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# High number of PD-1 positive intratumoural lymphocytes predicts survival benefit of cytokine-induced killer cells for hepatocellular carcinoma patients

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## Abstract

**Background & Aims**: Adjuvant cytokine-induced killer (CIK) cells treatment has shown potential in reducing the recurrence rate and prolonging the survival of patients with hepatocellular carcinoma (HCC). We aimed to identify the best predictive biomarker for adjuvant CIK cells treatment in patients with HCC after curative resection.

**Methods**: This study retrospectively included 145 pairs of HCC patients by one-to-one propensity score matching. One group received CIK cells transfusion after surgery (surgery-CIK group); the other one group underwent surgery only (surgery-only group). Immunohistochemistry (IHC) was used to measure PD-1, PD-L1, CD4, CD8 and Foxp3 expression in tumour tissues of surgery-CIK group; IHC of PD-1 and PD-L1 was conducted in the surgery-only group.

**Results**: The surgery-CIK group had a significantly higher disease-free survival (DFS) and overall survival (OS) rates compared to the surgery-only group. Of all the intratumoural biomarkers, in the surgery-CIK group, multivariate analysis showed that a high number of PD-$1^+$ tumour infiltrative lymphocytes (TILs) was the only factor that independently predicted favourable OS and DFS. By contrast, in the surgery-only group, no significant correlations between PD-1/PD-L1 expression and survival of patients were identified. Further correlation analysis showed a high number of PD-$1^+$ TILs associated with a high number of both $CD4^+$ and $CD8^+$ TILs in surgery-CIK group.

**Conclusions**: A high number of PD-$1^+$ TILs can serve as a potent biomarker for adopting CIK cells therapy in HCC patients after curative resection.

KEYWORDS
adoptive cellular immunotherapy, cancer, immunohistochemistry, prognosis

## 1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related mortality worldwide.[1] Although the efficacy of HCC treatment has significantly improved

**Abbreviations:** BCLC, Barcelona clinic liver cancer; CIKs, cytokine-induced killer cells; DFS, disease-free survival; HCC, hepatocellular carcinoma; IHC, Immunohistochemistry; MHC, major histocompatibility complex molecules; OS, overall survival; PD-1, programmed death-1; PD-L1, Programmed death-ligand 1; TILs, tumour-infiltrating lymphocytes.

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over the last decade due to the progress in resection criteria and locoregional treatment techniques, the prognosis of HCC remains poor, with a high incidence of local recurrence and distant metastases.[2] Currently, no adjuvant therapy for HCC is recommended after curative treatment in prevailing guidelines. There is also a pressing need for more effective treatment options that would result in more prolonged survival and in reducing tumour recurrence.

Recently, cancer immunotherapy has emerged as an appealing field in eliminating the micrometastatic and residual disease of cancer,[3] among which cytokine-induced killer (CIK) cells therapy is a promising option. CIK cells are activated lymphocytes, generated by expanding mononuclear cells in peripheral blood with cytokines comprising IFN-γ, IL-2 and anti-CD3 antibody.[4] Clinical studies have shown that adoptive CIK cells therapy has potent antitumour effects and can improve the prognosis of a variety of malignancies.[5]

For HCC, adjuvant CIK therapy has been demonstrated effective in prolonging patients' survival in accumulating study.[5] However, the survival benefit of CIK cells therapy varied significantly between HCC patients. Biomarkers that can differentiate between responders and non-responders are warranted for personalized treatment.

There is emerging evidence that immune escape and cancer immunoresistance play an essential role in tumour recurrence.[3] Programmed death receptor 1 (PD-1) is a transmembrane receptor, and high levels of PD-1 were mainly found in activated $CD8^+$, $CD4^+$ lymphocytes and NK cells. To date, two ligands have been identified for PD-1, namely the programmed death-ligand 1 (PD-L1) and PD-L2. The PD-1-PD-L1 engagement blocks T-cell receptor signalling, inhibits T-cell proliferation and contributes to T-cell exhaustion, which is a crucial mechanism of immune evasion by tumours.[6] The immunohistochemical expression of PD-1/PD-L1 has been correlated with the treatment response of PD-1 blockade immunotherapy in various malignancies.[7] In addition, the PD-1 expression on tumour-infiltrative lymphocytes (TILs) can predict antitumor responses of TILs, a kind of adoptive cellular immunotherapy.[8,9]

A recent study found that high intratumoral PD-L1 expression in HCC correlated with an improved treatment response of CIK cells therapy[10]; however, in their research, PD-L1 expression was the only immune-related factor investigated, the predictive value of other immunological factors in the local immune microenvironment like the expression of PD-1 on TILs and its association with different subpopulation of TILs were not assessed. A comprehensive investigation of the predictive values of tumoural PD-1/PD-L1 expression and various subpopulation of TILs can give us an insight into the potential mechanisms and help identify the best predictive biomarkers.

This study aimed to characterize the expression of PD-1 on TILs and PD-L1 on tumour cells and to determine whether the number of $PD\text{-}1^+$ TILs was a more potent biomarker than PD-L1 expression in predicting the response of CIK cells treatment.

