

# Proliferative Activity of Intratumoral CD8<sup>+</sup> T-Lymphocytes As a Prognostic Factor in Human Renal Cell Carcinoma: Clinicopathologic Demonstration of Antitumor Immunity<sup>1</sup>

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## ABSTRACT

Tumor-infiltrating lymphocytes, particularly CD8<sup>+</sup> T cells, could be a manifestation of antitumor immunity. We clinicopathologically analyzed the biological significance of tumor-infiltrating lymphocytes in 221 patients with renal cell carcinoma without preoperative treatments. More abundant infiltration of tumor tissue not only by CD8<sup>+</sup> but also CD4<sup>+</sup> T cells was associated with shorter survival of the patients, because of the positive correlation between the number of lymphocytes and representative tumor grade factors. This suggests that immune cell reactions are more pronounced as the tumor grade/biological malignancy progresses, probably because of increased antigenicity of tumor cells. We next analyzed the proliferative activity of CD8<sup>+</sup> T cells that infiltrated in tumor cell nests, which could also reflect antitumor immunity. Higher labeling index of Ki-67, a proliferation-associated antigen, among CD8<sup>+</sup> T cells in contact to tumor cells was associated with a longer survival by both uni- and multivariate analyses. Our data in human renal cell carcinoma suggest that infiltration of tumor tissue by T cells itself does not denote the efficacy of antitumor immunity because of its dependence on the biological malignancy of tumor cells, but infiltration of tumor tissue by CD8<sup>+</sup> T cells bearing more pronounced proliferative activity could reflect effective antitumor immunity. This concept would be important for future immunotherapy of human cancer.

## INTRODUCTION

The biological malignancy of human cancer is determined by the net effects of biological malignancy of tumor cells and host factors. The immune and vascular reactions are representatives of the host factors. In human cancers, however, such immune reactions are generally insufficient because of weak immunogenicity of cancer cells or impaired immunity in cancer-bearing patients (1). The presumptive mechanisms of these included absence of tumor-specific antigens, down-regulation of MHC class I molecule expression (2), or secretion of immunosuppressive cytokines (3). Recently, functional inactivation of potentially tumor-reactive T cells was analyzed as an important mechanism of immune evasion (4). Despite these obstacles, renal cell carcinoma has been known for the effectiveness of lymphokine-activated killer and cytokine therapies (5-7) and therapy using tumor cell-dendritic cell hybrids (8). This suggests that renal cell carcinoma is one of the targets of immunotherapy. However, previous histopathological reports failed to clarify prognostic significance of TILs<sup>3</sup> in renal cell carcinoma (9-12) despite the presence of tumor-

specific MHC class I-restricted cytotoxic T lymphocytes (13). To further analyze this point, the present study was designed to expand our previous analysis on CD8<sup>+</sup> T cells in gastrointestinal cancer, which showed that CD8<sup>+</sup> T cells infiltrated into cancer cell nests could reflect antitumor immunity (14, 15).

## MATERIALS AND METHODS

**Tissue Samples.** We analyzed 221 patients with renal cell carcinoma with complete 5-year follow-up data. They were histopathologically composed of 147 cases (67%) of clear cell type, 29 (13%) of granular cell type, 22 (10%) of mixed clear and granular type, 8 (4%) of chromophobe type, 12 (5%) of papillary type, and 3 (1%) of spindle cell type (16). None of these patients received preoperative immunotherapy or preoperative renal arterial embolization therapy. The age of patients ranged from 29 to 81 years of age (mean, 58.4 years), and the male:female ratio was 2.3:1.0. The patients received nephrectomy during 1985 and 1993 at Tohoku University Hospital in 108 cases, Ishinomaki Red Cross Hospital in 43 cases, and Sendai Social Insurance Hospital in 70 cases. Resected specimens were fixed in formalin and embedded in paraffin for the routine histopathological diagnosis. The follow-up data showed that 155 patients survived without cancer, 8 patients survived with cancer, and 58 patients died of cancer. We excluded patients who died of other causes. After surgery, 116 of 221 patients received immunotherapy with IFN- $\alpha$  in 114 patients or IFN- $\gamma$  in 2 patients. The effects of postoperative immunotherapy with IFNs were not significant by multivariate analysis (odds ratio, 0.97;  $P = 0.96$  by logistic model), consistent with previous studies (17, 18). The tumor stage was classified into four according to TNM classification (19). Cancer cells were graded into I, II, or III in each case as described previously (20).

