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Prognostic impact of tumor-infiltrating CD276/Foxp3-positive lymphocytes and associated circulating cytokines in patients undergoing radical nephrectomy for localized renal cell carcinoma

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Abstract. Renal cell carcinoma (RCC) is an immunogenic tumor and pathological specimen generally contain large quantities of tumor-infiltrating lymphocytes (TILs). Numerous cell types and cytokines could affect the immune escape mechanism of tumor cells. The aim of the present study was to investigate the prognostic impact of TILs and the associated circulating cytokines on localized clear cell RCC following radical nephrectomy. A total of 87 patients who had undergone radical nephrectomy and were pathologically diagnosed with localized clear cell RCC were included. The present study evaluated the profile of TILs with immunohistochemical analysis of tumor specimens using a panel of antibodies [cluster of differentiation (CD)-4, CD8, CD80, CD86, CD276, and Forkhead box p3 (Foxp3)]. Counts of each TIL were compared with clinicopathological variables. Based on the results of immunohistochemical analyses, putative cytokines, including interleukin (IL)-6, IL-10, IL-17, interferon-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, were selected, and their levels in preoperative serum were measured by ELISA. The levels were compared with TIL counts in tumor specimens. High counts of the $CD276^+$ and $Foxp3^+$ TILs were identified as independent factors for poor prognosis for metastasis and local recurrence following radical nephrectomy (P=0.033 and 0.006, respectively). A high $CD276^+$ TIL count was associated with preoperative serum levels of TNF-α and IFN-γ (P=0.027 and P=0.035, respectively), whereas a high count of $Foxp3^+$ TILs was associated with preoperative serum levels of TGF-β (P=0.021). High levels of TNF-α and TGF-β were associated with recurrence-free survival (P=0.035 and P=0.031, respectively). Topical intra-tumoral immunoreaction and systemic immune status may be associated with patients with localized RCC. The topical induction of the $CD276^+$ and $Foxp3^+$ TILs was suggested to be associated with high levels of serum TNF-α and IFN-γ. Preoperative serum levels of TNF-α and TGF-β could be simple and non-invasive biomarkers for risk stratification before radical surgery.

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Key words: renal cell carcinoma, cluster of differentiation 276, Foxp3, tumor-infiltrating lymphocyte, cytokine

Introduction

Approximately 70% of patients with renal cell carcinoma (RCC) are diagnosed with localized RCC, and incidental detection of asymptomatic RCC is increasing with the widespread use of ultrasonography and computed tomography (CT) (1). Localized RCCs are treated by radical nephrectomy or partial nephrectomy. After complete surgical resection for localized RCC, 20 to 30% of patients progress to metastatic disease (2). The prognosis for patients with RCC is primarily dependent on disease stage, and patients with a high TNM stage have a poorer prognosis. Once RCC has metastasized, the 5-year survival rate is <10% (3). The identification of prognostic markers would be useful to prevent localized RCC recurrence after surgery.

RCC is an immunogenic tumor and pathological specimens contain large numbers of tumor-infiltrating lymphocytes (TILs) (4). RCC can impair host antitumor immunity (5-7). Cancer cells express tumor-specific aberrant antigens and evade immune detection to survive by inducing immunosuppression or deriving survival signals from tumor-infiltrating immune cells (8,9). Various cells and cytokines are involved in the immune escape of tumor cells. For instance, regulatory T cells (Tregs) and the B7 family are associated with tumor immune escape. Tregs play an important role in maintaining the stability of the immune system and tumor immune tolerance. Forkhead box p3 (Foxp3) is a specific transcription factor expressed in Tregs (10). The B7 family is composed of cell-surface proteins that regulate immune responses by delivering co-stimulatory or co-inhibitory signals through their ligands.

The correlation of Foxp3-positive cells or B7 family with patient clinicopathological features have been investigated

in many cancers, including RCC (11-13). Tissue samples or blood samples only have tended to be used, with few studies examining both sample types. Furthermore, CD276 can increases the secretion of interferon-gamma (IFNγ) by activated T cells (14), and Foxp3 products interleukin (IL)-10, transforming growth factor-β (TGF-β), and tumor necrosis factor-alpha (TNFα) (15). Although the correlation between TILs and associated cytokines has been reported, there is no consensus on their interactions.