**Key points**

- CIK cell therapy improves the prognosis of HCC patients after curative resection.
- No association between $PD\text{-}1^+$ TILs and patient survival in the surgery-only group.
- $PD\text{-}1^+$ TILs could predict the efficacy of CIK treatment received by HCC patients.
- The correlation between high number of $PD\text{-}1^+$ TILs and the high number of both $CD4^+$ and $CD8^+$ TILs suggested that $PD\text{-}1^+$ TILs can reflect the existence of endogenous host immune response to tumours.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient selection

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by Sun Yat-sen University Cancer Center's Ethics Committee (No. YB2016-058). All patients gave written informed consent for the collection of tissue for research. Between March 2004 and December 2011, the medical records of consecutive HCC patients at Sun Yat-sen University Cancer Center were reviewed. The inclusion criteria were: (1) pathologically diagnosed HCC; (2) ECOG 0 or 1; (3) Child-Pugh Class A and (4) underwent curatively surgical resection as initial treatment. The exclusion criteria were: (1) history of cardiac disease; (2) previous or concurrent cancer; (3) central nervous system disease; (4) active infections other than chronic viral hepatitis; (5) history of organ allograft and (6) pregnant or breastfeeding women.

A total of 1404 patients met the criteria, of which 1259 patients received surgical resection alone (surgery-only group) and 145 patients received post-operative CIK cell immunotherapy (surgery-CIK group). To reduce selection bias between the two groups, we employed a quasi-experimental design to mimic randomized clinical trial using one-to-one propensity score matching. The selected variables entered into the propensity model included age, gender, Child-Pugh score, Barcelona clinic liver cancer (BCLC) stage, HBV history, serum AFP level, tumour size, tumour number, grades of differentiation, microscopic vascular invasion, microscopic satellite nodules, which covered all the typical clinicopathological factors that correlate with the decision in adopting of CIK cell treatment and the prognosis of HCC patients after surgery. Finally, a total of 145 pairs of patients were included in the final analysis (Figure S1).

### 2.2 | Surgical resection and CIK cells treatment

Curative resection was performed by experienced surgeons. For patients who suffered tumour recurrence, apart from a second resection, other therapies (TACE, RFA, SIRT and sorafenib) were decided by a multidisciplinary group, which consisted of physicians, interventional radiologists and oncologists.

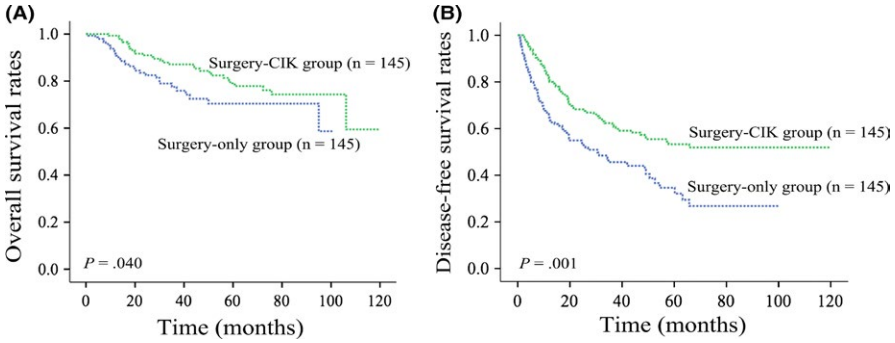


**FIGURE 1** Kaplan-Meier curves for the analysis of differences in survival between surgery-CIK group and surgery-only group in HCC patients. (A) Overall survival (OS) and (B) disease-free survival (DFS). *P* values were calculated by log-rank test

The preparation of CIK cells was described in our previous studies[11] and the CIK cells treatment assignment schedule is shown in Figure S2. More detailed information can be found in supplementary materials.

### 2.3 | Immunohistochemical (IHC) staining

All of the 290 patient tumour tissue samples were analysed immunohistochemically. The detailed method of IHC was provided in supplementary materials.

### 2.4 | Quantification of PD-1/PD-L1 expression and immune cell infiltration

The degree of immunostaining was scored independently by two observers in a blind manner. The interobserver agreements (Fleiss' kappa value) for each IHC staining factor between the two observers were shown in Table S1.

The proportion of PD-L1 positive cells of total tumour cells was scored as the percentages: ≥5%, 1%-5% and <1%; the tumour positivity was defined using the cut-off of 5%, based on previous studies.[10]

PD-1 was evaluated by the intensity of staining and the proportion of positively stained tumour-infiltrating lymphocytes. The staining index was calculated by the proportion of positive tumour-infiltrating lymphocytes multiply the staining intensity score, which was similar to other studies.[12,13] The proportion of lymphocytes was scored as 0 (no positive cells), 1 (0%-30% positive cells), 2 (30%-60% positive cells) and 3 (>60% positive cells). The intensity of staining was graded: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown). High number of PD-$1^+$ TILs was defined as scores of 6 or 9 and low number of PD-$1^+$ TILs was defined as scores of 4 or less (the median).

The number of Foxp$3^+$ TILs, CD$4^+$ TILs and CD$8^+$ TILs was evaluated according to the previous studies.[14,15] The detailed method was provided in Supporting information.

