

# Peripheral blood CD4<sup>+</sup> cell ATP activity measurement to predict HCC recurrence post-DCD liver transplant

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## SUMMARY

**Background and Aim:** Hepatocellular carcinoma (HCC) recurrence after orthotopic liver transplantation (OLT) continues to confound transplant surgeons and physicians. There are no effective methods to predict the patients at risk for recurrence so far although many studies have sought meaningful biomarkers. The ImmuKnow (IMK) assay is an immune cell function assay that detects cell-mediated immunity in an immunosuppressed population, mainly measuring peripheral blood CD4<sup>+</sup> adenosine triphosphate (ATP) release. The aim of this study was to assess the relationship between cellular immune function measured by the ImmuKnow assay and HCC recurrence post-OLT. **Methods:** A total of 76 HCC cases underwent Donation after Cardiac Death (DCD) liver transplant, which confirmed hepatocellular carcinoma by histology postoperatively. The ImmuKnow assay was prospectively performed in these cases at a range of 6–36 months post-OLT. Every test was repeated 1 week later, obtaining the average value for every patient. In addition, every case had liver imaging findings at approximately the exam time. **Results:** Fifteen cases with liver imaging findings showed HCC recurrence (19.7%) post-OLT, and the average ImmuKnow assay in these patients was 190 ± 48 ng/ml, which was less ( $p < 0.05$ ) than in patients without HCC recurrence, whose average ATP level was 313 ± 90 ng/ml. ATP levels post-OLT were found to be significantly associated with the risk of tumour recurrence. The ratio of T reg cells and the levels of TGFβ and IL-10 were higher in recurrence patients than in recurrence-free patients. **Conclusion:** Greater suppression of cellular immunity, as measured by the ImmuKnow assay, was associated with progression of HCC recurrence post-OLT. ImmuKnow assay was helpful in determining the risk of early recurrence of HCC postliver transplant. A pathway consisting of T reg cells, TGFβ and IL-10 might be the HCC recurrence-predominant pathway.

## Introduction

With the rapid progress in liver transplant surgical technique, short-term graft survival has been greatly improved in recent years. However, with the application of a variety of immunosuppressive agents, unsuitable immunosuppressed status leads to unsatisfactory long-term graft survival. Immunosuppressive status that is too low can lead to increased risks of rejection (1), whereas too much immunosuppression can cause malignant tumours, opportunistic infections and drug toxicities (2,3). The challenge in balancing the risks of over- and under immunosuppression is complicated by the lack of reliable means for predicting patients' immunosuppression needs. Most transplant centres assess the

immunological status of the graft liver by measuring trough levels of calcineurin inhibitors (CIs), combined with laboratory data (4,5), although neither of these indicators is sensitive nor specific for determining the current immunosuppressive status. Thus, one reliable method that can evaluate the patient's immunosuppressive status is urgently needed by clinicians. Cylex ImmuKnow has been approved by the Food and Drug Administration, and it measures the ability of CD4<sup>+</sup> T cells to respond to mitogenic stimulation by phytohemagglutinin-L *in vitro*.

Liver transplantation is an effective method for HCC thus far. However, tumour recurrence continues to confound clinicians, resulting in poor outcomes of LT. HCC recurrence or metastasis often arises and develops rapidly due to the lack of a

### What's known

- Immune function assay can predict post-transplant recurrence in patients with hepatocellular carcinoma.
- Lower cumulative CD4<sup>+</sup> T-cell function post-LT predicted a higher risk of HCC recurrence.

### What's new

- In this study, we found that the ratio of T reg cells and the levels of TGFβ and IL-10 were higher in recurrence patients than in recurrence-free patients.
- A pathway consisting of T reg cells, TGFβ and IL-10 might be an HCC recurrence-predominant pathway.
- The pathway for carcinogenesis in LT recipients with HCC recurrence, who were an immunosuppressed population, might be different from that in HCC patients without liver transplantation.

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specific and sensitive biomarker for tumour recurrence or immunity status measurements for immunosuppression that are too high.

It is well known that the cancer microenvironment, consisting of cancer and stromal cells, creates an immunosuppressive network through cytokine secretion, alterations in antigen-presenting cell subsets, and costimulatory and co-inhibitory molecule alterations, which can increase tumour recurrence post-transplant in patients with HCC (6). T reg cells have an immunosuppressive function, playing a key role in tumour immunologic escape. TGF- $\beta$  is believed to be directly related to the immunosuppressive function of T reg, while IL-10 could inhibit the responses of CD8<sup>+</sup> T cells, and both cytokines are essential for maintaining the function of T reg cells. Although there are different subgroups of CD4<sup>+</sup> T cells involving tumour promotion and tumour rejection in the process of immuno-editing, the immune function assay reflects the net state of immune activities (6). Previous studies have reported no correlations between ImmuKnow adenosine triphosphate (ATP) values and Th/Ts ratio in LT recipients with infections (7). However, the relationships among T reg cells, associated cytokines, immunologic status and the recurrence of HCC post-LT remain unknown.