Postoperative recurrence of RCC after radical surgery mostly depends on the presence of micrometastasis preoperatively. Here, we hypothesized the possible correlation of preoperative topical and systemic immunoreactions with the risk of micrometastasis. To evaluate topical and systemic preoperative immunoreactions, we investigated the correlation between the profile of TILs and preoperative serum cytokine levels. The aim of this study was to identify non-invasive and preoperative markers that could be valuable to predict the recurrence of localized clear cell RCC (ccRCC) after radical nephrectomy.

Materials and methods

Patients selection and data collection. Eighty seven patients who underwent radical nephrectomy for clinically localized ccRCC between January 2009 and December 2014 were included in this retrospective study. All patients underwent preoperative whole body computed tomography (CT) for tumor staging. The clinical stage was determined according to the TNM classification (16). Clinicopathological variables and laboratory data were extracted from medical records. The SSIGN score, which is an outcome prediction model for patients with ccRCC based on pathological stage, tumor size, nuclear grade, and necrosis (17), was evaluated as one of the clinicopathological variables. The histopathological review was conducted by an experienced uropathologist to determine the T category and Fuhrman grade (18), as well as the presence of necrosis, sarcomatoid variant, and lymphovascular invasion (LVI). All subjects gave their written informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Nara Medical University (Nara, China) (Project identification code: 1630, accepted: August 21, 2017).

Immunohistochemistry (IHC) staining. Resected tissue specimens were fixed in formalin, embedded in paraffin, and then subjected to IHC staining for the cell surface and immunological markers CD4, CD8, CD80 (B7-1), CD86 (B7-2), CD276 (B7-H3), and Foxp3 (a Treg marker). Paraffin blocks were cut and placed on Superfrost Plus microslides (Thermo Fisher Scientific, Inc., Yokohama, Japan). Sections were deparaffinized and citric acid buffer (pH 6.0) antigen retrieval was carried out with autoclaving. IHC staining was performed using the Histofine ABC kit (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's instructions. Briefly, slides were incubated overnight at 4˚C with monoclonal antibodies against CD4 (clone 4B12, ready-to-use; Nichirei Biosciences), CD8 (clone C8/144B, ready-to-use; Nichirei Biosciences), CD80 (clone EPR1157 (2), 1:500 dilution; Abcam, Cambridge, UK), CD86 (clone EP1158Y, 1:500 dilution; Abcam), CD276 (clone 6A1, 1:1000 dilution; Abcam), and Foxp3 (clone 236A/E7, 1:500 dilution; Abcam). The slides were counterstained with Mayer's hematoxylin, dehydrated, and sealed with a cover slide.