**Immunohistochemistry.** A biotin-streptavidin-peroxidase method was adopted using Histofine kit (Nichirei, Tokyo, Japan). The primary antibodies used were mouse monoclonal anti-human CD8 (clone C8/144b, DAKO, Glostrup, Denmark; diluted at 1:50), anti-granzyme B (clone GrB-7, Kamiya Biomedical Co., Seattle, WA; diluted at 1:30), and anti-Ki-67 (clone MIB-1, Immunotech, Cedex, France; diluted at 1:50). The pretreatment condition was microwave heating (95°C for 15 min) for CD8 and granzyme B and autoclave heating (120°C for 5 min) for Ki-67. The chromogen was 3,3'-diaminobenzidine tetrahydrochloride (brown). We evaluated granzyme B<sup>+</sup> cells with a sparsely granulated pattern as activated cytotoxic T cells, excluding densely positive natural killer cells. The internal positive control of CD8 staining was T cells distributed along the invasive margin, which were present in all cases examined.

**Double Immunohistochemistry for CD8 and Ki-67 (Performed in 78 Cases).** Double staining for CD8 and Ki-67 was performed as described previously (14, 15) in 78 cases in which the infiltration of intratumoral CD8<sup>+</sup> T cells was prominent. After autoclave heating, anti-CD8 monoclonal antibody (1:40) was applied for 90 min. Histofine kit (Nichirei) for alkaline phosphatase was used with new fuchsin as a chromogen (red). Tissue specimens were soaked in boiling water for 5 min to denature the antibody used in the first step. Anti-Ki-67 (1:50) was reacted overnight, followed by application of Envision with the chromogen 3,3'-diaminobenzidine (brown).

**Quantification Methods.** (a) Intratumoral CD8<sup>+</sup> T cells: We counted the number of those cells in a microscopic grid, 0.5 × 0.5 mm in size (0.25 mm<sup>2</sup>) using a microscopic field of ×200. Five areas with the most abundant distri-

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<sup>3</sup> The abbreviations used are: TIL, tumor-infiltrating lymphocyte; TNM, Tumor-Node-Metastasis.