We hypothesised that the ImmuKnow assay could be useful for the monitoring of immunological aspects post-LT for HCC. In this study, we focused on patients more than 1 year after LT in our centre. We used the IMK assay to study the immunosuppressive status of patients, as well as to supervise tumour recurrence postliver transplant and to explore the significance of the ImmuKnow assay for malignant recurrence.

## Patients and methods

Both outpatients and hospitalised patients were recruited from the Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, Zhejiang University School of Medicine. This study was approved by the appropriate ethics committees. Written informed consent was obtained from all of the patients before enrolment.

### Patients and collected data

Seventy-six patients who received DCD LTs from the First Affiliated Hospital and 50 healthy Chinese people were included in this study. The healthy people underwent one ImmuKnow detection for each person, whereas the LT patients underwent two ImmuKnow levels obtained by a repeat test 1 week later.

Detailed information about the LT patients is shown with the demographic data.

All of the patients received tacrolimus and a low dose of mycophenolate mofetil as baseline immunosuppression. The tacrolimus trough level was between 3 and 6 ng/ml. The diagnosis of HCC recurrence or metastasis relied on clinical features, tumour marker tests, and imaging diagnoses.

Transplantation was based on the Hangzhou criteria in patients without macrovascular invasion who met one of the two following criteria: (i) total tumour diameter  $\leq$  8 cm and (ii) total tumour diameter  $>$  8 cm with histopathological grade I or II and preoperative AFP level  $\leq$  400 ng/ml simultaneously. Other inclusion criteria consisted of LT patients with confirmed hepatocellular carcinoma by histology postoperatively and recorded baseline demographics. The exclusion criteria consisted of the presence of infection (existing infection focus or systematic infection manifestation), usage of steroids in the previous 2 weeks, leukopenia (WBC  $<$   $2 \times 10^9$ ), biliary complications and vascular complications. Samples were obtained at a median interval of 29.8 months after LT in stable LT patients and 18.4 months in recurrent LT patients.

### Immune function assay

CD4 T-lymphocyte function assay (ImmuKnow, Catalogue no. 4400; Cylex Inc., Columbia, MD) was performed as follows: whole blood samples were collected in sodium heparin tubes; the blood was stored at room temperature and was tested within 6 h of blood draw. Briefly, 250  $\mu$ l of whole blood were diluted with sample diluent, added to the wells of a 96-well microtiter plate, and incubated overnight (15–18 h) with phytohemagglutinin as a stimulant at 37 °C in a 5% CO<sub>2</sub> incubator. Using magnetic particles coated with anti-human CD4 monoclonal antibodies, CD4<sup>+</sup> T cells were magnetically selected within the microwells and then were lysed to release intracellular ATP. The addition of a luciferin or luciferase reagent to this ATP produced lights, which was measured by a luminometer. The amount of light produced was proportional to the concentration of ATP expressed in nanograms per millilitre.

Before stimulation, part of the blood sample was also detected by ELISA to confirm the levels of cytokines (TGF- $\beta$ , IL-10, IL-1 $\beta$  and IL-17) in the serum and by fluorescence-activated cell sorting to confirm the T reg ratio in isolated white cells.

### Statistical analysis

All of the data were recorded as the mean  $\pm$  SD or the median with interquartile ranges. Continuous variables were compared using Student's *t*-test

**Table 1** Demographic data of liver transplant recipients

Clinical variables	Recurrence LTs	Stable LTs	p-value
Age (year)	51.5	50	0.91
Sex (M:F)	14:1	59:2	0.41
Child–Pugh score	7.5 ± 2.1	8.0 ± 2.3	0.49
MELD score	12.3 ± 3.4	12.0 ± 4.1	0.65
Range (months)	18.4	29.8	0.13

M, male; F, female; MELD, model for end-stage liver disease.

between two groups or one-way ANOVA among multiple groups. Categorical variables were compared using the chi-squared test or Fisher's exact test. The Kaplan–Meier method was used to calculate survival ratios, and the differences were assessed by log-rank analysis.  $p$ -values < 0.05 were considered statistically significant.