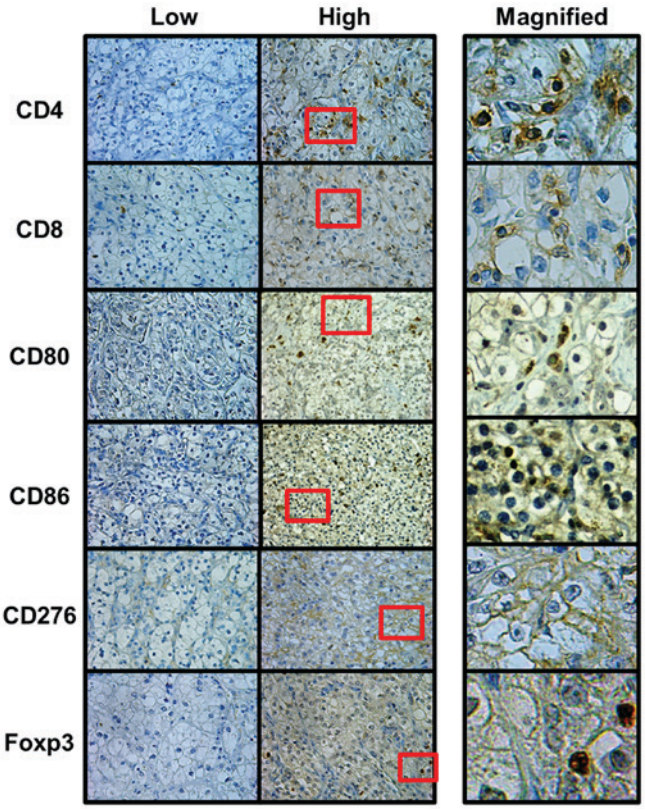

Figure 1. Representative images of tumors stained for each marker. Low group (left-hand column) and high group (middle column) are shown. The magnified images of the red squared area in the high group column are also shown on the right-hand side. Magnification, x1,000. CD, cluster of differentiation; Foxp3, Forkhead box p3.

Expression of markers. All stained tissue samples were evaluated by two investigators (KO and YI) without knowledge of the patients' clinical records. One sample was divided into four randomly selected tumor areas at x400 magnification. The percentage of stained cells was calculated by taking the mean of percentage calculated by dividing a positive cell count by the total cell count of positive cell count and negative cell count. Specimens were classified into two groups (low and high) based on the staining population. The cutoff level of CD4 and CD8 was set at 20%, and the cutoff level of CD80, CD86, CD276, and Foxp3 was set at 10% (13,19). The expression of Foxp3 was evaluated with the nucleus of tumor cells exhibiting immunoreactivity, and the expressions of other markers were evaluated with the cell membrane (Fig. 1).

Measurement of serum cytokines. Serum was collected from every patient in tubes before the operation and centrifuged at 1,000 x g for 15 min. The supernatant was recovered and stored at -80˚C until analysis. Based on the result of the IHC analysis, six cytokines (IL-6, IL-10, IL-17, IFN-γ, TNF-α, and TGF-β) were selected. Their profiles in preoperative sera were determined using the following ELISA kits: human IL-6

