RESEARCH ARTICLE

# Effect of Peripheral Blood CD4 + CD25 + Regulatory T Cell on Postoperative Immunotherapy for Patients with Renal Carcinoma

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## Abstract

**Objective: To investigate the effect of peripheral blood CD4 + CD25 + regulatory T cell on postoperative immunotherapy in patients with renal carcinoma. Methods: 38 patients with renal cell carcinoma were recruited, and 20 patients from the operation group purely underwent the radical nephrectomy therapy, 18 patients from the combined group successively underwent the radical nephrectomy therapy and IFN-α adjuvant immunotherapy. Additionally, 12 healthy subjects were recruited in the same period of time and regarded as the control group. Flow cytometry was used to detect CD4 +, CD8 +, CD4 + CD25+ T lymphocyte subset content and the ratio of all parts in the pre-operative period, in the first post-operative week and in the third post-operative month, compare and analyze its variation trend. Results: The CD4+CD25+ T lymphocyte subset content of individual renal carcinoma patients was significantly higher than that of the control group, also increases with the progression in the tumor stage (P<0.05). The post-operative CD4 + CD25+T lymphocytes of individual operation group and combined group patients showed different degrees of increment, but the increment of the combined group was significantly lower than that of the operation group (P<0.05). For the combined group patients with less pre-operative CD4 + CD25+T lymphocytes, their levels would increase after the immunotherapy, while the pre-operative patients with more CD4 + CD25+ T lymphocytes were the opposite situation. Conclusion: The detection of peripheral blood CD4+CD25+ regulatory T lymphocyte subset can reflect the anti-tumor immune status of renal cell carcinoma patient body. It can contribute to predict the prognosis of immunotherapy and provide reference for the choice of renal carcinoma post-operative adjuvant immunotherapy.**

**Keywords:** Renal carcinoma - Immunotherapy - CD4+CD25+ regulatory T cell

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## Introduction

The onset and progression of cancer could be closely related to immunological status (Jemal A et al., 2011). There is certain relationship between regulatory T cell (Treg)-mediated immune tolerance and the growth of tumor cells. Treg detection could reflect immunological status and help to determine the effect of therapeutic regimen (Chodon T et al., 2015). CD4+CD25+ Treg has the immunomodulatory effect, which can induce the body to show low-responsiveness and immune suppression, and then form the immune tolerance (Chen R et al., 2014). Renal cell carcinoma (Renal carcinoma) is a common urologic tumor with relatively high degree of malignancy. Even for the patients who are undergoing the radical surgical treatment, there are still about 40% of patients with metastasis or recurrence (Abe H et al., 2013; Bigot *P* et al., 2013), also with poor prognosis. This study detected the CD4+CD25+ Treg of renal carcinoma patients and discussed its effect of reflecting the body's anti-tumor immune status to provide relevant reference for the choice of renal carcinoma post-operative adjuvant immunotherapeutic regimen.

## Materials and Methods

*Clinical Materials*

From August 2013 to October 2015, our hospital's Department of Urology diagnosed 38 renal cell carcinoma patients who underwent the surgical therapy during hospitalization. And 20 cases from the operation group have purely undergone the radical nephrectomy therapy, Male: 12 cases; Female: 8 cases; Age: 35-68 years; Average: 52.3±4.2 years. Pathological type: 5 cases of granular cell carcinoma, 14 cases of clear cell carcinoma, 1 case of mixed cell carcinoma. Clinical stage: 6 cases in Stage I, 14 cases in Stage II. After radical nephrectomy, 18 patients from the combined group underwent IFN-α adjuvant immunotherapy. Male: 11 cases; Female: 7 cases; Age: 33-70 years; Average: 51.7±5.4 years; Pathological type: 3 cases of granular cell carcinoma, 14 cases of clear cell carcinoma, 1 case of mixed cell carcinoma; Clinical

