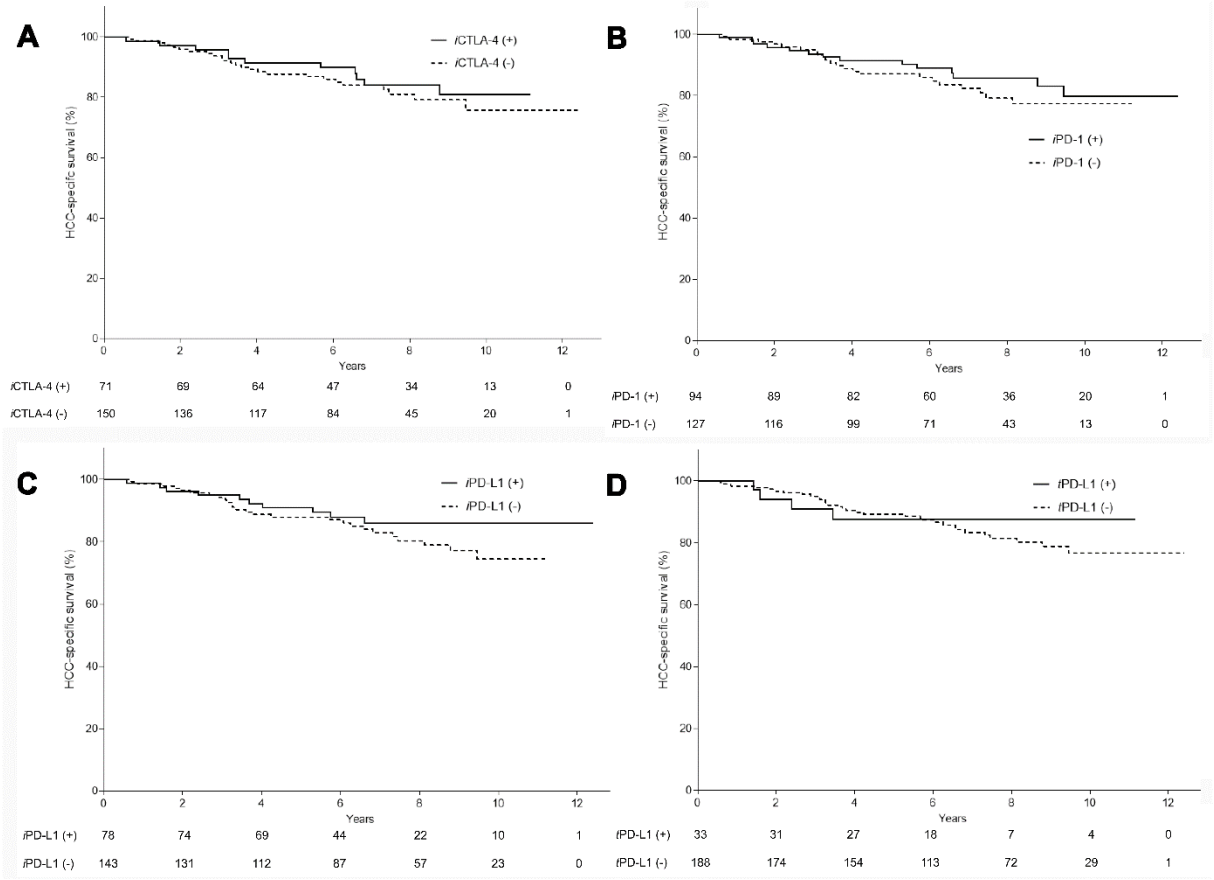


**Figure 3. Expression of immune checkpoint proteins and cancer-specific survival. None of the immune checkpoint proteins was significantly associated with cancer-specific survival. (all *P*s>0.05). (A) *i*CTLA4, (B) *i*PD-1, (C) *i*PD-L1, and (D) *t*PD-L1.**