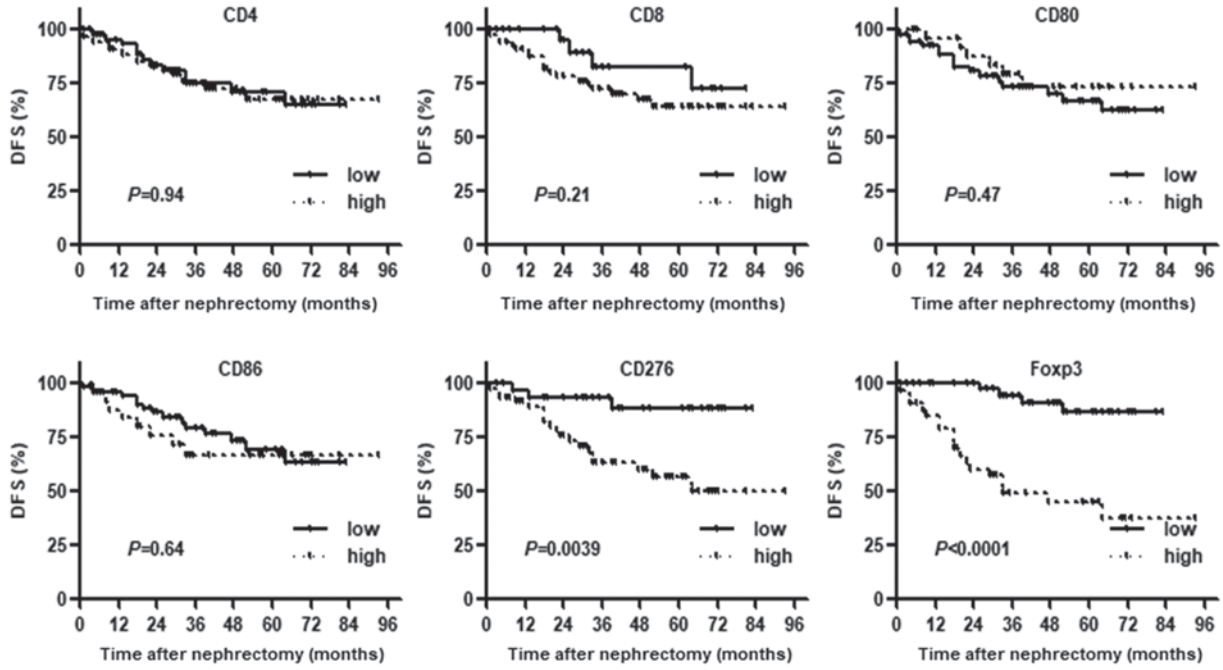

Figure 2. Kaplan-Meier curves for disease-free survival in all 87 cases. The patients with high counts of CD276$^+$ or Foxp3$^+$ tumor-infiltrating lymphocytes had a significantly higher risk of recurrence when compared with patients in the low group. CD, cluster of differentiation; Foxp3, Forkhead box p3; DFS, disease-free survival.

(950.030.096, Diaclone SAS, Besancon, France), human IL-10 (950.060.096, Diaclone SAS), human IL-17A (850.940.096, Diaclone SAS), TNF-α (950.090.096, Diaclone SAS), IFN-γ (950.000.096, Diaclone SAS), and TGF-β1 (DB100B, R&D Systems, Minneapolis, MN, USA). A microplate reader (Tecan Systems Inc., San Jose, CA, USA) was used to measure the absorbance at 450 nm.

Statistical analyses. The statistical analyses were performed with SPSS for Windows (version 20.0; IBM, Corp., Armonk, NY, USA). Figure plotting was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The correlations between the counts of TILs and clinicopathological characteristics were analyzed using the Mann-Whitney U test, Chi-square test, or Fisher's exact test as appropriate. The correlations between the counts of TILs and serum levels of cytokines were analyzed using the Mann-Whitney U test. Disease-free survival (DFS) was used as an endpoint, and Kaplan-Meier survival curves were plotted and compared using the log-rank test for univariate analysis. The Cox regression model was used for multivariate DFS analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The median follow-up period after radical nephrectomy for the DFS analysis was 39.9 months (range, 4-93 months). During the follow-up period, 22 patients (25.3%) experienced recurrence, which was defined as local recurrence or metastasis involving lymph node, bone, lung, or other sites. The median follow-up period after radical nephrectomy for the overall survival analysis was 48.8 months (range 6-100 months). During the follow-up period, 14 patients (16.1%) died.

Expression of TIL counts and clinical course. Patients were classified into low and high groups based on staining (Fig. 1). Univariate analysis with Kaplan-Meier curves and log-rank test analysis showed that patients with high counts of CD276$^+$ or Foxp3$^+$ TILs had a significantly higher risk of recurrence after nephrectomy compared with patients with low counts of CD276$^+$ or Foxp3$^+$ TILs. With regard to the other markers, there were no significant differences in DFS between the two groups (Fig. 2).

Correlation between CD276 or Foxp3 counts and clinicopathological variables. The baseline clinicopathological variables for the 87 cases and their association with the counts of CD276$^+$ or Foxp3$^+$ TILs are summarized in Table I. Patients with high counts of CD276$^+$ TIL had a significantly high pT stage and were linked with the presence of LVI compared to patients in the low group (Table I). Moreover, patients with high counts of the Foxp3$^+$ TIL had a significantly high pT stage and high SSIGN score compared to patients in the low group.

Prognostic factors of recurrence after radical nephrectomy. Cox univariate analyses showed that high pT stage, high SSIGN score, high counts of CD276$^+$ TILs, and high counts of Foxp3$^+$ TILs were factors for poor prognosis for recurrence (Table II). Multivariate analyses showed that high counts of the CD276$^+$ and Foxp3$^+$ TILs were independent factors for poor prognosis due to recurrence.

Correlation between TIL counts and preoperative serum level of cytokines. Based on the result of the

Table I. Characteristics of clear cell renal cell carcinoma patients in dependent of CD276 and Foxp3 expression.

