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Lack of clinical significance of the ImmuKnow™-Cylex assay for the detection of cellular immune function in patients with renal cell carcinoma

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ABSTRACT. This study aimed to explore the clinical value of the $CD4^+$ T cell ATP levels in patients with renal cell carcinoma through the application of the ImmuKnow™-Cylex® assay. We recruited 104 patients with renal cancer who had undergone surgery at Fuzhou General Hospital from March 2009 to June 2012, and were subsequently treated by dendritic cell and cytokine-induced killer cell bio-therapy or interferon-α therapy. The changes in $CD4^+$ T cell ATP levels were detected at the perioperative period and at 10 days, 1 month, 3 months, and 1 year after the surgery using the ImmuKnow assay. In addition, the differences in ATP levels in different therapy groups were compared and the prognosis conditions were analyzed. Our results demonstrated that no significant difference in the ATP levels occurred at different time points; furthermore, there were no obviously different ATP levels between the different therapy groups, and the ATP levels were found

to have no clinical significance for the assessment of renal cancer prognosis. Overall, this study suggested that $CD4^+$ T cell ATP levels as detected by the ImmuKnow assay have no obvious clinical value in patients with renal cancer.

Key words: Renal carcinoma; ImmuKnow assay; ATP; Prognosis

INTRODUCTION

Renal carcinoma is the most common malignant tumor in the urinary system. Overall, there are approximately 271,000 new confirmed patients with renal cancer every year worldwide, making renal cancer representative of 2% of the total cancers and 80% of renal primary malignant carcinomas (Ferlay et al., 2010; Jemal et al., 2011). Previous studies have found that the occurrence, development, metastasis, and prognosis of kidney cancer were closely related to immune function (Pohla et al., 2012; Polimeno et al., 2013). Thus, most scholars, such as Kobayashi et al. (2013) and Saroha et al. (2013), thought that monitoring immune functions might provide clinical evidence toward the determination of prognosis and individualized treatment of kidney cancers. However, the assays currently used to assess the indicators of cellular immune function, such as the mixed lymphocyte reaction and determination of phytohemagglutinin stimulated peripheral blood lymphocytes and anti-CD3 monoclonal antibodies, have some disadvantages including a high price for detection, complicated monitoring processes, and poor sensitivity (De Paolis et al., 2011; Martínez-Flores et al., 2014). The ImmuKnow™-Cylex® assay was developed by the Cylex company (Columbia, MD, USA) to detect immune function via measurement of adenosine triphosphate (ATP) levels from peripheral blood $CD4^+$ T cells, to evaluate the cellular immune status. In addition, as a Food and Drug Administration approved *in vitro* assay, the ImmuKnow™-Cylex® assay provides a global assessment of cellular immune function to help monitor the immune status of immunosuppressed patients (Uemura et al., 2011). At present, the ImmuKnow test serves as a valid tool for assessing the cellular immune status of organ transplant recipients (Nishikawa et al., 2014; Sood and Testro, 2014). Our center introduced the ImmuKnow assay to monitor the immunologic status of transplant patients in 2007. Thus, in this study we attempted to use ImmuKnow™-Cylex® assay to monitor the change in the ATP levels of $CD4^+$ T cells from patients with renal cancer to investigate the potential clinical application of the ImmuKnow™-Cylex® assay on assessing the immune status of patients with renal carcinoma.

MATERIAL AND METHODS

Subjects

We included 104 patients with renal carcinoma (74 men and 30 women aged from 23-82 years) in the Fuzhou General Hospital of the Nanjing Military Command from March 2009 to June 2012 in this study. All patients had received nephrectomy combined with dendritic cell and cytokine-induced killer cell (DC-CIK) therapy (Mesiano et al.,

2012) or interferon-α (IFN-α) therapy (George et al., 2011). Postoperative pathologic results showed there were 83 patients with suprarenal epithelioma, 7 with papillary cellular carcinoma, 8 with chromophobic cellular tumor, and 3 with other cellular tumors. According to the American Joint Committee on Cancer stage grouping (Edge et al., 2009), the patients included 82 at stage I, 11 at stage II, 7 at stage III, and 4 at stage IV. This study was conducted in accordance with the declaration of Helsinki, and with approval from the Ethics Committee of Fuzhou General Hospital. Written informed consent was obtained from all participants.