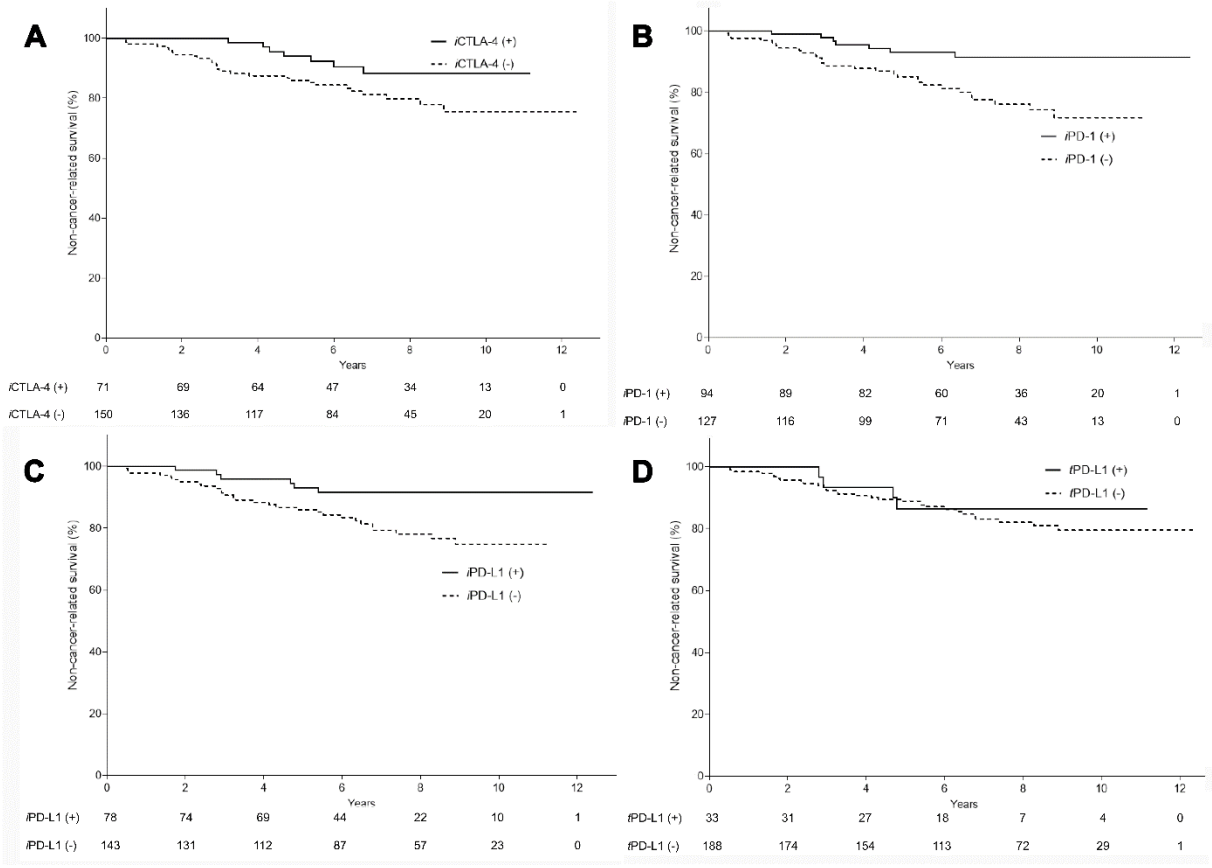


**Figure 4. Expression of immune checkpoint proteins and non-cancer-related survival. Enhanced expression of Immune checkpoint molecules in tumor-infiltrating mononuclear cells was associated with a better outcome in terms of non-cancer-related survival ([A] *i*CTLA4, [B] *i*PD-1, and [C] *i*PD-L1, all *Ps*<0.05), whereas tumoral PD-1 ([D] *t*PD-L1) did not have any impact on non-cancer-related mortality.**

After resection, HCC reoccurred in 45.7% of the total patients (n=101). Median time-to-recurrence was 4.6 years (IQR, 1.1-6.7 years). Microscopic vessel invasion and multiple tumor number were significantly associated with shorter time-to-recurrence

after resection (adjusted HRs [95% CI], 0.53 [0.32–0.88] and 0.52 [0.29–0.91], respectively; both *P*s<0.05). There were no correlations between expression of immune checkpoints and time-to-recurrence (all *P*s>0.05; Table 4 and Fig. 5).