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**Table 1. Comparison of T Lymphocyte Subset Proportions under Different Factors (x±s, %)**

| Item | | CD4+ | CD8+ | CD4+/CD8+ | CD4+CD25+ |
|---|---|---|---|---|---|
| Age | <60 years (n=20) | 36.83±5.27 | 18.79±3.42 | 1.92±0.24 | 17.12±2.73 |
| | ≥60 years (n=18) | 36.56±5.54 | 18.63±3.51 | 1.95±0.27 | 17.24±2.69 |
| t | | 0.154 | 0.142 | -0.363 | -0.136 |
| P | | 0.8785 | 0.8877 | 0.7190 | 0.8924 |
| Sex | Male (n=23) | 36.72±5.46 | 18.65±3.36 | 1.93±0.31 | 17.16±2.57 |
| | Female(n=15) | 36.77±5.35 | 18.74±3.25 | 1.94±0.26 | 17.20±2.65 |
| t | | -0.028 | -0.082 | -0.103 | -0.046 |
| P | | 0.9780 | 0.9353 | 0.9183 | 0.9633 |
| Stage | Stage I (n=11) | 36.43±5.28 | 18.57±3.43 | 1.92±0.23 | 16.12±2.58 |
| | Stage II (n=27) | 36.71±5.62 | 18.70±3.39 | 1.96±0.28 | 18.87±3.44 |
| t | | -0.142 | -0.107 | -0.149 | -2.384 |
| P | | 0.8882 | 0.9155 | 0.6779 | 0.0225 |

stage: 5 cases in Stage I; 13 cases in Stage II. Additionally select 12 cases of healthy subjects in the same period as the control group, Male: 7 cases; Female: 5 cases; Age: 33-70 years; Average: 51.2±4.8 years. The baseline material's comparative difference between two groups of renal carcinoma patients had no statistical significance ($P$>0.05).

*Inclusion Criteria*

The post-operative pathological result has proven to be renal cell carcinoma. The patients underwent no immunotherapy before hospitalization and took no immunomodulatory drugs recently.

*Exclusion Criteria*

Recent serious bacterial infection. Serum creatinine higher than 180umol/L. The increment of aspartate aminotransferasen and alanine aminotransferase beyond three times of normal upper limit. Suffering from severe metabolic diseases. Recent radiochemotherapy history. Combined hepatitis.

*Methods*

Therapy Methods All of 38 renal carcinoma patients underwent the radical nephrectomy. Specific manipulations were finished by the same group of physicians. The combined group was administered with post-operative immunotherapy: Administered with IFN-α subcutaneous injection since the 15th post-operative day, 3MIU in the first post-operative week and 3 times per week; 6MIU in the second post-operative week and 3 times per week; 9MIU in the third post-operative week and 3 times per week, and then continue this dose for three post-operative months. During this period, regularly re-examine the liver functions and blood routine.

Specimen Collection the patients in the operation group and combined group collected the cubital vein blood with limosis in the morning with conventional EDTA anticoagulant as backup respectively in the pre-operative period, in the first post-operative week and in the third post-operative month. The patients in the control group collected the cubital vein blood with limosis for once.

Detection Method (1) Fluorescent Antibody Labeling and preparation of PB-WC (Peripheral Blood White Cell) suspension: Take three test tubes which are marked as A, B and C, respectively add 100ul of whole blood into them. A is negative control. In B, add 20ul of mouse anti-human CD3FITC/CD8PE two-color fluorescence-labeled antibody (Nanjing JianCheng Bioengineering Institute). In C, add 20ul of mouse anti-human CD25FITC/CD4PE/CD3PC5 tricolor fluorescence-labeled antibody (Nanjing JianCheng Bioengineering Institute), fully oscillate and homogeneously mix it, incubate in darkness in the room temperature environment for 15min. Add 1ml of red blood cell lysis buffer (Beijing CellChip Biotechnology Co., Ltd) respectively into A, B and C, homogenously mix it and incubate again for 10min. After centrifugation at 1000r/min for 5 min, discard the supernatant, add 1ml of PBS and homogenously mix it. Then, centrifugation at 1000r/min for 5 min, discard the supernatant and add 300-600ul of PBS, you can obtain the fluorescent antibody-labeled peripheral blood WBC (white blood cell) suspension. (2) Flow cytometry: Place the prepared suspension into the flow cytometry channel. There are 10, 000 cells in each tube, then CELLQUEST software will generate a two-dimension scatter diagram. The data such as CD4+%, CD8+%, CD4+CD25+% and CD4+/CD8+ ratio will be calculated.