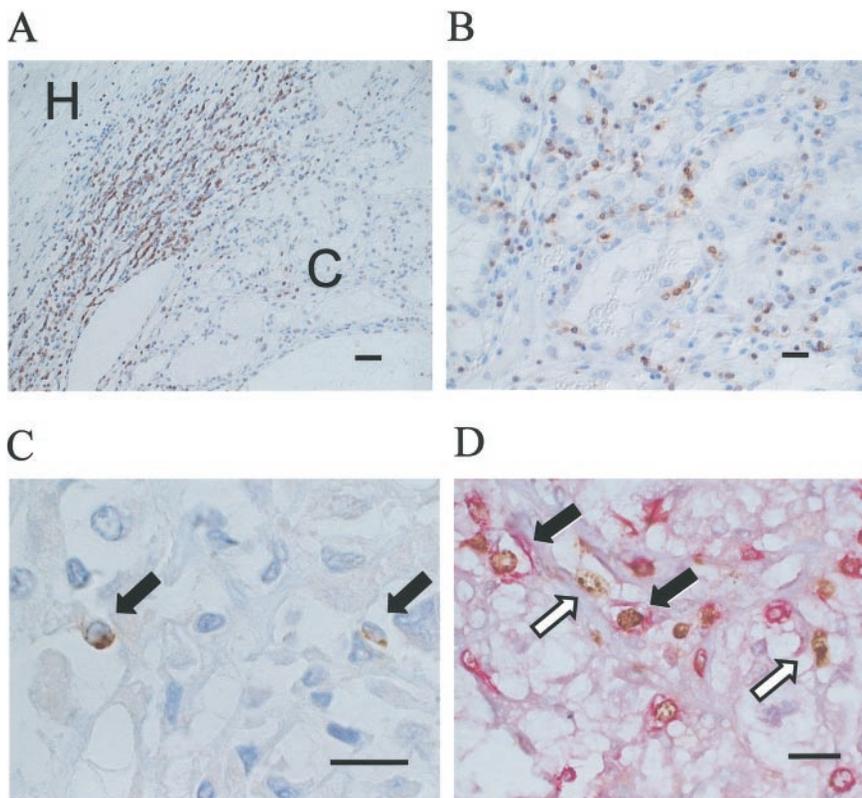


Fig. 1. Immunohistochemistry for CD4 (A), CD8 (B), granzyme B (C), and double immunohistochemistry for CD8/Ki-67 (D). In A–C, the immunoreactivity is shown by the color brown. Nuclear counterstain is shown by hematoxylin (blue). In A, CD4<sup>+</sup> cells are most abundant along the invasive margin (tumor-host interface). C, cancer cells; H, host tissue. In B, note the presence of CD8<sup>+</sup> cells in contact with tumor cells, designated as “intratumoral CD8<sup>+</sup> T cells” in the present study. In C, cancer tissue is infiltrated by granzyme B<sup>+</sup> lymphocytes (arrows). In D, black arrows indicate double positive cells for CD8 (red, membrane pattern) and Ki-67 (brown, nuclear staining pattern). White arrow, Ki-67<sup>+</sup> cancer cells; Red cells, CD8<sup>+</sup> cells without Ki-67 reactivity. Scale bar, 40 μm in A and 20 μm in B–D.

bution were selected in each case. The area of microscopic field in the present study corresponds to 27% of that of our previous study (15).

(b) Peritumoral CD8<sup>+</sup> and CD4<sup>+</sup> T cells and TILs by H&E stain: We semiquantified those cells into three groups (0, nil or mild; 1, moderate; and 2, abundant) as described previously (15). Tumor-host interface was clearly confirmed in 201 cases. Of these, we obtained clear results for CD4 in 178 cases. Two independent observers judged the distribution of these three variables. When the results were not consistent, the two observers discussed to reach a consensus on the semiquantification scoring.

(c) The labeling index of Ki-67 in cancer cells: The number of Ki-67<sup>+</sup> cancer cells was counted among 1,000 cancer cells using ×200 microscopic field. The area of most abundant distribution was chosen.

(d) Labeling index for Ki-67 in CD8<sup>+</sup> T cells: This was defined as the percentage of the number of Ki-67<sup>+</sup>/CD8<sup>+</sup> cells among 200 CD8<sup>+</sup> intratumoral lymphocytes. Areas with most abundant distribution of CD8<sup>+</sup> cells were chosen for this observation. Two independent observers counted immunoreactive cells. The interobserver variation was checked with the correlation coefficients 0.86 ( $P < 0.0001$ ) for the single immunohistochemistry and 0.65 ( $P < 0.0001$ ) for the double immunohistochemistry for CD8 and Ki-67. The intraobserver variation (interassay variation) was also checked with the correlation coefficients 0.93 ( $P < 0.0001$ ) for the single immunohistochemistry and 0.76 ( $P < 0.0001$ ) for the double immunohistochemistry. All of the counting was done in a blind fashion; the observers were not informed of the outcome of the patients or of the results of other observers.

**Statistical Analysis.** The univariate analysis was done by the Kaplan-Meier method, and the test of significance was checked by the log-rank test. Statistical differences of the values among different groups were tested by Kruskal-Wallis and Tukey tests using software Dr. SPSS 8.0J (SPSS Japan, Inc., Tokyo, Japan). The multivariate analysis was performed by the backward stepwise method using Cox proportional hazards model and logistic model (Dr. SPSS 8.0J).

**RESULTS**

We retrospectively analyzed 221 patients with renal cell carcinoma without preoperative therapy. TILs recognized by conventional H&E stain were mainly detected along the tumor-host interface. We desig-

nated these cells as peritumoral TILs. TILs were also detected within cancer cell nests or present in the stroma in contact to cancer cells. These lymphocytes were designated as intratumoral (intraepithelial) TILs in the present study. Peritumoral TILs were comprised of both CD4<sup>+</sup> (Fig. 1A) and CD8<sup>+</sup> cells, whereas CD8<sup>+</sup> cells predominated over CD4<sup>+</sup> cells among intratumoral T cells (Fig. 1B). We first counted the number of CD8<sup>+</sup> T cells to explore their biological significance. Our previous study showed that a larger number of CD8<sup>+</sup> T cells in tumor cell nests was associated with a longer survival in colorectal cancer (15). Contrary to our assumption, the overall and disease-free survival rates were shorter in patients with renal cell carcinoma with abundant intratumoral CD8<sup>+</sup> T cells than in those having a smaller number of intratumoral CD8<sup>+</sup> cells (Fig. 2). Furthermore, semiquantitative analyses of peritumoral CD8<sup>+</sup> T cells, peritumoral CD4<sup>+</sup> T cells, and peritumoral TILs recognized by H&E staining showed the same results: the more lymphocytes, the shorter the patients’ 5-year survival (Fig. 3). These results were, however, not