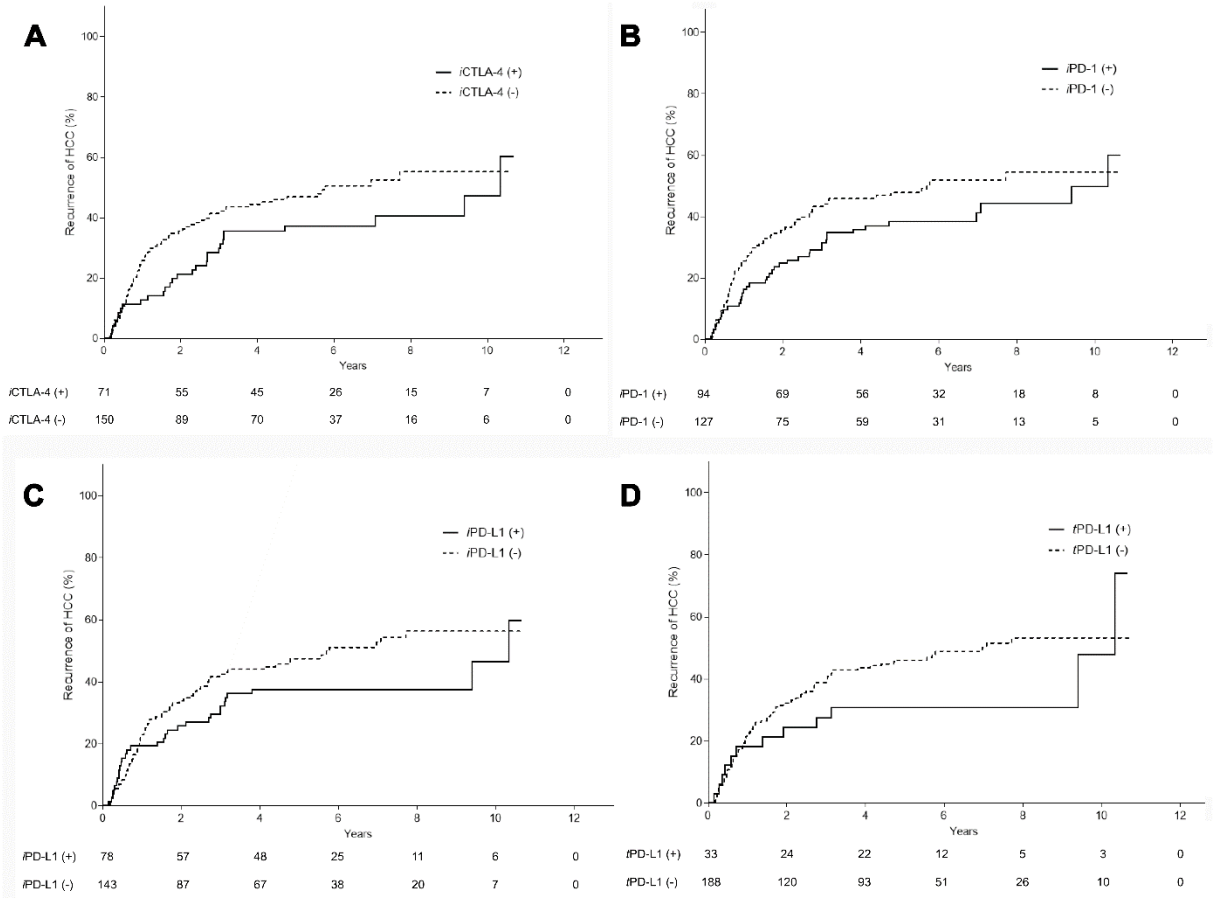


**FIG. 3. Expression of immune checkpoint proteins and time-to-recurrence. None of the immune checkpoint proteins was significantly associated with time-to-recurrence in the HCC patients. (all *P*s>0.05). (A) *i*CTLA4, (B) *i*PD-1, (C) *i*PD-L1, and (D) *t*PD-L1.**