		CD276			Foxp3		
Variables	Total n	Low	High	P-value	Low	High	P-value
n	87	36	51		53	34	
Sex				0.89[a]			0.92[a]
Male	67	28	39		41	26	
Female	20	8	12		12	8	
Age, median (range), years	67	68.5 (36-87)	66.0 (39-85)	0.96[b]	67 (36-87)	67.5 (42-85)	0.61[b]
MSKCC				0.60[a]			0.60[a]
Good	44	17	27		28		
Intermediate/poor	43	19	24		25	18	
pT category				0.0021[a]			0.01[a]
T1	32	21	11		26	6	
T2	3	1	2		2	1	
T3/4	52	14	38		25	27	
SSIGN score				0.065[a]			0.0083[a]
≤4	58	28	30		41	17	
>4	29	8	21		12	17	
LVI				0.0039[a]			0.91[a]
LVI-	42	24	18		33	9	
LVI+	45	12	33		20	25	

[a]Chi-square test or Fisher's exact test, [b]Mann-Whitney U test. MSKCC, Memorial Sloan Kettering Cancer Center; LVI, Lymphovascular invasion; CD, cluster of differentiation; Foxp3, Forkhead box p3.

immunohistochemical analysis, we focused on the association between CD276/Foxp3-positive lymphocytes and the circulating cytokines. Relationships with various cytokines have been reported in CD276/Foxp3-positive lymphocytes, where CD276 increases IFNγ secretion by activated T cells (15) as well as Foxp3 products IL-10, TGF-β, and TNFα (16). Furthermore, levels of IL6 and IL17, which were associated with Th17, were also assessed because the balance of Th17 and Treg can be skewed in patients with RCC (20). Therefore, putative cytokines including IL-6, IL-10, IL-17, IFN-γ, TNF-α, and TGF-β were selected and their levels in the preoperative serum were measured. Patients with high counts of CD276$^+$ TILs had significantly high serum levels of TNF-α and IFN-γ compared to patients in the low group. Patients with high counts of Foxp3$^+$ TILs had a significantly high serum level of TGF-β1 compared to patients in the low group (Table III).

Prognostic value of preoperative serum level of TNF-α, IFN-γ, and TGF-β1. To identify preoperative blood-based tests useful in predicting prognosis, the association of TNF-α, TGF-β1, and IFN-γ with prognosis was analyzed, since these three cytokines have been linked with the expression of CD276$^+$ or Foxp3$^+$ TILs. Kaplan-Meier analysis revealed that patients with high serum levels of TNF-α and TGF-β1 had a significantly higher risk of recurrence after nephrectomy compared to patients with low serum levels. There was no significant difference in IFN-γ between the two groups (Fig. 3).

Discussion

In this study, we found that high counts of CD276$^+$ and Foxp3$^+$ in tumor tissues were independent factors for the poor prognosis of visceral metastasis or local recurrence after radical nephrectomy for localized ccRCC. Moreover, we found that high preoperative serum levels of TNF-α and TGF-β1 were also factors for poor prognosis.

Foxp3 belongs to the transcription factor Forkhead box family and is a master gene of Treg differentiation and a specific transcription factor expressed in Tregs. Foxp3 exists in the tumor microenvironment and is a negative factor that controls antitumor immunity by disrupting T cell increase (10). It has also been reported that the expression of tumor-infiltrating Foxp3-positive lymphocytes was associated with poor prognosis in several cancers, including lung (21), breast (22), liver (23), pancreatic (24), ovarian (25), and kidney cancer (26).

CD276 is a member of the B7 family, which comprises cell-surface proteins that regulate immune responses by delivering co-stimulatory or co-inhibitory signals through their ligands. B7-H3 is one of the most recently described members of the B7 family, so its function and binding partners have not yet been elucidated. Tumor-infiltrating CD276-positive lymphocytes have been associated with poor prognosis in several cancers, including lung (27), colon (28), and kidney cancer (29).

In this study, high counts of Foxp3$^+$ TILs were associated with pT stage and SSIGN score, and high counts of CD276$^+$

Table II. Univariate and multivariate analyses for disease-free survival of clinicopathological variables in patients with clear cell renal cell carcinoma.