Samples

Blood samples were collected from 104 patients with renal carcinoma before the surgery and at 10 days, 1 month, 3 months, and 1 year after the surgery. There were 520 samples and each sample contained 3 mL blood.

Protocols

The ImmuKnow™-Cylex® assay contains three steps: cell stimulation, $CD4^+$ T cell selection, and ATP release, thus allowing the calculation of the ATP value (Mizuno et al., 2013). Briefly, whole blood was diluted with sample diluent, added to 96-well plates, and incubated for 15-18 h in a 37°C, 5% CO_2 incubator (Sanyo, Osaka, Japan). The following day, $CD4^+$ T cells were positively selected by magnetic separation (Cylex Magnet tray 1050, Cylex Inc., Columbia, MD, USA) using anti-human CD4 or CD3 monoclonal antibody-coated magnetic beads. Cells were then washed to remove residual cells and lysed. ATP release was measured using luciferin/luciferase and a luminometer (Tuner Biosystems, Sunnyvale, CA, USA). The immune response was assessed by the concentration of ATP release (ng/mL) comparing stimulated to non-stimulated samples. Data Analysis Calculator V2.2 Software (Cylex Inc.) was used to judge the results.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software (SPSS, Chicago, IL, USA) and all results are reported as means ± SD. One-way analysis of variance was used for comparisons between different groups. The Kaplan-Meier method was used to calculate progression-free survival rate for five years and then the Log-rank was used to compare the differences in the survival rates in different groups. $P < 0.01$ denoted a high statistically significant difference.

RESULTS

Change of ATP levels

Intracellular ATP levels from $CD4^+$ T cells were not significantly different preoperatively and at 10 days, 1 month, 3 months, and 1 year post-operation ($P > 0.01$). However, a highly significant difference in the ATP levels was detected between 10 days and 1 month after the surgery ($P < 0.01$) (Figure 1).

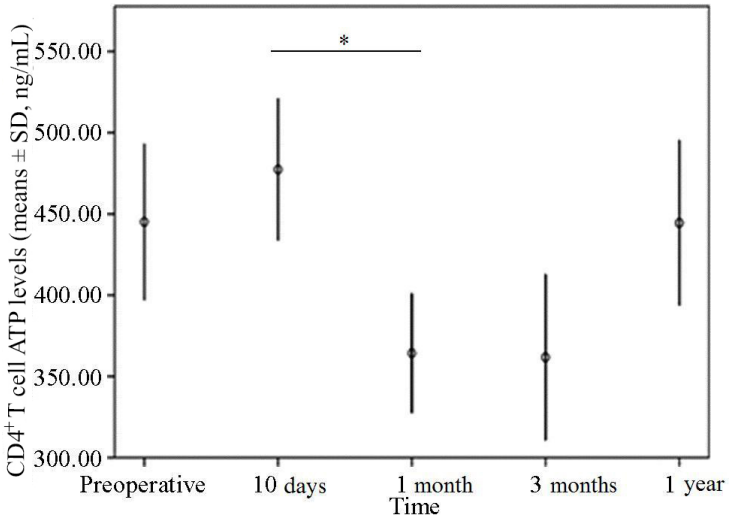

Figure 1. Changes in $CD4^+$ T cell ATP level over time. ATP levels had no significant difference overall at different times ($P > 0.01$). A significant difference was observed between the 10-day and 1-month post-surgery groups (*$P < 0.01$).

Intracellular ATP levels in the different therapies

After 104 patients had undergone surgery, 49 received DC-CIK therapy and 55 received IFN-α therapy. Intracellular ATP levels from $CD4^+$ T cells in patients undergoing the two therapy methods were compared. ImmuKnow™-Cylex® assay results showed that there was no significant difference between DC-CIK and IFN-α therapy ($P > 0.01$) (Table 1). Furthermore, the intracellular ATP levels of $CD4^+$ T cells from the five different time points were not significantly different between the DC-CIK and IFN-α therapy groups ($P > 0.01$, Table 1).

Table 1. $CD4^+$ T cell ATP levels of patients treated by DC-CIK or IFN-α therapy (means ± SD, ng/mL).