**Prognostic Effect of Combined Expression of the PD-1/PD-L1 and CTLA-4 Pathways**

We further examined whether the PD-1/PD-L1 and CTLA-4 axes had a combined effect on survival. The patients were divided into three groups based on the expression of immune checkpoints: the first group was positive for both iPD-1 and/or *i*PD-L1, and *i*CTLA-4 (n=58; 26.2%), the second was positive for only one of the two pathways (n=71; 32.1%), and the third group was negative for both pathways (n=92; 41.6%). Kaplan-Meier estimates showed that the difference between the overall survival curves of the three groups was marginally significant (*P*=0.060; Fig. 4). When further analyzed, a significant difference was observed for non-cancer-related survival (*P*=0.025), but not for cancer-specific survival (*P*=0.807; Fig. 6)

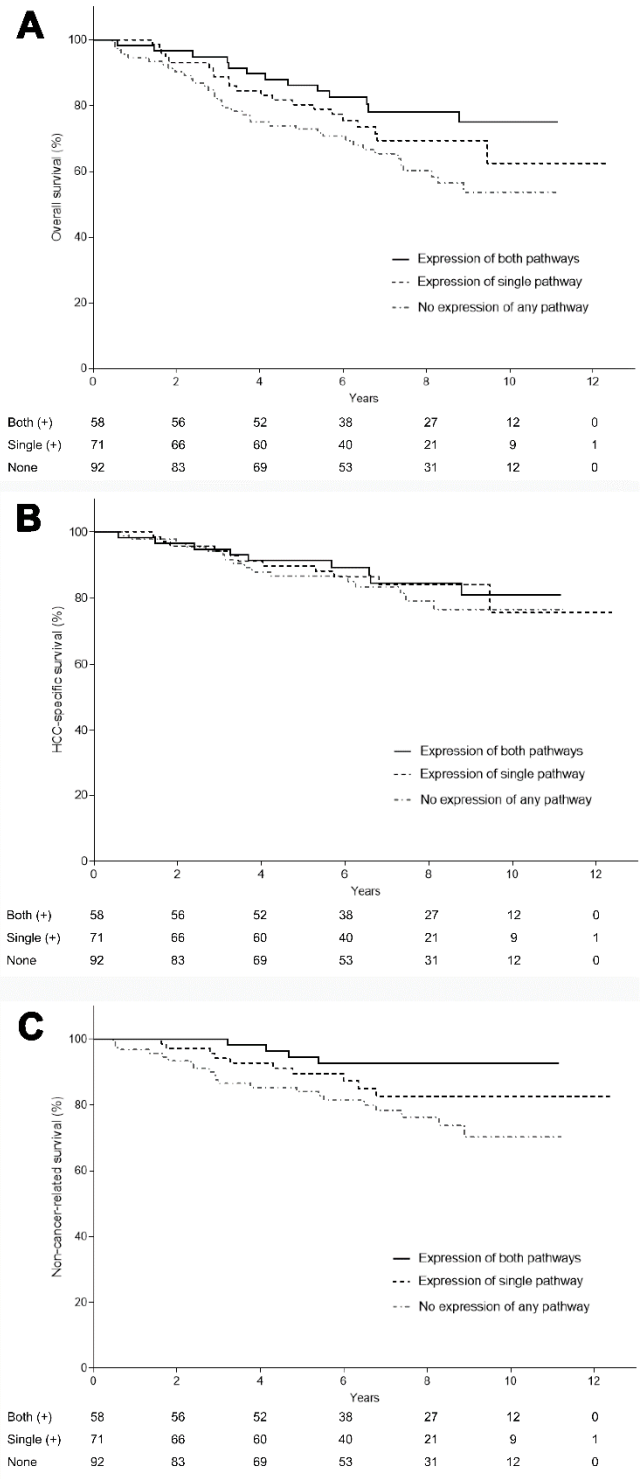


**FIG. 4. Effect of combined expression of the PD-1/PD-L1 and CTLA-4 pathways on overall survival. The patients were divided into the following three groups based on the expression of immune checkpoint proteins: activation of both**

**pathways (PD-1:PD-L1 & CTLA-4; group 1), activation of only one of the two pathways (group 2), and activation of neither pathway (group 3). The difference between the overall survival of the three groups was marginally significant (*P*=0.06). When further analyzed, a significant difference was observed for non-cancer-related survival (C), but not for cancer-specific survival (B).**

## DISCUSSION

Most HCCs arise on a background of chronic inflammatory liver, and thus are considered pathophysiologically typical immunogenic cancers.[29-31] Based on the immunological mechanisms thought to be acting during hepatocarcinogenesis, the effects of diverse immunomodulatory regimens such as therapeutic vaccination, immune checkpoint inhibitors, and transfer of adoptive cellular immunity, have been investigated.[32-34] Encouraging results are expected from ongoing clinical trials of immune checkpoint blockers in advanced HCC, as these blockers have already shown great promise in intractable cancers including advanced melanoma and lung cancer.[33,35-38] However, the efficacy of targeted immune checkpoints in patients with chronic liver disease and related HCC is unclear.