## Results

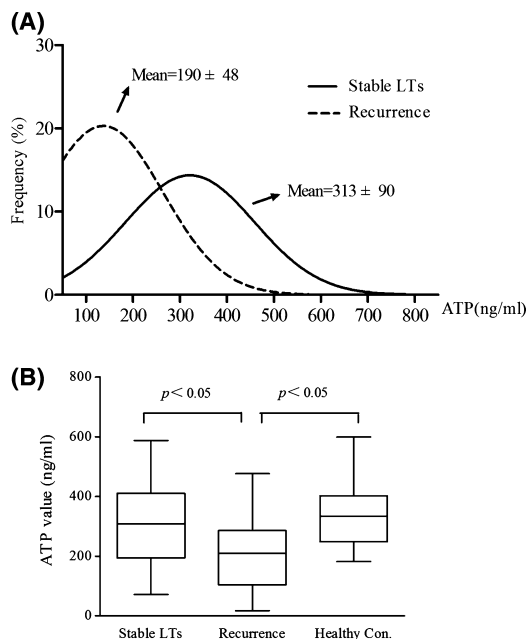
A total of 76 LT patients from January 2011 to December 2013 were included in this investigation. Of these patients, 19.7% (15/76) developed HCC recurrence or metastasis with average age of 50 years old. The remainder were stable LT patients, accounting for 80.3% (61/76), with average age of 51.5 years old. The demographics of the patients are presented in Table 1.

### Comparison of immune response in healthy Chinese adults and liver transplant recipients

A population-based study was conducted, comparing the immune responses characteristic of apparently healthy controls, stable LT patients and HCC recurrence LT patients. As shown in Figure 1A, the ImmuKnow ATP levels in the stable and recurrent LTs patients were normally distributed, yielding stimulated responses on average of 337 ± 105 ng/ml ATP in apparently healthy controls (50 samples from 50 controls), 313 ± 90 ng/ml ATP in stable patients (122 samples from 61 recipients) and 190 ± 48 ng/ml ATP in HCC recurrent LT patients (30 samples from 15 recipients). Statistically, the immune response characteristics of HCC recurrence LT patients were significantly lower ( $p < 0.05$ ) than both the healthy controls and stable LT patients.

### Comparison of T reg cells in stable and recurrent HCC in liver transplant recipients

The T reg ratio was 6.78% in LT patients with HCC recurrence and 3.98% in stable LT patients (Figure 2). Statistically, the T reg ratio of the recurrent

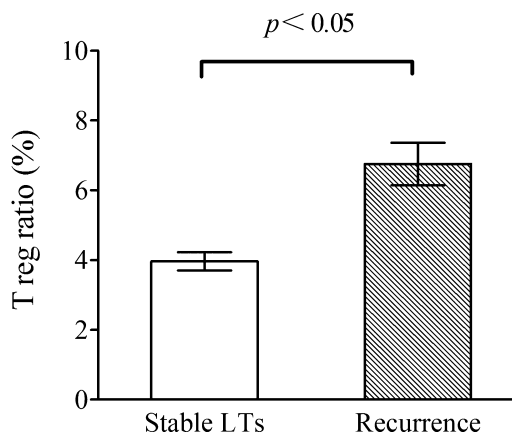


**Figure 1** (A) Histograms of immune function in recurrence LTs and stable LTs. Overall distributions of these two groups were tested by ImmuKnow assay. Curves were generated by SPSS 19.0 (SPSS Inc., Chicago, IL, USA), and were confirmed to be normally distributed. (B) ImmuKnow values from recurrence LTs were significantly less than those from stable LTs and apparent healthy Chinese controls ( $p < 0.05$ )

LT patients was significantly higher than that of stable LT patients ( $p < 0.05$ ).

### Comparison of cytokines in stable and recurrent HCC in liver transplant recipients

The TGF- $\beta$  and IL-10 concentrations in the HCC recurrent LT patients were significantly higher than those in stable LT patients ( $p < 0.05$ ), whereas the



**Figure 2** The T reg ratio from stable LTs was significantly less than that from recurrence LTs ( $p < 0.05$ )

IL-1 $\beta$  and IL-17A concentrations in the HCC recurrence LT recipients showed no differences from the stable LT patients statistically (Figure 3).