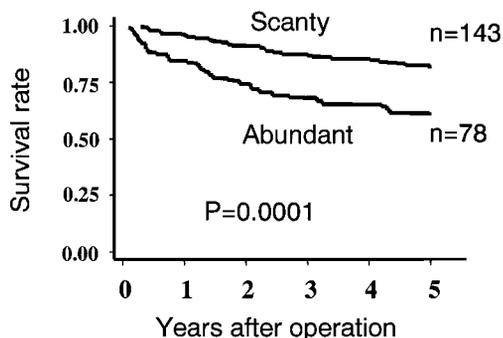


Fig. 2. Cause-specific, overall survival curves by intratumoral CD8<sup>+</sup> T cells. The patients were divided into two groups (dichotomy) at 50 (near the average value, 48.3). No significant differences were noted between overall and disease-free survival rates (data not shown). Dichotomy of the patients at the median value (30) showed the same results [ $P = 0.0004$  (Logrank test), data not shown].

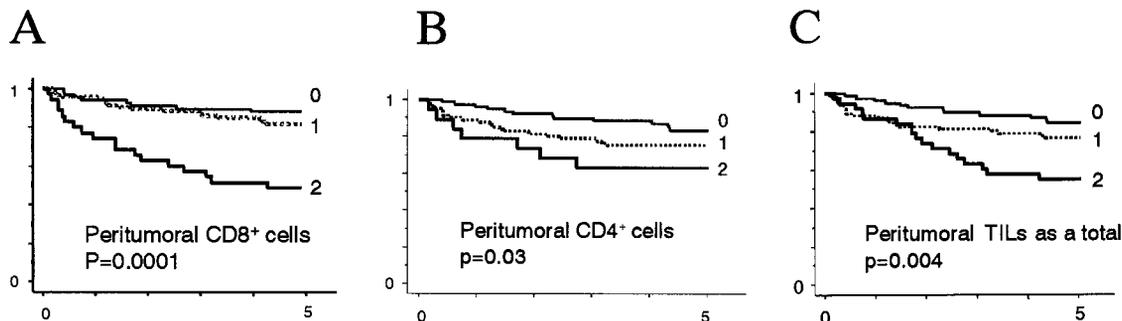


Fig. 3. Cause-specific, overall survival curves by peritumoral CD8<sup>+</sup> cells (A), by peritumoral CD4<sup>+</sup> cells (B), and by TILs by H&E staining (C). All of the parameters were semiquantified into 0 (scanty), 1 (moderate, discontinuously positive), and 2 (abundant, continuously positive). Vertical and horizontal axes, the survival rate and years after operation, respectively.

contradictory to previous reports describing that TILs analyzed by frozen sections are related to tumor grade in renal cell carcinoma (9, 10). Furthermore, our data were consistent with previous reports on Hodgkin's lymphoma (21) and anaplastic large cell lymphoma (22); the presence of granzyme B<sup>+</sup>/CD8<sup>+</sup> T cells was associated with a worse prognosis. Thus, we were confronted with results suggesting that neither CD8<sup>+</sup> and CD4<sup>+</sup> T cells nor TILs as a whole are relevant to effective tumor immunity in human renal cell carcinoma.