Previous studies have suggested that abnormal regulation of immune checkpoint proteins in tumors is linked to better or to worse prognosis depending on the type of cancer.[13-15,18,19] Quantitative meta-analyses of data on solid tumors has suggested that overexpression of PD-L1 in tumor cells, or PD-1 in tumor infiltrating immune cells, is associated with poor prognosis in patients with malignant neoplasms of epithelial

origin such as esophageal, gastric, colorectal, breast and ovarian cancers.[13] This could be due to the ability of the PD-1/PD-L1 pathway to inhibit T cell-mediated antitumor immunity and to act as an anti-apoptotic receptor on cancer cells. On the other hand, tumoral expression of PD-L1, rather than PD-1, has been shown to have beneficial effects in patients with non-small cell lung cancer, metastatic urothelial carcinoma, colorectal cancer of the proficient mismatch repair type, and laryngeal cancers.[14,19,39] It appears that CD8-positive T cells recruited from the tumor microenvironment induce a partial tumoricidal immune response, and promote upregulation of PD-L1 by secreting interferon-*r*.[40-42] This antitumor potential of the PD-1/PD-L1 axis was also observed in our HCC series. However French and Chinese studies of immune checkpoints in HCC have yielded inconsistent results.[21,22,24]

The role of CTLA-4 signaling, another immune checkpoint pathway, in the clinical setting of malignancy has been little investigated, and there are conflicting results about its impact on outcomes in patients with different forms of cancer.[17,27,28,43] Unlike a previous study on esophageal cancer,[28] our findings indicate that positive CTLA-4 expression in lymphocytes is associated with a better prognosis in HCC, as it is in breast cancer [43]: it seems that a high density of CTLA-4-positve lymphocytes is a secondary product of an increase of effector T cells that promotes immune invasion fighting against cancer.[39,43,44] Curiously, CTLA-4 was not detected within tumor cells in our and others' studies of HCC. In addition, in animal experiments, at least one third of the tumor-infiltrating lymphocytes expressed CTLA-4 along with PD-1, which might lead to synergistic immune modulation.[45] Interestingly, co-expression of the two signaling pathways and their additive effect on survival was also noted in our study.