### Correlation of T cell-mediated immunity with HCC recurrence

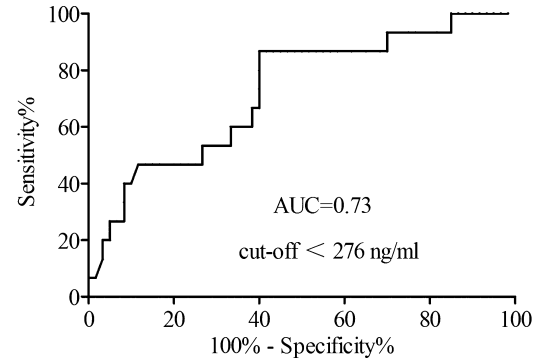
ImmuKnow ATP data were analysed by ROC curve analysis to establish optimal cut-off values for recurrence based on the strength of immune response. A liver recipient with an ImmuKnow ATP value less than 276 ng/ml was more likely to experience recurrence or metastasis (AUC = 0.73,  $p < 0.01$ ) (Figure 4), and the odds ratio evaluated by Fisher's exact tests was 9.75 (95% confidence interval 2.02–47.15,  $p < 0.01$ ).

The sensitivity and specificity of ImmuKnow in the diagnosis of recurrence or metastasis were 87% and 60%, respectively.

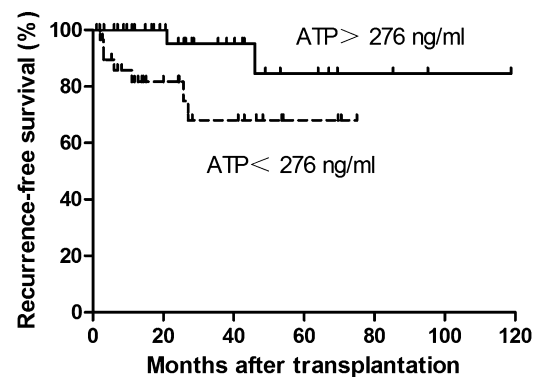
The patients were stratified into a high immune group (ATP > 276 ng/ml) and a low immune group (ATP < 276 ng/ml). There was 10% (4/40) of patients who developed tumour recurrence in the high immune response group, compared with 31% (11/36) in the low immune group. Recurrence-free survival was significantly better in patients with a high immune response than in patients with a low immune response ( $p < 0.05$ ) (Figure 5).

### Discussion

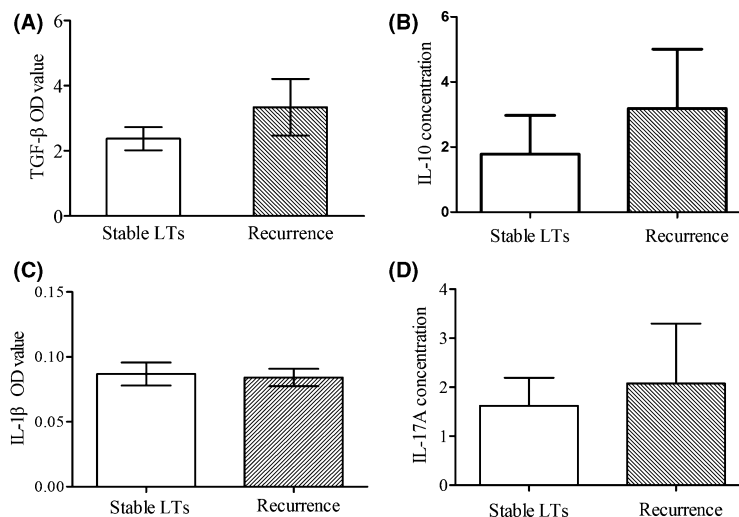
ImmuKnow does not correlate with CD4<sup>+</sup> cell numbers, and the assay is theorised to provide an independent variable (8). Studies in orthotopic liver transplantation (OLT) recipients have reported



**Figure 4** In ROC analysis, the AUC was 0.73, and the optimum cut-off value was 276 ng/ml for recurrence



**Figure 5** Survival difference between the groups of HCC patients after liver transplantation with high- and low immune responses. A superior recurrence-free survival rate ( $p < 0.05$ ) was observed in the high immune response group, compared with the low immune response group



**Figure 3** TGF- $\beta$  values and IL-10 concentrations from recurrence LTs were significantly higher than those from stable LTs ( $p < 0.05$ ) (A, B). IL-1 $\beta$  values and IL-17A concentrations from HCC recurrence LTs showed no difference from those from the stable LTs (C, D)

contradictory results with ImmuKnow (9–15). Most of these studies were retrospective, had limited follow-ups, were heterogeneous in study design, and often included multiple solid organ transplants in the analysis, despite immunosuppression protocols and clinical event risks differing substantially among different transplant populations.