To search for the reasons of this apparent discrepancy from our previous study, we next analyzed the correlation between the number of lymphocytes and other tumor cell factors (19, 23). As shown in Fig. 4, the number of intratumoral CD8<sup>+</sup> T cells was positively correlated with both tumor grade and with the labeling index of tumor cells for Ki-67, a representative cell proliferation marker. This indicated that intratumoral CD8<sup>+</sup> cells were more abundant in tumors with severer cellular atypia and with a higher growth rate. No correlation was found between TNM stage and the number of intratumoral CD8<sup>+</sup> T cells (data not shown). Semiquantitative scoring of peritumoral CD8<sup>+</sup> and peritumoral CD4<sup>+</sup> cells and TILs recognized by H&E staining showed the same results; all these parameters positively correlated with tumor grade and the labeling index of tumor cells for Ki-67 (Table 1). The results on tumor grade are consistent with previous flow cytometric analyses on TILs in renal cell carcinoma (11, 12). Multivariate analysis showed that TNM stage, labeling index of cancer cells for Ki-67, and tumor grade had significant, deteriorating effects on cause-specific, 5-years' survival rate (Table 2). Histological type (clear cell *versus* nonclear cell type) was not significant by multivariate analysis, although univariate analysis showed that clear cell type showed a longer survival (Table 2). None of four lymphocytic parameters [intratumoral CD8<sup>+</sup> cells (Table 2), peritumoral CD8<sup>+</sup> T cells, peritumoral CD4<sup>+</sup> T cells, and peritumoral TILs] were significant for patients' survival by multivariate analyses.

Table 1 Correlation coefficients between tumor cell factors and peritumoral lymphocytes

	Labeling index of Ki-67 (cancer cells) <sup>a</sup>	Tumor grade <sup>b</sup>
Peritumoral CD8 <sup>+</sup> cells <sup>c</sup>	0.325	0.285
Peritumoral CD4 <sup>+</sup> cells <sup>c</sup>	0.307	0.326
Peritumoral TILs (HE) <sup>c</sup>	0.367	0.362

<sup>a</sup> Quantified.  
<sup>b</sup> Scored into three.  
<sup>c</sup> Semiquantified into three.

Taken together, the apparent adverse effects of lymphocytes on patients' survival rates were caused by the dependency on tumor grade or proliferative activity of tumor cells, which are more potent prognostic factors than lymphocytes. We have thus clarified that lymphocytic reactions in tumor tissue become more prominent as the malignant potential of the tumor progresses in renal cell carcinoma.

Granzyme B was detected in a part of intratumoral lymphocytes as a sparsely granular pattern (Fig. 1C), suggesting the presence of activated, cytotoxic T cells (24). The proportion of these sparsely granular granzyme B<sup>+</sup> cells to intratumoral CD8<sup>+</sup> cells ranged from 7 to 41% (19.6% in average) in representative 12 cases. The presence of granzyme B<sup>+</sup> cells among intratumoral T cells was basically the same as that in EBV-associated gastric cancer (14) and in colorectal cancer (15). Therefore, we next analyzed the proliferative activity of intratumoral lymphocytes by double immunohistochemistry for CD8 and Ki-67 (Fig. 1D), because this parameter may reflect the magnitude of tumor-specific T-cell response (14). We analyzed 78 cases with more abundant intratumoral CD8<sup>+</sup> lymphocytes, which were composed of 45 (58%) cases of clear cell type, 15 (19%) of granular cell type, 11 (14%) of mixed type, none of chromophobe type, 5 (6%) of papillary type, and 2 (3%) of spindle cell type. The median values, 75 and 25 percentiles of the labeling index of Ki-67 in intratumoral CD8<sup>+</sup> T cells, were 9, 16, and 5%, respectively, in this group. As shown in Fig. 5, higher labeling index of Ki-67 in intratumoral CD8<sup>+</sup> T cells was associated with a longer survival of patients. Of particular importance, multivariate analyses confirmed that the labeling index of Ki-67 in intratumoral CD8<sup>+</sup> T cells was significant for patients' survival, together with the tumor stage and proliferative activity of tumor cells (Table 3). The hazard ratio (0.3) and odds ratio (0.05) of CD8/Ki-67 are below 1, indicating that this parameter was related to a longer survival of patients. These two multivariate analyses indicated that the proliferative activity of intratumoral CD8<sup>+</sup> T cells is an independent prognostic factor in renal cell carcinoma. This could be a clinicopathological demonstration of an occurrence of antitumor immunity.