### 2.5 | Follow-up and statistical methods

The median follow-up time for the surgery-CIK group and the surgery-only group was 98.7 months (9-120 months) and 83.0 months (4-105 months) respectively. The primary endpoint was disease-free survival (DFS) and the second endpoint was overall survival (OS). DFS was defined as the time from surgery to the time of recurrence (local or distant) or the date of the last follow-up. OS was calculated from the date of surgery to either the date of death or the last follow-up. Statistical comparisons of categorical data were carried out with the Pearson $\chi^2$ test. Rates of OS and DFS were estimated using the Kaplan-Meier method and compared with the subgroups using the log-rank test. The interobserver agreements were calculated by Fleiss' kappa statistics. To determine the efficacy of adjuvant CIK cells treatment after surgery, one-to-one propensity score matching using the nearest neighbour matching method was utilized, with a caliper of 0.25.

The backward method of the multivariate Cox regression model for OS and DFS was utilized to determine the independent prognostic factors for the population after matching, with covariates used in PSM included. To evaluate and compare the prognostic values of PD-1/PD-L1 expression in the surgery-CIK group and surgery-only group, the backward method of the Cox regression model for OS and DFS was utilized. Cox regression model including the two primary effect variables (PD$1^+$ TILs and treatment) and their interaction effect parameter was used to evaluate the interaction effect between PD$1^+$ TIL and therapy. $P < .05$ in all cases was considered statistically significant. All data were analysed with SPSS 24.0, GraphPad 5.0 and R 2.13.2 software.

The authenticity of this data has been validated by uploading the critical raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number as RDDB2017000137.

## 3 | RESULTS

### 3.1 | Baseline characteristics and prognosis of surgery-CIK and surgery-only groups

The baseline data for the population are described in Table S2. After one-to-one propensity score matching, 145 pairs of patients from the surgery-CIK group and surgery-only group were matched (Table S3). All the patients in the surgery-CIK group finished the four cycles of CIK infusion; almost 50% of patients received additional cycles of CIK infusions. 26 (17.9%) patients received more than 16 cycles CIK infusions (Table S4). There were no significant differences in the

distribution of all clinicopathological characteristics between the two groups except the HBV history ($P$ = .031).

Univariate analysis suggested that the surgery-CIK group had a significantly higher DFS ($P$ = .001) and OS ($P$ = .040) rates as to the surgery-only group (Figure 1). Multivariate analysis showed that adjuvant CIK treatment was independently associated with improved OS (HR 0.55, 95% CI 0.33-0.92) and DFS (HR 0.59, 95% CI 0.42-0.83) for HCC patients after surgery (Table S5-S6).

Further analysis showed patients receiving CIK immunotherapy could significantly reduce the risk of intrahepatic distant recurrence (beyond 2 cm from margin and less than 2 years after resection, $P$ = .047) and intrahepatic de novo origins (more than 2 years after resection, $P$ = .016) (Figure S3)

### 3.2 | Adverse effects of CIK cells therapy

Adverse effects of CIK treatment was shown in supplementary materials.

### 3.3 | PD-1 and PD-L1 expression pattern in HCC tissue samples

To investigate the expression of PD-1 and PD-L1 in tumours, we conducted further IHC of tissue samples in both groups. Representative IHC images of PD-L1 and PD-1 are shown in Figure 2. PD-1 positivity was observed in both tumour-infiltrating lymphocytes and stromal lymphocytes (Figure 2B and F). PD-L1 staining was predominantly