	Disease-free survival					
	Univariate			Multivariate		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Sex			0.93			
Male	1					
Female	0.96	0.36-2.58				
Age, years			0.73			
<70	1					
≥70	1.16	0.50-2.73				
MSKCC			0.26			
Good	1					
Intermediate/poor	1.69	0.68-4.18				
pT category			0.02			0.16
≤T2	1					
T3	2.74	1.17-6.42		0.32	0.069-1.54	
SSIGN score			0.0023			0.30
≤4	1					
>4	4.19	1.67-10.54		1.65	0.64-4.26	
LVI			0.13			
-	1					
+	1.92	0.83-4.44				
CD276 expression			0.0039			0.033
Low	1					
High	3.48	1.49-8.12		3.76	1.12-12.67	
Foxp3 expression			<0.0001			0.006
Low	1					
High	8.05	3.33-19.44		2.92	1.35-6.31	

HR, hazard ratio; CI, confidence interval; MSKCC, Memorial Sloan Kettering Cancer Center; LVI, Lymphovascular invasion; CD, cluster of differentiation; Foxp3, Forkhead box p3.

TILs were associated with pT stage and LVI. The SSIGN score is an outcome prediction model for patients with ccRCC treated with radical nephrectomy. The score is based on pathological stage, tumor size, nuclear grade, and necrosis (18). LVI, pT stage, and SSIGN score are important prognostic factors of RCC, but they cannot be evaluated preoperatively. Therefore, we focused on preoperative serum levels of several cytokines, which have been reported to be correlated with the recruitment of $CD276^+$ cells and $Foxp3^+$ cells.

The relationships between Tregs or CD276 and several cytokines have been reported previously. von Boehmer *et al* (30) reported that Tregs have several modes of suppressive action at their disposal that may depend on the microenvironment in which the suppressor cells are activated, and may be differentially used to suppress different forms of immunopathology. The secreted factors, such as IL-10 (an inhibitor for dendritic cells) and TGF-β1 (which directly act on T cells) participate in the suppressive action. B7-H3 reportedly costimulates the proliferation of both $CD4^+$ and $CD8^+$ T cells, enhances the induction of cytotoxic T cells, and selectively stimulates IFNγ production in the presence of T cell receptor signaling (14). In contrast, inclusion of antisense B7-H3 oligonucleotides decreases the expression of B7-H3 on dendritic cells and inhibits IFNγ production by dendritic cell-stimulated allogeneic T cells. The over-expression of B7-H3 and B7-H4 induce T cells to secrete TGF-β1 and the immunosuppressive cytokines IL-2, IL-6, and IL-17 (31). The authors concluded that TGF-β1 leads to T cell-mediated tumor evasion through the increased expression of B7-H3 and B7-H4.

In this study, high counts of $CD276^+$ TILs were linked with high levels of TNF-α and IFN γ in the preoperative serum, and high counts of $Foxp3^+$ TILs were linked with the preoperative high serum level of TGF-β1. These three cytokines were compared with the clinical course as candidate prognosis predictors. High serum levels of TNF-α and TGF-β1 were significantly correlated with the higher risk of recurrence.

Table III. Correlation between the peritumoral immune associated antigens (CD276 and Foxp3) and preoperative serum levels of cytokines.

	CD276			Foxp3		
Variables	Low	High	P-value	Low	High	P-value
Total n	36	51	-	53	34	-
TNFα, median (range)	0.024 (0.016-0.072)	0.058 (0.016-0.49)	0.03	0.049 (0.016-0.49)	0.036 (0.016-0.16)	0.19
TGFβ, median (range)	0.066 (0.15-1.19)	0.065 (0.098-1.18)	0.57	0.61 (0.15-1.18)	0.71 (0.098-1.19)	0.02
IFNγ, median (range)	0.031 (0.018-0.070)	0.034 (0.015-0.082)	0.04	0.031 (0.015-0.078)	0.034 (0.018-0.082)	0.32
IL-6, median (range)	0.71 (0.071-3.80)	0.66 (0.071-3.82)	0.23	0.66 (0.071-3.82)	0.71 (0.073-3.80)	0.38
IL-10, median (range)	0.11 (0.023-0.88)	0.018 (0.019-3.33)	0.39	0.11 (0.019-1.43)	0.20 (0.023-3.33)	0.18
IL-17, median (range)	0.11 (0.025-1.28)	0.12 (0.025-1.06)	0.82	0.090 (0.025-0.80)	0.15 (0.025-1.28)	0.95

Mann-Whitney U test, with units of pg/ml. TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon; IL, interleukin.