*Statistical Method*

SPSS19.0 statistical software was adopted, one-way ANOVA was used for intergroup comparison, SNK-q test was used for pair-wise comparison, and the difference with $P$<0.05 had statistical significance.

## Results

*Influential factors of T lymphocyte subset proportion*

Respectively group 38 renal carcinoma patients based on age, gender, and pathological stage, compare the pre-operative levels of CD4+, CD8+, CD4+/CD8+ and CD4+CD25+ Treg. The result showed that age and sex have no significant influence on the above indicators. While, for the patients across different stages, the CD4 + CD25+ Treg levels of individual Stage I patients were lower than those of Stage I patients. The difference had statistical significance. Please see Table 1 for details.

*Variation of T lymphocyte subsets before and after therapy*

In the pre-operative period, there was no significant difference between the operation group and the combined group's all T lymphocyte subset proportions ($P$>0.05). But their overall average CD4+CD25+ Treg was higher than the control group ($P$<0.05), while CD4+ and CD4+/CD8+

**Table 2. Variation of T Lymphocyte Subsets before and after Therapy (x±s, %)**

| Group Category | CD4+ | CD8+ | CD4+/CD8+ | CD4+CD25+ |
|---|---|---|---|---|
| Operation Group | | | | |
| Pre-operative | 36.64±5.08* | 18.78±3.46 | 1.88±0.27* | 17.18±2.57* |
| The first post-operative week | 36.74±5.25 | 18.79±3.38 | 1.93±0.45 | 17.26±2.44 |
| The first post-operative month | 39.83±5.75# | 18.87±3.15 | 2.17±0.62# | 18.46±3.26# |
| Combined Group | | | | |
| Pre-operative | 36.61±5.13 | 18.81±3.44 | 1.89±0.25 | 17.14±2.64* |
| The first post-operative week | 36.77±5.17 | 18.82±3.32 | 1.92±0.37 | 17.02±2.52 |
| The first post-operative month | 39.97±5.82# | 18.92±2.79 | 2.19±0.42# | 15.14±2.75# |
| Control Group | 39.76±1.18 | 17.32±0.68 | 2.19±0.13 | 14.02±0.62 |

Note: #$P$<0.05 VS pre-operative; *$P$<0.05 VS Control Group

ratio lower than the control group ($P$<0.05). However, among 38 renal carcinoma patients, there are 7 cases (3 in the operation group and 4 cases in the combined group) with CD4+CD25+ Treg level lower than the control group ($P$<0.05). Furthermore, there is no significant difference between its CD4+ and CD4+/CD8+ ratio and those of the control group ($P$>0.05). .

In the first post-operative week, in comparison with the pre-operative period, there was slight increase in the operation group's CD4+, CD4+CD25+ and CD4+/CD8+ CD4+ ratio, but slight decrease in the combined group's CD4+CD25+Treg, with no significant difference in comparison with the pre-operative period ($P$>0.05). There was no obvious variation in the CD8+ of 2 groups of patients.

In the third post-operative month, in comparison with the pre-operative period, there was obvious increase in the operation group's CD4+, CD4+CD25+ and CD4+/CD8+ CD4+ ratio, also increase in the combined group's CD4 and CD4+/CD8+ CD4+ ratio ($P$<0.05), but decrease in its CD4+CD25+ ($P$<0.05). There was slight increase in the CD8+ of both groups, but the difference had no statistical significance ($P$>0.05).

Among 7 patients with the pre-operative CD4+CD25+ Treg level lower than the control group, 6 cases showed higher post-operative CD4+CD25+ Treg level ($P$<0.05). While among other 31 patients, 19 cases showed lower post-operative CD4+CD25+ Treg level. Please see Table 2 for details.

## Discussion

Renal carcinoma has relatively high incidence and mortality. Even if radical resection therapy is implemented, there are still relatively high proportion of patients with post-operative metastasis or recurrence. Therefore, comprehensive therapy is of great significance to improving the prognosis of patients with renal and other sites of cancer (Liu et al., 2015; Liu et al., 2014; Li et al., 2015; Ji et al., 2014; Huang et al., 2014; Huang et al., 2015; Huang et al., 2015; Cui et al., 2015; Cao et al., 2014). Renal carcinoma has relatively poor sensitivity to conventional chemotherapy, radiotherapy and hormone therapy. However, current studies have proven that it has relatively good response rate to the immunotherapy (Hinotsu S et al., 2013; Wang D et al., 2014), and then provides new approach to the post-operative therapy of renal carcinoma.