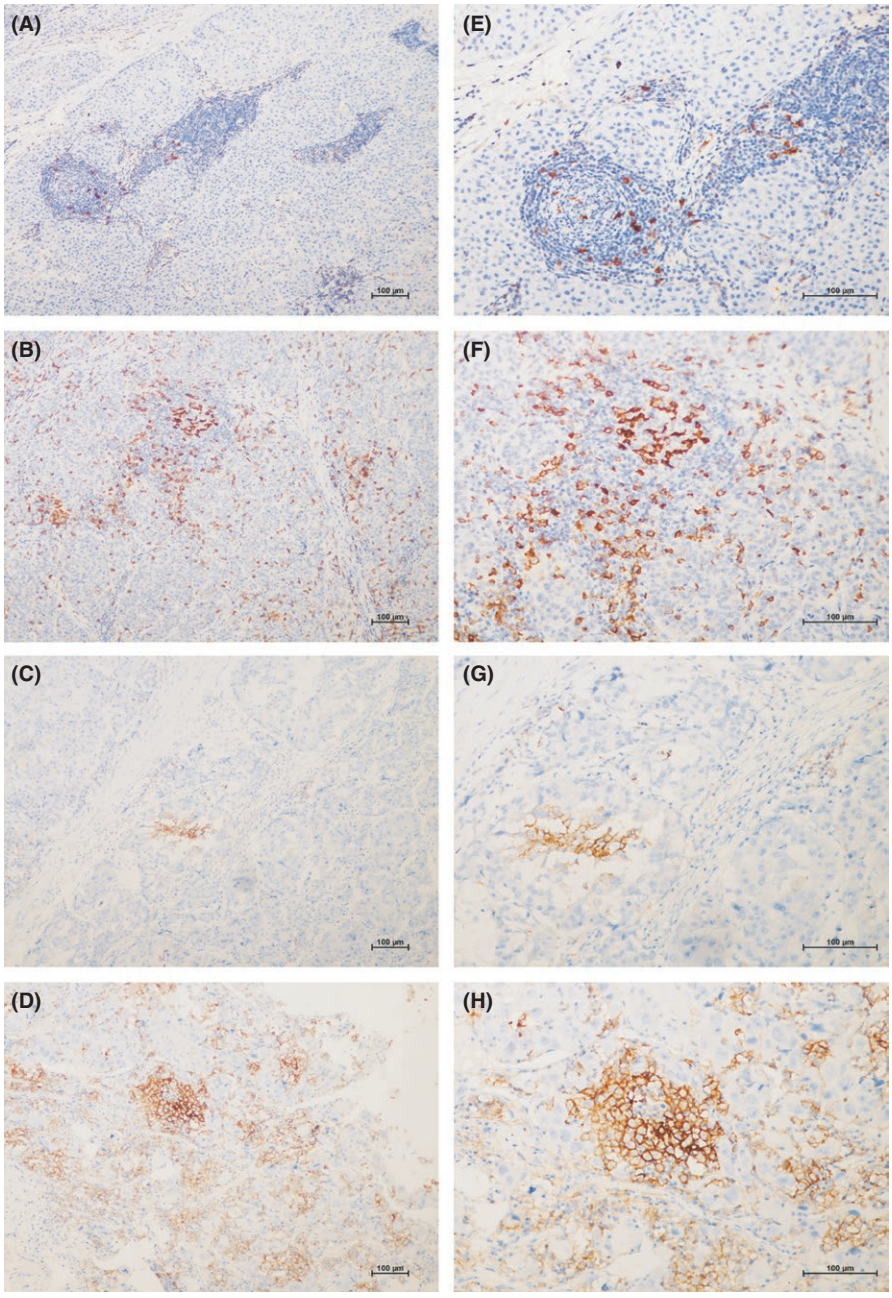


**FIGURE 2** Immunohistochemical staining of PD-1 and PD-L1 in primary hepatocellular carcinoma surgical specimens. A,E, PD-1-low tumour-infiltrating lymphocytes; (B,F) PD-1-high tumour-infiltrating lymphocytes; (C,G) PD-L1-negative hepatocellular carcinoma; (D,H) PD-L1-positive hepatocellular carcinoma; (A-D, 100× magnification; E-H, 200× magnification)