It is well known that while the CD8+ T cell-mediated immune response during infections by hepatitis B and C viruses contributes to viral clearance, it also has a cytotoxic effect on the host liver.[9,12] Moreover, T cells also affect the progression of liver disease by inducing inhibitory immune checkpoint proteins.[10,11] This immune dampening during the chronic phases of liver disease may have a protective effect by limiting excessive necroinflammatory and fibrogenic responses in the liver, so reducing the chance of cancer development.[12] Such a process may account for our observation that immune checkpoint expression consistently reduced deaths unrelated to HCC rather than cancer-related deaths due to recurrence.

In our series, tumors expressing immune checkpoint proteins were more aggressive, with higher levels of AFP and greater immature and invasive pathology, which is in line with data from a prior French report.[21] The invasive effect of tumor immune checkpoints has also been observed in other solid neoplasms such as melanoma, renal cell carcinoma and breast cancer.[15,46,47] Despite the positive impact of the attenuation of hepatic inflammation by immune checkpoints, their injurious effect in interfering with antitumor immunity may justify blocking them as a therapeutic option in incurable HCC patients.

Our study has some limitations. The tissue microarray method used may not reflect the potential heterogeneity of immune checkpoint expression, although it can evaluate multiple samples rapidly. This weakness discouraged us from evaluating numbers of tumor infiltrating lymphocytes.[48] Another consideration is that the roles played by immune checkpoint pathways in more advanced tumors could not be clarified in our surgical cohort. In addition, immune checkpoint signaling has been found to be

upregulated under hypoxic conditions, and such conditions were often induced during treatment of the HCCs by chemoembolization or with sorafenib.[49,50]

## Conclusion

In conclusion, our investigation reveals the individual and additive effects of immune checkpoint molecules upregulated in the tumor-infiltrating mononuclear cells of HCC tissues in prolonging the survival time of patients, specifically survival not related to cancer recurrence. Because of the beneficial effects of immune checkpoints on survival, careful selection of therapeutic interventions will be required in future to avoid any harmful effects, particularly in the presence of active hepatitis.

## REFERENCES


1. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137-148.
2. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565-1570.
3. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252-264.
4. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. Am J Clin Oncol 2016;39:98-106.
5. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev 2008;224:166-182.
6. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med 1995;182:459-465.
7. Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol 2009;10:1185-1192.
8. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677-704.
9. Boonstra A, Woltman AM, Janssen HL. Immunology of hepatitis B and hepatitis C virus infections. Best Pract Res Clin Gastroenterol 2008;22:1049-1061.
10. Ye B, Liu X, Li X, Kong H, Tian L, Chen Y. T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance. Cell Death Dis 2015;6:e1694.

11. Bertoletti A, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? Curr Opin Microbiol 2000;3:387-392.

12. Kassel R, Cruise MW, Iezzoni JC, Taylor NA, Pruett TL, Hahn YS. Chronically inflamed livers up-regulate expression of inhibitory B7 family members. Hepatology 2009;50:1625-1637.

13. Wu P, Wu D, Li L, Chai Y, Huang J. PD-L1 and Survival in Solid Tumors: A Meta-Analysis. PLoS One 2015;10:e0131403.

14. Vassilakopoulou M, Avgeris M, Velcheti V, Kotoula V, Rampias T, Chatzopoulos K, et al. Evaluation of PD-L1 Expression and Associated Tumor-Infiltrating Lymphocytes in Laryngeal Squamous Cell Carcinoma. Clin Cancer Res 2016;22:704-713.

15. Choueiri TK, Fay AP, Gray KP, Callea M, Ho TH, Albiges L, et al. PD-L1 expression in nonclear-cell renal cell carcinoma. Ann Oncol 2014;25:2178-2184.

16. McLaughlin J, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, et al. Quantitative Assessment of the Heterogeneity of PD-L1 Expression in Non-Small-Cell Lung Cancer. JAMA Oncol 2016;2:46-54.