It is currently considered that T-cell-mediated cellular immunity has important functions in the malignant tumor immunity. By detecting the ratio variation of T lymphocyte subset, the body's anti-tumor immunocompetence could be reflected (Zhao X et al., 2015). CD8+T cell is main anti-tumor effector cell, while CD4+T cell is important helper cell. For malignant tumor patients, CD4+/CD8+ can reflect the body's immune disorder status. When the body's immune function is suppressed, CD8+ will increase, CD3+ and CD4+, as well as CD4+/CD8+ ratio will decrease.

CD4+CD25+ Treg has low reactivity and immunosuppressive effect on alloantigens or autoantigens. It mainly suppresses the effector T cell's activation, proliferation and effector function. While tumor immunity is mainly cellular immunity, and then CD4+CD25+Treg can cause tumor immunosuppression and immune evasion (Wang S et al., 2015; Mao Q et al., 2015). This study demonstrated that the pre-operative CD4+ and CD4+/CD8+ ratio of individual renal carcinoma patient was lower than those of normal population, while their CD4+CD25+Treg was at a high level. The above result reflected the body's potential immunosuppressive status. In addition, individual patient's age and sex had no significant influence on CD4+CD25+Treg. While the analysis of patients in different pathological stages showed that CD4+CD25+Treg of Stage I renal carcinoma patients was lower than that of Stage II, renal carcinoma patient's immune function would continuously worsen with the progression of patient conditions, while Treg could reflect the worsened tumor immune function to some extent.

There was certain relationship between immunotherapy reaction and individual patient's peripheral blood lymphocyte ratio. The increase of CD4+/CD8+ CD4+/CD8+ showed better prognosis (Anguille S et al., 2015; Schmeel FC et al., 2014), indicating that immunotherapy could regulate the status of in-vivo immune system, and then bring a positive influence on the therapy result. In this study, both CD4+ and CD4+/CD8+ ratio of individual renal carcinoma patients increased after therapy, but the combined group more significantly. The result indicated that all renal carcinoma patients had different degrees of immunosuppression. Under the operative effect and (or) therapeutic effect, the body's anti-tumor immune activation and inflammation would cause CD4 + and CD4 + / CD8 + ratio to increase. The CD4 + CD25 + Treg of the combined group decreased after therapy,

because CD4 + CD25 + Treg may suppress the body's anti-tumor immune effect and immune status, as well as CD4 + lymphocytes. The decrease of CD4 + CD25 + Treg indicated immunotherapy would contribute to improving the body's immune suppression status, and then benefit for the playing the role of cellular immunity function, clearing the post-operative residual lesions.

In addition, the study also found that the CD4 + CD25 + Treg level of a small number of (n=7) renal carcinoma patients was lower than the control group, indicating that Treg had anti-tumor immune suppression effect on most renal carcinoma patients except some patients. Specific reasons may be related to the diversity and uncertainty of malignant tumor causes. The CD4+CD25+ Treg levels of the above seven patients increased after therapy, while 19 of the remaining 31 patients showed lower CD4 + CD25 + Treg levels after therapy, indicating that the existence of immunosuppression status had great influence on the result of post-operative immunotherapy. Therefore, immune status assessment should be actively executed for pre-operative renal carcinoma patients, and then the appropriate adjuvant immunosuppressive therapy would be determined.

In summary, the detection of peripheral blood T-lymphocyte can reflect the anti-tumor immune status of individual renal carcinoma patient's body, contribute to predict the prognosis of immunotherapy and provide reference for the choice of renal carcinoma post-operative adjuvant immunotherapeutic regimen. But it is worth noting that the result may have certain deviation caused by fewer cases incorporated into this study, shorter follow-up time, as well as no definite control and analysis for different pathological types of renal cell carcinoma. The above problems need further prospective and large-sample randomized controlled clinical study.

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