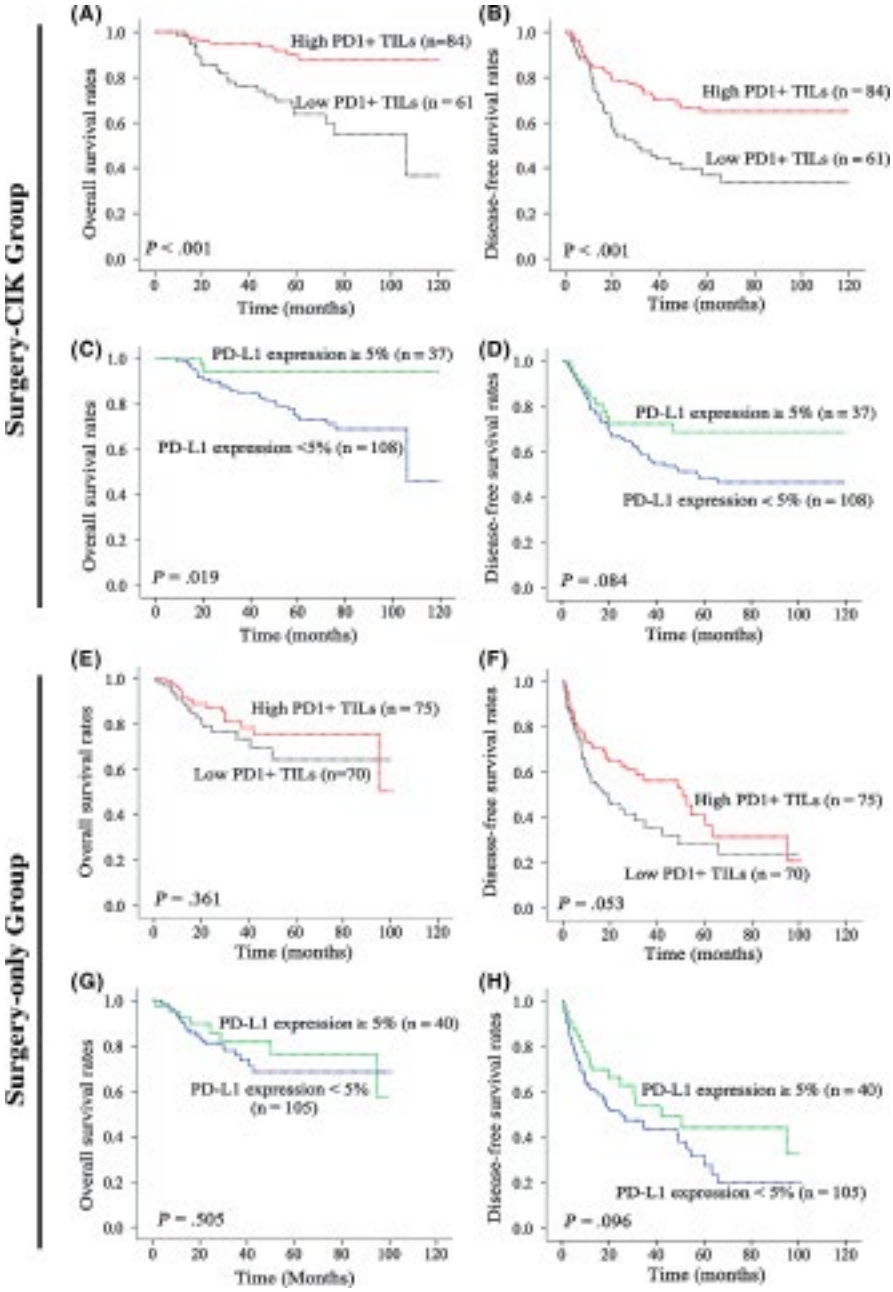


**FIGURE 3** Correlation of the number of PD-1$^+$ TILs and the level of PD-L1 expression on tumour cells with the survival benefit from adjuvant CIK cells immunotherapy. Kaplan-Meier (KM) curve for overall survival (OS, A) and disease-free survival (DFS, B) according to the number of PD-1$^+$ TILs as well as the OS (C) and DFS (D) by the PD-L1 expression level on tumour cells in the surgery-CIK group. KM curves for OS (E) and DFS (F) according to the number of PD-1$^+$ TILs as well as the OS (G) and DFS (H) by PD-L1 expression level on tumour cells in the surgery-only group. *P* values were calculated by log-rank test

observed in tumour cell membranes and also on some tumour-infiltrative lymphocytes (Figure 2D and H).

In Figure S4, three IHC expression types for PD-L1 were observed: no expression (Figure S4A and D), focal expression (Figure S4B and E) and diffuse expression (Figure S4C and F). Among the surgery-CIK group, 108 (74%) patients had a low PD-L1 expression (<5% PD-L1$^+$ cells), and 37 (26%) had a high PD-L1 expression (≥5% PD-L1$^+$ cells); 61 (42.1%) had a low number of PD-1$^+$ TILs and 84 (57.9%) patients exhibited a high number of PD-1$^+$ TILs.

In the surgery-only group, 105 (72.4%) had a low PD-L1 expression and 40 (27.6%) had a high PD-L1 expression; 70 (48.3%) had a low number of PD-1$^+$ TILs and 75 (51.7%) patients exhibited a high number of PD-1$^+$ TILs. A high PD-L1 expression was associated with a high number of PD-1$^+$ TILs in both groups ($P < .001$).

### 3.4 | PD-1/PD-L1 expression, clinical and immunological features and survival benefit from adjuvant CIK cells immunotherapy

The prognostic values of PD-1/PD-L1 expression in both the surgery-CIK group and surgery-only group were compared. Regarding OS, univariate analysis showed that, in the surgery-CIK group, both PD-L1 expression and number of PD-1$^+$ TILs were significantly associated with improved OS (Figure 3A and C). In the surgery-only group, these associations were insignificant (Figure 3E and G). Regarding DFS, in the surgery-CIK group, univariate analysis showed that high number of PD-1$^+$ TILs was associated with improved DFS (Figure 3D). While in the surgery-only group, the association was insignificant (Figure 3H).

**TABLE 1** Correlation of clinicopathologic and immunological findings with intratumoral PD-1 expression in HCC patients receiving CIK treatment after surgery

| Variables | PD-1$^+$ TILs | | *P* value |
|---|---|---|---|
| | Low | High | |
| Sex | | | |
| Male | 55 (90.2) | 73 (86.9) | .547 |
| Female | 6 (9.8) | 11 (13.1) | |
| Age | | | |
| <50 | 43 (70.5) | 47 (56.0) | .075 |
| ≥50 | 18 (29.5) | 37 (44.0) | |
| BCLC stage | | | |
| A | 55 (90.2) | 72 (85.7) | .422 |
| B | 6 (9.8) | 12 (14.3) | |
| HBV history | | | |
| No | 7 (11.5) | 13 (15.5) | .490 |
| Yes | 54 (88.5) | 71 (84.5) | |
| AFP (ng/mL) | | | |
| ≤25 | 30 (49.2) | 19 (22.6) | <.001 |
| >25 | 31 (50.8) | 65 (77.4) | |
| Tumour size | | | |
| ≤5 cm | 35 (57.4) | 56 (66.7) | .253 |
| >5 cm | 26 (42.6) | 28 (33.3) | |
| Tumour number | | | |
| Single | 56 (91.8) | 76 (90.5) | .782 |
| Multiple | 5 (8.2) | 8 (9.5) | |
| Grades of differentiation | | | |
| Low | 11 (18.0) | 15 (17.9) | .784 |
| Medium | 43 (70.5) | 56 (66.7) | |
| High | 7 (11.5) | 13 (15.5) | |
| Microscopic vascular invasion | | | |
| Absent | 55 (90.2) | 73 (86.9) | .547 |
| Present | 6 (9.8) | 11 (13.1) | |
| Microscopic satellite nodules | | | |
| Absent | 57 (93.4) | 77 (91.7) | .690[a] |
| Present | 4 (6.6) | 7 (8.3) | |
| PD-L1 expression | | | |
| Low | 56 (91.8) | 52 (61.9) | <.001 |
| High | 5 (8.2) | 32 (38.1) | |
| Foxp3$^+$ TILs | | | |
| Low | 49 (80.3) | 49 (58.3) | <.001 |
| High | 12 (19.7) | 35 (41.7) | |
| CD4$^+$ TILs | | | |
| Low | 47 (77.0) | 38 (45.2) | <.001 |
| High | 14 (23.0) | 46 (54.8) | |
| CD8$^+$ TILs | | | |
| Low | 51 (83.6) | 36 (42.9) | <.001 |
| High | 10 (16.4) | 48 (57.1) | |