The average level for the ImmuKnow assay in the 15 recurrent patients was  $190 \pm 48$  ng/ml, which was significantly less than that in the non-recurrent population and the apparently healthy controls. However, there were a few recurrence LT patients with ImmuKnow ATP levels greater than 300 ng/ml – even exceeding 400 ng/ml – suggesting the complexity of tumour immunity. Although there have been a few studies that assessed functional immunity with the Cylex ImmuKnow assay in liver transplant recipients and significant associations have been found between low ImmuKnow ATP levels and HCC recurrence in accordance with our results (6,16); to our knowledge, this study was the first to assess functional immunity together with CD4<sup>+</sup> T-cell subgroup and cytokine levels in Chinese DCD liver transplant recipients. Importantly, we found significantly higher T reg ratios and TGF- $\beta$  and IL-10 levels with HCC recurrence compared with non-recurrence in our LT recipients. In the tumour progression pathway in LT recipients, CD4<sup>+</sup> T cells develop into T regs under the influence of TGF- $\beta$ , which actively blocks tumour immunity by suppressing tumouricidal CD8<sup>+</sup> T cells where predominant, rather than the pathways for IL-17 produced by Th17 cells, which in turn induce cytokines and chemokines that promote inflammation, and IL-10 produced by M2 macrophages, which polarise immunity towards a tumour-promoting response (17).

Our average ImmuKnow ATP level was  $337 \pm 105$  ng/ml in apparently healthy controls, which was less than that of Americans ( $433 \pm 148$  ng/ml) (8). The difference might be partially due to racial differences (18) and might also be due to our small sample size. Conversely, our ImmuKnow ATP results in stable LTs were  $313 \pm 90$  ng/ml, which was higher than in previous reports (8,19). As it was a pilot study, we observed the LT patients mainly in the period post transplant, at medians of months 29.8 and 18.4, whereas the previous study focused on a distinct period, which might partly explain the difference in ATP levels. Mender et al. (15) also observed a significant trend of decreased ImmuKnow ATP levels in liver recipients over time after LT. In addition, different immunosuppressive regimens used between Chinese and American recipients after LT might also have contributed to the differences observed in cellular

immunity and in the results of ATP levels, although the Chinese healthy controls had relatively lower levels.

Typically, the measurement of peripheral blood trough levels of immunosuppressive drugs after transplantation has been a critical means of therapeutic management used to prevent toxicity and to provide effective immunosuppression. The scatter distribution of trough tacrolimus levels in ATP values showed no correlation with therapeutic drug dosages (7). This immune function assay could provide supplemental information that could guide physicians' decision-making in post-transplant management.

Multivariate analysis was not performed to investigate whether the ATP value was an independent prognostic factor among various parameters, such as tumour differentiation, maximal tumour size, bilobar involvement, lymph node positivity, total tumour volume, CD4<sup>+</sup> T-cell subgroup ratio, cytokine levels and so on. The reasons were as follows. First, several studies have already indicated that the immune response value is an indicator of global post-transplant immunology independent of pretransplant tumour morphology or characteristics (16). Second, given the small number of HCC recurrences, multivariate analysis could not be performed, which would not allow for the control of potential confounding variables. Finally, we identified the CD4<sup>+</sup> T-cell subgroup ratio and cytokine levels to investigate whether there was a predominant pathway for carcinogenesis in LT recipients with HCC recurrence, who were an immune-suppressed population, compared with HCC patients without liver transplant as a preliminary experiment.

However, our study had the limitations of a retrospective study from a single centre and the employment of single time point measurements, as well as the risk of potential bias and the effects of confounders. More than 75% of our patients underwent the ImmuKnow assay more than 12 months after transplantation, although the value of ImmuKnow assay was significantly different, depending on the length of time after transplantation within 1 year (20); whether the functional immune response differs over time in the late phase requires further investigation. A single result cannot be expected to predict the long-term immune function of the patient. Ideally, serial measurements, correlated with changes in immunosuppressant dosing, would be needed to assess the immune response adequately post-OLT. A randomised, controlled trial (21) preliminarily answered the question of whether the study group had better 1-year survival and lower immunosuppressant dosages and blood levels. Nevertheless, a



large, formal, multicentre, randomised, controlled trial is still needed to resolve many questions regarding ImmuKnow with regard to its ability to be an objective biomarker of immune function in OLT patients.

In conclusion, we showed that Cylex ImmuKnow assay was helpful in monitoring LT recipients with HCC recurrence. With the advent of this technique, the paradigm of post-transplant monitoring will shift, and we anticipate that an improved assessment of the net immune state might well lead to better patient management and evidence-based individualisation. Further investigation will determine the role of the ImmuKnow assay in tailoring immunosuppression and preemptive manoeuvres, such as systemic chemotherapy and targeted therapies (e.g. VEGF inhibitors), and in reducing immunosuppressive agent doses and concentrations, based on low ATP values, to reduce recurrence in LT recipients.

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