We next confined the cases of this group to clear cell type, because clear cell type might be more immunogenic as shown by allogenic

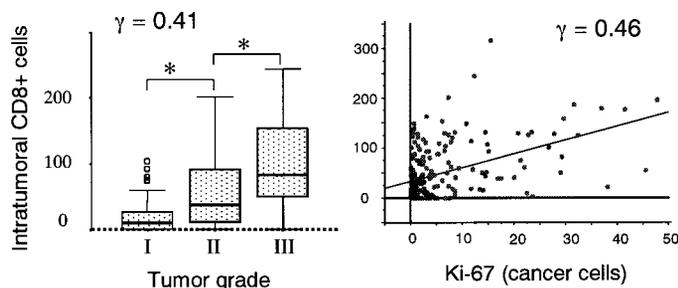


Fig. 4. Intratumoral CD8<sup>+</sup> T cells were positively correlated with tumor grade (left) and labeling index for Ki-67 in cancer cells (right). Vertical axis (left and right), number of intratumoral CD8<sup>+</sup> T cells in 0.25 mm<sup>2</sup>. Grade I, mild cellular atypia (n = 68); Grade II, moderate atypia (n = 124); and Grade III, severe cellular atypia (n = 29; left). \*P < 0.0001 by Tukey's test.

Table 2 Multivariate analysis in all 221 cases

	Kaplan-Meier		Multivariate analysis/Cox proportional hazards model					
	Log-rank test; <i>P</i>	Parameter estimate	SE	<i>P</i>	Hazard ratio	95% C.I. <sup>a</sup>	<i>P</i>	
Stage	<0.0001	0.98	0.15	<0.001	2.7	2.0–3.7	<0.0001	
Ki-67 (cancer) <sup>b</sup>	<0.0001	1.4	0.5	0.002	→ <sup>c</sup>	4.5	1.8–11	
Grade	<0.0001	0.43	0.27	0.12	1.7	1.0–2.8	0.04	
Intratumoral CD8 <sup>+</sup> T cells <sup>d</sup>	0.0002	0.17	0.28	0.5				
Histology <sup>e</sup>	<0.0001	0.26	0.3	0.38				

<sup>a</sup> C.I., confidence interval.

<sup>b</sup> Categorized into 0 (<1.5% = median value) and 1 (>1.5%).

<sup>c</sup> Arrow, step-down parameter selection.

<sup>d</sup> Categorized into 0 (<50) and 1 (>50).

<sup>e</sup> Categorized into clear cell type and others.

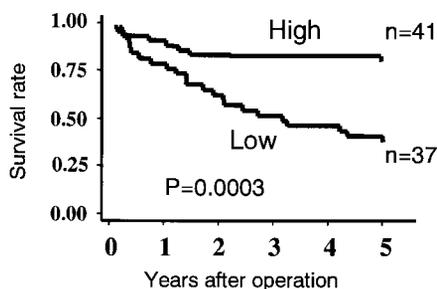


Fig. 5. Cause-specific survival curves by labeling index of Ki-67 in intratumoral CD8<sup>+</sup> T cells in 78 cases. Dichotomy of the patients was done at the median value (9%).

peripheral-blood stem-cell transplantation treatment (25) and by treatment by monoclonal antibodies (26). The total number of cases of this group was 45. The impact of the labeling index of Ki-67 among intratumoral CD8<sup>+</sup> cells of this group was essentially the same, because the *P* was 0.0004 by Logrank test, and the hazard ratio was 0.15 (95% confidence interval 0.03–0.71) with the *P* of 0.02 by Cox proportional hazards model.

**DISCUSSION**

This is the first study that demonstrates positive correlation between the tumor grade and lymphocytic reactions and further clarifies the biological significance of proliferative activity of intratumoral CD8<sup>+</sup> cells. The difference between the present study and the previous one dealing with CD8<sup>+</sup> T cells in colorectal cancer (15) should be

discussed first. One of the most prominent features in renal cell carcinoma is the heterogeneity of malignant potential among individual tumors. This was clearly demonstrated by the findings showing that not only TNM stage but also tumor grade and proliferative activity of tumor cells are prognostic factors by multivariate analysis. We demonstrated that the number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells infiltrated within or around renal cell carcinoma was positively correlated with these cancer cell factors. This situation is totally different from that in colorectal cancer, which is usually uniform in histological differentiation; most cases are well or moderately differentiated adenocarcinoma. This positive correlation between intratumoral CD8<sup>+</sup> T cells and tumor grade (atypia) further suggests a possible increase in the number and/or amounts of tumor-associated antigens that can be recognized by T cells in more dedifferentiated cancer. Therefore, our results suggested that infiltration of tumor tissue by lymphocytes itself does not denote the efficacy of antitumor immunity, probably because their effects, if present, may be canceled by more advanced aggressiveness of tumor cells. These results in renal cell carcinoma are essentially the same as in lung cancer, because we already observed a similar increase of CD8<sup>+</sup> lymphocytes in poorly differentiated-type cancer than in differentiated-type cancer of the lung (27).