Data are numbers of patients and data in parentheses are percentages.
[a]Fisher' s exact test.

In addition to the PD-L1 expression that had been reported by a previous study,[10] the number of PD-1$^+$ TILs also seems to be a potential efficacy predictor for CIK treatment. Therefore, we conducted further IHC of CD4, CD8 and Foxp3 for the surgery-CIK group (Figure S5) and assessed the association between the number of PD-1$^+$ TILs with clinical and immunological factors (Table 1). Correlative analysis showed a high number of PD-1$^+$ TILs associated with a high AFP level ($P < .001$), a high number of CD4$^+$ TILs ($P < .001$), a high number of CD8$^+$ TILs ($P < .001$) and a high number of Foxp3$^+$ TILs ($P < .001$). No significant association between the number of PD-1$^+$ TILs and age, sex, BCLC stage, tumour number, tumour size, HBV history, pathological grades, microscopic vascular invasion and microscopic satellite nodules was identified.

To compare the prognostic impact of these immunological factors in the surgery-CIK group, multivariate analysis including PD-1$^+$ TILs, PD-L1 expression, CD4$^+$ TILs, CD8$^+$ TILs, Foxp3$^+$ TILs and all clinicopathological factors was conducted. The results showed that high number of PD-1$^+$ TILs was strongly associated with favourable OS (HR 0.19, 95% CI 0.08-0.45) and DFS (HR 0.40, 95% CI 0.24-0.66) for HCC patients receiving CIK treatment after surgery (Tables 2 and 3); while PD-L1 expression was excluded in the final model for both outcomes. In the surgery-only group, multivariate analysis including PD-L1 expression and PD-1$^+$ TILs and clinicopathological factors for OS and DFS were also conducted while both PD-1$^+$ TILs and PD-L1 expressions were excluded in the final model (Table S7-S8). The PD-1$^+$ TIL × treatment interaction terms were further tested for DFS ($P = .048$) and OS ($P = .003$) in the whole cohort, which confirmed the differential impact of PD-1$^+$ TILs on DFS and OS of patients in these two groups.

## 4 | DISCUSSION

Although several studies reported that adjuvant CIK cells treatment might improve the prognosis of HCC patients after curative therapies, contradictory evidence exists on the efficacy of this systemic therapy. To determine the efficacy of adjuvant CIK therapy and reduce the impact of selection bias, we included a relatively large number of HCC patients receiving CIK therapy after curative resection and utilized one-to-one propensity score matching to balance the covariates between the two groups. Our results support the conclusion that adjuvant CIK cells therapy can improve the OS and DFS for HCC patients after curative resection.

Our study as well as the previous trials consistently showed that CIK cells treatment could reduce the risk of intrahepatic distant recurrence (beyond 2 cm from margin and <2 years after resection).[5] As early recurrence of HCC is related closely to the metastasis of remaining neoplastic cells, the benefit of clearing residual HCC cells using CIK cells might explain the reduced rates of early recurrence.[5] Additionally, in our study, patients in the surgery-CIK group were also found to have lower rates of late recurrence (beyond 2 years) than those in surgery-only group. A possible explanation for the inconsistency of late recurrence survival benefit between our and previous