Therapy	Preoperative	10 days	1 month	3 months	1 year	P (within group)
DC-CIK	448.2 ± 126.9	464.2 ± 212.7	376.3 ± 119.3	344.7 ± 127.4	444.3 ± 86.8	>0.01
IFN-α	441.8 ± 196.7	487.8 ± 205.9	345.8 ± 152.7	436.6 ± 117.4	444.7 ± 131.4	>0.01

Comparison between two groups, $P > 0.01$. DC-CIK = dendritic cell and cytokine-induced killer cell.

Relationship between ATP levels and prognosis of renal cancer

According to the reported ATP levels from $CD4^+$ T cells (Hooper et al., 2005), the ImmuKnow assay zones (in ng/mL ATP) of strong, moderate, and low immune function correspond to >525 ng/mL, 225 to 525 ng/mL, and <225 ng/mL. In this study, the median 375 ng/mL was taken as the critical point for moderate immune function, and there were 65 patients whose ATP levels were over 375 ng/mL and 39 patients whose ATP levels were less than 373 ng/mL before the surgery. In addition, there were two patients who suffered from metastasis or recurrence and 7 deaths occurred during the 60 months of follow-up; 1 patient suffered from metastasis or recurrence, 4 deaths occurred, and the 5-year progression-free survival rate was 88.9% in the ≥375-ng/mL ATP group, and 1 patient suffered from metastasis or recurrence, 3

deaths occurred, and the 5-year progression-free survival rate was 80.9% in the <375-ng/mL ATP group. There was no significant difference between the two groups (P > 0.01) (Figure 2).

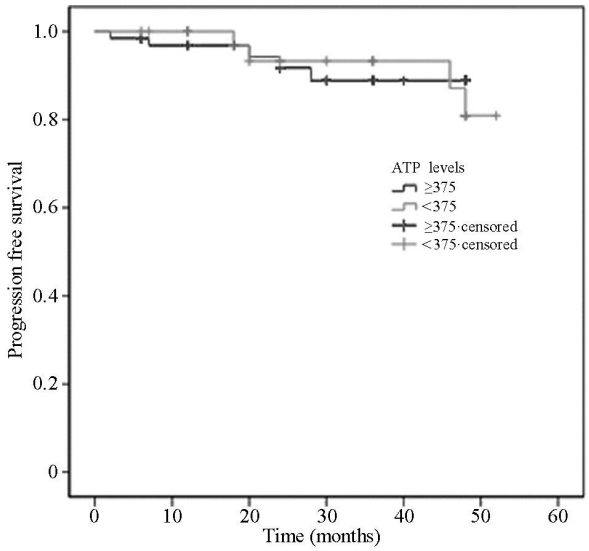

Figure 2. Kaplan-Meier survival of patients with renal cell carcinoma according to ATP levels. The two groups had no significant difference in progression-free survival (P > 0.01).

DISCUSSION

The occurrence, development, metastasis, and prognosis of kidney cancer have been shown to be closely related to immune functions. Cellular immunity is an important mechanism of anti-tumor immunity, and CD4+ T lymphocytes represent the main effector cells in this response. Sottong et al. (2000) found that the ATP content inside CD4+ T lymphocytes could reflect immune functions and suggested that the ATP assay had clear applications in monitoring the response to cancer therapy. Therefore, the assessment of the postoperative immune status of patients with renal cancer and regulation of the immune status to provide personal therapy are important strategies to control tumor recurrence and metastasis (Kobayashi et al., 2013). This study aimed to identify a new detection method for the assessment of cellular immune functions, through application of the ImmuKnow method to monitor the changes in ATP content inside the CD4+ T lymphocytes of patients with renal cancer, and to explore its clinical value in detecting the immune functions of patients with renal cancer. However, the results of this study indicated that there was no significant change in the ATP content of CD4+ T cells at the different time points analyzed as detected by the ImmuKnow method in the patients. Furthermore, we determined that there was no significance of the ATP levels toward the development of individual treatment and prognosis of patients with renal cancer.