Analysis of proliferative activity of lymphocytes is based on the assumption that higher proliferative activity of lymphocytes could be related to stronger antigenic stimuli to lymphocytes and/or higher responsiveness of T cells. The present results suggest that T cells in the tumor tissue bearing proliferative activity could eventually work as antitumor effector cells. This further suggests that TILs with lower proliferative activity may not exert effector function because of im-

Table 3 Multivariate analysis in 78 patients with abundant intratumoral CD8<sup>+</sup> cells

A.	Kaplan-Meier		Multivariate analysis/Cox proportional hazards model					
	Log-rank test; <i>P</i>	Parameter estimate	SE	<i>P</i>	Hazard ratio	95% C.I. <sup>a</sup>	<i>P</i>	
Stage	<0.0001	0.9	0.24	0.0001	2.5	1.5–3.8	0.0001	
CD8/Ki-67 <sup>b</sup>	0.0003	−0.99	0.42	0.02	→ <sup>c</sup>	0.3	0.15–0.78	
Ki-67 (cancer) <sup>d</sup>	<0.0001	0.77	0.58	0.18	2.5	0.86–7.4	0.09	
Grade	0.0006	0.54	0.44	0.22				
Histology <sup>e</sup>	0.004	−0.2	0.47	0.62				

B.	Kaplan-Meier		Multivariate analysis/logistic model					
	Log-rank test; <i>P</i>	Parameter estimate	SE	<i>P</i>	Odds ratio	95% C.I. <sup>a</sup>	<i>P</i>	
Stage	<0.0001	1.6	0.43	0.0003	4.8	2.0–11.3	0.0003	
CD8/Ki-67 <sup>b</sup>	0.0003	−3	1	0.0030	→ <sup>c</sup>	0.007–0.34	0.002	
Ki-67 (cancer) <sup>d</sup>	<0.0001	2	0.97	0.04	6.4	1.3–32	0.02	
Grade	0.0006	−0.13	0.98	0.87				
Histology <sup>e</sup>	0.004	−0.04	1	0.97				

<sup>a</sup> C.I., confidence interval.

<sup>b</sup> Labeling index of intratumoral CD8<sup>+</sup> cells for Ki-67 categorized into 0 (<9.0% = median value) and 1 (>9.1%).

<sup>c</sup> Arrow, step-down parameter selection.

<sup>d</sup> Categorized into 0 (<1.5% = median value) and 1 (>1.5%).

<sup>e</sup> Categorized into clear cell type and others.

munosuppressive milieu in renal cell cancer tissue (28, 29). The presence of proliferating lymphocytes in cancer tissue, on the other hand, indicates that not all lymphocytes are fully matured, given step-wise functional maturation of cytotoxic T cells (30). In this regard, analysis of antigen-presenting cells in cancer tissue and/or regional lymph nodes will be the next-step study to examine whether or not putative proliferative signals for T cells within cancer tissue are antigen specific. Peritumoral CD4<sup>+</sup> T cells are also important to assist CD8<sup>+</sup> effector cell differentiation, because cytokine profile of TILs from renal cell carcinoma is polarized to be like type 1 (Th1/Tc1) despite the influence of down-modulatory effects by IL-6 and IL-10 (13). In the present study, proliferative activity of CD4<sup>+</sup> T cells was not analyzed because of rather inconsistent staining results for CD4.

In conclusion, we have shown that lymphocytic reaction itself is associated with tumor grade/biological malignancy and that the presence of intratumoral CD8<sup>+</sup> T cells with proliferative activity, presumably cytotoxic effector cells, is important in human renal cell carcinoma. These results could pave the way for more effective immunotherapy of human cancer.

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## Proliferative Activity of Intratumoral CD8<sup>+</sup> T-Lymphocytes As a Prognostic Factor in Human Renal Cell Carcinoma: Clinicopathologic Demonstration of Antitumor Immunity

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