**TABLE 2** Univariate and multivariate analysis of OS for surgery-CIK patient group

| | | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| **Variable** | **No. of cases** | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** |
| Sex (Female vs Male) | 17/128 | 0.55 (0.13-2.32) | .417 | - | - |
| Age (≥50 vs <50) | 55/90 | 0.73 (0.34-1.57) | .420 | - | - |
| BCLC stage (B vs A) | 18/127 | 2.20 (0.90-5.42) | .086 | - | - |
| HBV history (Yes vs No) | 125/20 | 5.30 (0.72-38.96) | .101 | - | - |
| AFP (>25 vs ≤25) | 96/49 | 1.14 (0.53-2.44) | .733 | - | - |
| Tumour size (>5 cm vs ≤5 cm) | 54/91 | 2.00 (0.97-4.10) | .059 | - | - |
| Tumour number (multiple vs single) | 13/132 | 1.03 (0.41-2.57) | .955 | - | - |
| Microscopic vascular invasion (present vs absent) | 17/128 | 2.54 (0.96-6.75) | .061 | - | - |
| Microscopic satellite nodules (present vs absent) | 11/134 | 4.42 (1.79-10.9) | .001 | 6.58 (2.53-17.08) | <.001 |
| PD-L1 expression (high vs low) | 37/108 | 0.21 (0.05-0.88) | .033 | - | - |
| PD-1$^+$ TILs (high vs low) | 84/61 | 0.23 (0.10-0.52) | <.001 | 0.19 (0.08-0.45) | <.001 |
| Foxp3$^+$ TILs (high vs low) | 47/98 | 0.32 (0.11-0.91) | .032 | - | - |
| CD4$^+$ TILs (high vs low) | 60/85 | 0.44 (0.20-0.99) | .048 | - | - |
| CD8$^+$ TILs (high vs low) | 58/87 | 0.31 (0.13-0.77) | .011 | - | - |

studies could be related to the number of infusion cycles and the duration of maintenance CIK treatment used.[4] In our study, almost 50% of patients received additional cycles of CIK infusions and 26 (17.9%) patients received more than 16 times CIK infusions (Table S4). Pan et al.[16] have reported that more than eight cycles of CIK transfusions could exhibit significantly better disease control than less than eight cycles for HCC patients. In addition to the direct tumour-killing effect of CIK cells treatment, there also could be an indirect mechanism reducing late tumour recurrence by controlling the replication of HBV. It has been reported that autologous CIK cells could suppress HBV replication,[17] which is the predominant cause of HCC in our study; hence, a long-term maintenance of CIK therapy could reduce late recurrence.

In our study, the survival of individuals in the surgery-CIK group varied significantly (OS range, 9-120 months), indicating that biomarkers that can differentiate between responders and non-responders are warranted. As an adoptive immunotherapy, various immunological factors in the immune microenvironment may influence the efficacy of CIK cells treatment. Our study showed that a high number of PD-1$^+$ TILs were independently associated with favourable OS and DFS for HCC patients receiving CIK treatment after surgery, while the expression of PD-L1 was excluded in the final model for both outcomes. In the surgery-only group, both PD-1$^+$ TILs and PD-L1 expressions were excluded in the Cox models for OS and DFS. These results indicated that of all the immunological factors included, the PD-1 expression on TILs is the most associated with the clinical efficacy of CIK cells immunotherapy and that it may be a specific biomarker for adopting CIK therapy.

The correlation between the high number of PD-1$^+$ TILs and favourable treatment response by CIK cells therapy we found was interesting.

**TABLE 3** Univariate and multivariate analysis of DFS for surgery-CIK patient group

| | | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| Variable | No. of cases | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Sex (female vs male) | 17/128 | 0.58 (0.23-1.44) | .240 | - | - |
| Age (≥50 vs <50) | 55/90 | 0.80 (0.48-1.33) | .394 | - | - |
| BCLC stage (B vs A) | 18/127 | 1.27 (0.63-2.57) | .505 | - | - |
| HBV history (Yes vs No) | 125/20 | 4.13 (1.30-13.17) | .016 | 4.57 (1.43-14.66) | .010 |
| AFP (>25 vs ≤25) | 96/49 | 0.80 (0.49-1.33) | .395 | - | - |
| Tumour size (>5 vs ≤5 cm) | 54/91 | 1.77 (1.08-2.88) | .023 | 1.93 (1.17-3.18) | .010 |
| Tumour number (multiple vs single) | 13/132 | 1.33 (0.61-2.92) | .476 | - | - |
| Microscopic vascular invasion (present vs absent) | 17/128 | 2.94 (1.56-5.53) | .001 | 3.96 (2.07-7.60) | <.001 |
| Microscopic satellite nodules (present vs absent) | 11/134 | 2.80 (1.38-5.68) | .004 | - | - |
| PD-L1 expression (high vs low) | 37/108 | 0.57 (0.30-1.09) | .089 | - | - |
| PD-1$^+$ TILs (high vs low) | 84/61 | 0.43 (0.26-0.70) | .001 | 0.40 (0.24-0.66) | <.001 |
| Foxp3$^+$ TILs (high vs low) | 47/98 | 0.68 (0.39-1.18) | .172 | - | - |
| CD4$^+$ TILs (high vs low) | 60/85 | 0.87 (0.54-1.46) | .635 | - | - |
| CD8$^+$ TILs (high vs low) | 58/87 | 0.54 (0.31-0.92) | .023 | - | - |