In this study, 104 samples were collected under the steady immune status of patients, whose data were in accordance with a recent study (Ben-Youssef et al., 2009). Recent studies that reported ImmuKnow assay measures at a single time point did not identify individuals at risk for the future development of clinically significant events; therefore, it was suggested that dynamic monitoring of ATP levels from CD4+ T cells might better reflect the cellular immune

function of patients with renal cancer (Huskey et al., 2011). Here, our study monitored the dynamic change of ATP levels from $CD4^+$ T cells at the perioperative period and at 10 days, 1 month, 3 months, and 1 year after the surgery in 104 patients with renal cancer. Immunity was further compromised because of trauma, blood loss, anesthesia, and blood transfusion after patients with renal cancer underwent the surgery. After the surgery, with the general recovery of bodily functions, immunity would also rise, so we therefore expected that there would be significant changes in the ATP content of $CD4^+$ T lymphocytes at the different time points (Coffey et al., 2003). However, as shown in Figure 1, our results demonstrated that the ATP levels were not significantly different at the different time points. A possible reason for this might be that the ImmuKnow method has been found to exhibit low sensitivity toward the detection of ATP content (Husain et al., 2009; Ling et al., 2012); this assay might not therefore recognize the immune function changes in patients with renal cancer. We expected that patient immunity would have decreased 10 days after the surgery; however, Figure 1 shows that the intracellular ATP levels from $CD4^+$ T cells at this time were significantly higher than at other time points. We suggest that this finding might be because postoperative patients were administrated thymopeptide, interleukin, and IFN to enhance immunity. In order to confirm this, it would be necessary to detect the ATP content on the exact day after surgery, and compare this value with that at 10 days later to show whether a statistically significant difference exists between these two time points.

Demicheli et al. (2008) thought that the post-operative decrease in immune functions of patients with renal cancer might lead to the diffusion and metastasis of cancer. We should therefore assess the immune status of post-operative patients with renal cancer. Current standards of care include the administration of bio-therapy and certain immunoregulatory drugs to maintain higher levels of immune status to prevent or delay the recurrence of tumors (George et al., 2011; Mesiano et al., 2012). Therefore, we treated patients post-operatively with DC-CIK bio-therapy and IFN-α therapy. The DC-CIK therapy protocol involves the initiation of DC and CIK cell infusion 10 days after surgery. The treatment was repeated once at 1 month intervals, considered as one cycle. The protocol for IFN- α therapy included injection of 3 MIU IFN-α three times per week beginning at postoperative day 10, then injection of 6 MIU three times per week and 9 MIU three times per week, with each injection series lasting 10 weeks. Many studies have indicated that the numbers of $CD4^+$ T cells were increased after treatment with DC-CIK or INF-α therapy (Characiejus et al., 2001; Ranieri et al., 2007). Thus, we predicted that the ATP levels from $CD4^+$ T cells would be increased in postoperative patients with renal cancer compared to the levels in these patients before surgery. In contrast to this, Table 1 shows that there was no significant difference in the ATP levels from $CD4^+$ T cells before and after the therapy. The results were not consistent with our expectation; a possible reason might be that the sensitivity of the ImmuKnow method has been reported to be low (Husain et al., 2009; Ling et al., 2012), and thus might not detect the change associated with an increase in immune function. Therefore, we concluded that the detection of ATP levels from $CD4^+$ T cells by the Immuknow™-Cylex® assay did not reflect the actual immune status of patients with renal cancer after surgery.

Myslik et al. (2014) demonstrated that the results from ImmuKnow method detection of ATP levels before the transplantation surgery were significantly correlated with the determination of prognosis. We aimed to investigate the relationship of ATP content in the $CD4^+$ T lymphocytes with renal cancer prognosis. Therefore, we defined the median ATP value of 375 ng/mL as the critical value in our study, and we followed up the prognosis of patients with

ATP ≥ 375 ng/mL and those with ATP < 375 ng/mL) (Hooper et al., 2005). Our results showed that the 5-year-progression-free survival rate was not significantly different between the two groups. Thus, we demonstrated that ATP levels from CD4+ T cells could not be used to predict the prognosis of patients with renal cancer.

Monitoring postoperative immune status has been useful to determine prognosis and to outline personal therapy regimens. Our results showed that the ATP levels as measured by the ImmuKnow ™-Cylex® assay did not reflect the actual immune status of the patients and therefore did not have clinical value. Consequently, we need to explore a method with higher sensitivity that reflects the actual immune status of patients with renal cancer.

Conflicts of interest

The authors declare no conflict of interest.

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