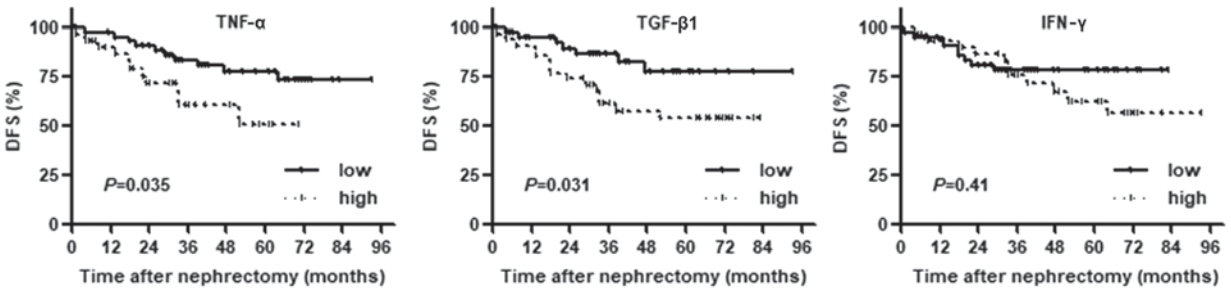

Figure 3. Kaplan-Meier curves for disease-free survival in all 87 cases. Patients with high serum levels of TNF-α and TGF-β1 had a significantly higher risk of recurrence than patients with low serum levels. TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon; DFS, disease-free survival.

One possible scenario is that tumor cells increase the production of TNF-α and TGF-β1 to help tumor cells progress. Tumor cells may increase the expression of B7-H3 and promote differentiation from T cells to Tregs. As a result, the production of TNF-α and TGF-β1 are increased, which may support the immune escape and progression of tumor cells.

Moreover, based on the present results, it can be suggested that in patients with high serum levels of TNF-α and TGF-β1, the topical immunoreaction in the tumor site might have some kind of influence on systemic immunoreactions preoperatively, leading to poor prognosis in patients. Thus, preoperative serum levels of TNF-α and TGF-β could be good candidate risk stratification biomarkers of localized ccRCC.

Some limitations exist in this study. First, this study was a retrospective design, had a relatively small number of cases, and the follow-up period was short. The findings need further validation in forthcoming studies in prospective controlled large sampled clinical trials. Secondly, we described one possible scenario for the progression of tumor cells, but we did not inspect these results in animal or cell experiments. In addition, other types of TILs and cytokines were not evaluated. Further studies should inspect the processes via *in vivo* or *in vitro* studies, and evaluate other types of TILs and cytokines.

In conclusion, our findings have demonstrated the possible association of topical intratumoral immunoreaction and systemic immune status in patients with localized RCC. The topical induction of the $CD276^+$ and $Foxp3^+$ TILs was suggested to be linked with high levels of serum TNF-α and IFN-γ. Preoperative serum levels of TNF-α and TGF-β could be simple and non-invasive biomarkers for risk stratification before radical surgery.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

KI, MM and KF contributed to the design of study and writing of the manuscript. KO, SH, YM, DG, YI, and SO conducted the molecular biology studies. YN, SA and NT performed the statistical tests. MM and KF assisted with the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Nara Medical University (Nara, China) approved this protocol. (Project identification code:

1630, accepted: August 21, 2017). All subjects gave their written informed consent for inclusion before they participated in the study.

Patient consent for publication

All subjects gave their informed consent for inclusion before they participated in the study.

Competing interests

The authors declare that they have no competing interests.

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