Our study hypothesize that a specific pre-existing tumour microenvironment immune phenotype (high number of PD-1$^+$ TILs) is important for the identification of benefit to adoptive immunotherapy. At present, there is growing evidence that high number of PD-1$^+$ TILs could be a valid biomarker for predicting favourable efficacy of PD-1 blockade therapy[18] as well as the adoptive cellular immunotherapy of TILs.[8,9] Although PD-1 is generally regarded as a negative regulatory role in T cells, its expression is not necessarily synonymous with tumour immune evasion; the PD-1 expression may be a marker of activated TILs and reflect an ongoing antitumour immune response.[19,20] In our study, a high number of PD-1$^+$ TILs was closely linked with a high number of CD4$^+$ TILs and CD8$^+$ TILs, indicating that a high number of PD-1$^+$ TILs reflects the existence of immunosurveillance or endogenous antitumour immunity (adaptive antitumour immune response) within the tumour microenvironment, although accompanies with an immunosuppressive environment. On the other hand, adoptive CIK cells are endowed with non-major histocompatibility complex (MHC)-restricted cytotoxic activities and act via Fas-Fas ligand interactions, perforin-dependent cell killing and production of tumoricidal cytokines,[21] to some extent, which may potentially compensate the aforementioned limitation of immunosuppression. A plausible explanation for the benefit from CIK cell therapy in the patients with high number of PD-1$^+$ TILs is that high number of PD-1$^+$ TILs marks the existence of adaptive antitumour immune response, namely a specific pre-existing tumour microenvironment immune phenotype susceptible to CIK immunotherapy, and additional CIK therapy, to some extent, could exert a synergistic antitumour effect against HCC through the non-MHC-restricted way and compensate the impairment of MHC-restricted cytotoxic immunity.

Our data suggested that PD-1$^+$ TILs was a more potent and specific biomarker than PD-L1 in predicting the response of CIK cells treatment, whose predictive value has been reported recently by Chen et al.[10] In their study, they demonstrated that PD-L1 expression marked the existence of adaptive immune response in HCC microenvironment; therefore, the HCC patients with high PD-L1 expression had an active antitumor immunity, and hence, they are more susceptible to CIK immunotherapy. However, currently, there were two general mechanisms proposed for explaining the increased PD-L1 in malignancies. One is innate immune resistance (type I, a subtype insusceptible to immunotherapy), which proposed that the PD-L1 expression may be driven by intrinsic cellular changes caused by carcinogenesis, which can lead to inhibition of lymphocytes infiltration. The other one is called TILs driving adaptive immune resistance (type II, a subtype susceptible to immunotherapy), which proposed that tumours upregulate PD-L1 in response to IFN-γ released by TILs as an adaptive immune resistance mechanism to suppress local effector T-cell function; in this sense, immunosurveillance exists in these tumours.[22] Therefore, high PD-L1 expression, theoretically, may represent either an absence (type I) or presence (type II) of the anticancer immunity and it is not a direct marker of adaptive immune resistance. Hence, selecting target population for CIK immunotherapy just based on the PD-L1 expression will possibly include the type I patients insusceptible to the CIK therapy. In our study, additional CD4 and CD8 staining were conducted in 145 HCC patients (surgery-CIK group), and close correlations between high number of PD-1$^+$ TILs and the high number of both CD4$^+$ and CD8$^+$ TILs were observed, indicating that high number of PD-1$^+$ TILs directly represents the existence of adaptive antitumor immune response in HCC. Overall, PD-1$^+$ TILs is a better biomarker than PD-L1 expression in predicting survival benefit of adjuvant CIK therapy for HCC patients.

A recent review summarized that a high PD-L1 expression in tissue samples was a poor prognostic indicator for HCC after surgical resection.[7] This inconsistency between our data and previous studies may be due to the use of different antibodies and assay protocols as well as interpretative subjectivity. Firstly, evaluating the expression of PD-L1 in the previous study on tissue microarray might underestimate or overestimate the PD-L1 expression levels due to intratumoural heterogeneity.[23] In our study, the whole-tissue sections were used to assess PD-L1 staining. Secondly, PD-L1 is a type I transmembrane molecule and inhibits lymphocyte function when it is engaged by its receptors. It is believed that cell-surface expression of PD-L1 is the most immediately biologically relevant; however, the antibody used in previous two studies did not distinguish cytoplasmic and membranous patterns of PD-L1 staining.[23,24] In our study, a newly developed anti-PD-L1 antibody was used (clone E1L3N, Cell Signaling Technology), which has been validated by recent studies.[25,26]

Our study has several limitations. Firstly, this is a single-centre-based retrospective study. Secondly, although propensity score matching could be beneficial in assessing the efficacy of CIK cells treatment, it might also cause selection bias. In this study, we reviewed all the early stage HCC patients who received CIK cells therapy after surgery in our centre from 2004 to 2011 to minimize this bias.

In conclusion, our study demonstrated that PD-1$^+$ TILs was a potent and specific biomarker in predicting the survival benefit of CIK immunotherapy in HCC patients. The correlation between high number of PD-1$^+$ TILs and the high number of both CD4$^+$ and CD8$^+$ TILs suggested that PD-1$^+$ TILs can reflect the existence of endogenous host immune response to tumours, a specific pre-existing tumour microenvironment immune phenotype susceptible to CIK immunotherapy although accompanies with immunosuppression, and additional CIK therapy, to some extent, could compensate for immunosuppressive environment and exert a synergistic antitumour effect against HCC.

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## CONFLICTS OF INTEREST

The authors do not have any disclosures to report.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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