17. Kim JW, Nam KH, Ahn SH, Park do J, Kim HH, Kim SH, et al. Prognostic implications of immunosuppressive protein expression in tumors as well as immune cell infiltration within the tumor microenvironment in gastric cancer. Gastric Cancer 2016;19:42-52.

18. Fusi A, Festino L, Botti G, Masucci G, Melero I, Lorigan P, et al. PD-L1 expression as a potential predictive biomarker. Lancet Oncol 2015;16:1285-1287.

19. Bellmunt J, Mullane SA, Werner L, Fay AP, Callea M, Leow JJ, et al. Association of PD-L1 expression on tumor-infiltrating mononuclear cells and overall survival in patients with urothelial carcinoma. Ann Oncol 2015;26:812-817.

20. Umemoto Y, Okano S, Matsumoto Y, Nakagawara H, Matono R, Yoshiya S, et al. Prognostic impact of programmed cell death 1 ligand 1 expression in human leukocyte antigen class I-positive hepatocellular carcinoma after curative hepatectomy. J Gastroenterol 2015;50:65-75.

21. Calderaro J, Rousseau B, Amaddeo G, Mercey M, Charpy C, Costentin C, et al. Programmed death ligand 1 expression in hepatocellular carcinoma: Relationship With clinical and pathological features. Hepatology 2016;64:2038-2046.

22. Shi F, Shi M, Zeng Z, Qi RZ, Liu ZW, Zhang JY, et al. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. Int J Cancer 2011;128:887-896.

23. Willimsky G, Schmidt K, Loddenkemper C, Gellermann J, Blankenstein T. Virus-induced hepatocellular carcinomas cause antigen-specific local tolerance. J Clin Invest 2013;123:1032-1043.

24. Gao Q, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 2009;15:971-979.

25. Kim HR, Ha SJ, Hong MH, Heo SJ, Koh YW, Choi EC, et al. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. Sci Rep 2016;6:36956.

26. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 2013;139:667-676.

27. Huang PY, Guo SS, Zhang Y, Lu JB, Chen QY, Tang LQ, et al. Tumor CTLA-4 overexpression predicts poor survival in patients with nasopharyngeal carcinoma. Oncotarget 2016;7:13060-13068.

28. Zhang XF, Pan K, Weng DS, Chen CL, Wang QJ, Zhao JJ, et al. Cytotoxic T lymphocyte antigen-4 expression in esophageal carcinoma: implications for prognosis. Oncotarget 2016;7:26670-26679.

29. Hato T, Goyal L, Greten TF, Duda DG, Zhu AX. Immune checkpoint blockade in hepatocellular carcinoma: current progress and future directions. Hepatology 2014;60:1776-1782.

30. Makarova-Rusher OV, Medina-Echeverz J, Duffy AG, Greten TF. The yin and yang of evasion and immune activation in HCC. J Hepatol 2015;62:1420-1429.

31. Breous E, Thimme R. Potential of immunotherapy for hepatocellular carcinoma. J Hepatol 2011;54:830-834.

32. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, et al. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 2012;18:3686-3696.

33. Duffy AG, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, et al. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. J Hepatol 2017;66:545-551.

34. Shimizu K, Kotera Y, Aruga A, Takeshita N, Katagiri S, Ariizumi S, et al. Postoperative dendritic cell vaccine plus activated T-cell transfer improves the survival of patients with invasive hepatocellular carcinoma. Hum Vaccin Immunother 2014;10:970-976.

35. Kudo M. Immune Checkpoint Inhibition in Hepatocellular Carcinoma: Basics and Ongoing Clinical Trials. Oncology 2017;92 Suppl 1:50-62.

36. Ribas A, Kefford R, Marshall MA, Punt CJ, Haanen JB, Marmol M, et al. Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. J Clin Oncol 2013;31:616-622.

37. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. J Clin Oncol 2014;32:1020-1030.

38. Rizvi NA, Mazieres J, Planchard D, Stinchcombe TE, Dy GK, Antonia SJ, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. Lancet Oncol 2015;16:257-265.

39. Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer 2013;49:2233-2242.

40. Wimberly H, Brown JR, Schalper K, Haack H, Silver MR, Nixon C, et al. PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer. Cancer Immunol Res 2015;3:326-332.

41. Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 2014;20:5064-5074.

42. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 2012;4:127ra137.

43. Yu H, Yang J, Jiao S, Li Y, Zhang W, Wang J. Cytotoxic T lymphocyte antigen 4 expression in human breast cancer: implications for prognosis. Cancer Immunol Immunother 2015;64:853-860.

44. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor microenvironment. Cell Death Dis 2015;6:e1792.

45. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res 2013;73:3591-3603.

46. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer 2010;116:1757-1766.

47. Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J. Prognostic significance of PD-L1 and PD-L2 in breast cancer. Hum Pathol 2016;47:78-84.

48. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26:259-271.

49. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, et al. PD-L1 is a novel direct target of HIF-1alpha, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. J Exp Med 2014;211:781-790.

50. Prieto J, Melero I, Sangro B. Immunological landscape and immunotherapy of hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 2015;12:681-700.

**국문요약**

T 림프구가 관여하는 항종양 면역을 조절하는 면역 관문 단백질들이 다양한 종양에서 임상 경과에 영향을 줄 수 있다는 보고들이 있었다. 따라서 본 연구에서는 수술적 치료를 받은 간세포암 환자들을 대상으로 면역 관문 단백질의 조직학적 발현정도가 예후에 미치는 영향을 보고자 하였다. 근치적 간암 절제술을 시행받은 221명의 환자들이 연구에 참여하였고, 종양 세포 및 종양 미세 환경 내 단핵구의 Programmed-cell death ligand-1 (PD-L1)의 발현, 종양 미세 환경내 단핵구의 Programmed-cell death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4)의 발현 정도를 면역조직화학염색법을 사용하여 평가하였다. 221명의 환자들 중 종양 미세 환경내 CTLA4, PD-1, PD-L1의 발현율은 각각 32.1% (n=71), 42.5% (n=94), 35.3% (n=78)였고, 종양내 PD-L1의 발현율은 14.9% (n=33) 였다. 다중로지스틱 회귀 분석에 따르면 성별 및 종양 크기 5cm 초과가 CTLA4 양성과 유의한 관계가 있었다 (오즈비 0.46과 1.94; *P*s<0.05). 불량한 조직 분화도는 종양 세포 및 종양 미세 환경에서의 PD-L1 발현과 연관이 있었다 (오즈비 2.88 and 3.46, *P*s<0.05). 미세혈관침범(Microvascular invasion)은 종양미세환경내 PD-L1의 발현과 관계가 있는데 반해, 혈청태아단백의 상승은 종양내 PD-L1의 발현과 연관이 있었다 (오즈비

2.24 and 2.45; *P*s<0.05). 다변량 생존 분석시 종양미세환경 내 면역 관문 단백질의 발현은 전체 생존 및 비-암 관련 생존율 (non-cancer-related survival)과 유의한 연관이 있었지만 (*Ps*<0.05), 재발까지의 시간이나 암 관련 생존율과는 상관관계를 보이지 않았다 (all *Ps*>0.05). PD-1:PD-L1 신호전달체계와 CTLA-4 신호전달체계의 동시 활성화는 전체 생존율 및 비-암 관련 생존율의 향상과 관련이 있었다. 결론적으로 면역 관문 단백질이 간세포암의 종양미세환경에서 활성화되는 경우 환자의 좋은 예후, 특히 암과 관련되지 않은 생존율 연장과 연관이 있는 것으로 확인되었다.

## 중심단어

간암, 예후, CTLA-4, PD-L1, PD-1