

**Original contribution**

Pathological significance and prognostic roles of densities of CD57+ cells, CD68+ cells, and mast cells, and their ratios in clear cell renal cell carcinoma^{☆,☆☆}



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Summary The immune system is closely associated with malignant behavior in renal cell carcinoma (RCC). Therefore, understanding the pathological roles of immune cells in tumor stroma is essential to discuss the pathological characteristics of RCC. In this study, the clinical significance of densities of CD57+ cells, CD68+ cells, and mast cells, and their ratios were investigated in patients with clear cell RCC. The densities of CD57+, CD68+, and mast cells were evaluated by immunohistochemical techniques in 179 patients. Proliferation index, apoptotic index, and microvessel density were evaluated by using anti-Ki-67, anti-cleaved caspase-3, and anti-CD31 antibodies, respectively. The density of CD57+ cell was negatively correlated with grade, pT stage, and metastasis, although densities of CD68+ cell and mast cell were positively correlated. Ratios of CD68+ cell/CD57+ cell and mast cell/CD57+ cell were significantly correlated with grade, pT stage, and metastasis. Survival analyses showed that the CD68+ cell/CD57+ cell ratio was a significant predictor for cause-specific survival by multivariate analyses (hazard ratio = 1.41, 95% confidence interval = 1.03–1.93, $P = .031$) and was significantly correlated with proliferation index, apoptotic index, and microvessel density ($r = .47, P < .001$; $r = -.31, P < .001$; and $r = .40, P < .001$, respectively). In conclusion, CD57+ cells, CD68+ cells, and mast cells played important roles in malignancy in clear cell RCC. The CD68+ cell/CD57+ cell ratio was strongly correlated with pathological features and prognosis in these patients because this ratio reflected the status of cancer cell proliferation, apoptosis, and angiogenesis.

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1. Introduction

Renal cell carcinoma (RCC) is one of the major urological cancers; immunological function affects the malignant behavior of RCC cells. RCC is known to have high metastatic potential, and prognosis of patients with metastatic RCC is poor despite the development of various types of anticancer drugs [1]. On the other

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hand, if curative surgery is performed for nonmetastasis patients, 20%-30% suffer relapse or metastasis [2]. Therefore, more detailed information on the pathological mechanisms of tumor growth and progression in RCC is essential to explore treatment and observation strategies. In recent years, many investigators have suggested that interactions between cancer cells and the surrounding stromal microenvironment play important roles in the malignant aggressiveness in many types of solid tumors, including RCC. Previous studies have shown that fibroblasts, endothelial cells, and tumor-infiltrating cells (TICs) exist in stromal tissues, and TICs are composed of various cells of the hematopoietic system including lymphocytes, mast cells, and macrophages. In addition, these stromal cells act as oncogenic factors by complex mechanisms via regulation of cancer cell growth and angiogenesis [3,4]. In fact, the pathological characteristics of RCC are closely dependent on the immune system, and various immunotherapies are useful in patients with RCC [5]. Currently, the use of novel immune checkpoint inhibitors can result in improved survival compared to that with molecular targeted therapy [6]. Thus, understanding the pathological roles of immune cells in tumor stroma is essential to discuss the observations and therapeutic strategies in patients with RCC.

In the present study, we focused on the pathological roles of TICs, such as CD57+ cells (commonly recognized as natural killer [NK] cells) [7-11], CD68+ cells (known as *macrophages*) [10-15], and mast cells, in human clear cell RCC tissues. In addition, the relationships between their densities and cancer cell proliferation, apoptosis, and angiogenesis were analyzed.

2. Materials and methods

2.1. Patients

Formalin-fixed and paraffin-embedded sections were obtained from surgical specimens of 179 clear cell RCC patients in Nagasaki University Hospital. Although consecutive specimens were examined, some specimens were excluded because of low cancer cell numbers following previous investigations. We also excluded patients who received neoadjuvant therapy. In the study population, all patients were those who had received some molecular targeted therapies; however, no patient was treated with immune checkpoint inhibitors. All patients were evaluated by chest radiograph, computed tomography, and bone scanning. The tumor-node-metastasis system was used for staging, with the grade determined using the criteria of Fuhrman et al [16]. The median (interquartile range) follow-up period was 39 (19-62) months. The study protocol was approved by the Human Ethics Review Committee of Nagasaki University Hospital.

2.2. Immunohistochemistry

For immunohistochemical staining, the antibodies used were anti-Ki-67 antibody (Dako Corp, Glostrup, Denmark),

anti-CD57 antibody (Lab Vision Corp, Fremont, CA), anti-mast cell tryptase antibody (NeoMarkers, Fremont, CA), and anti-CD31 and anti-CD68 antibody (Leica Biosystems, New Castle, United Kingdom). In situ labeling for apoptosis was also performed to detect the apoptotic

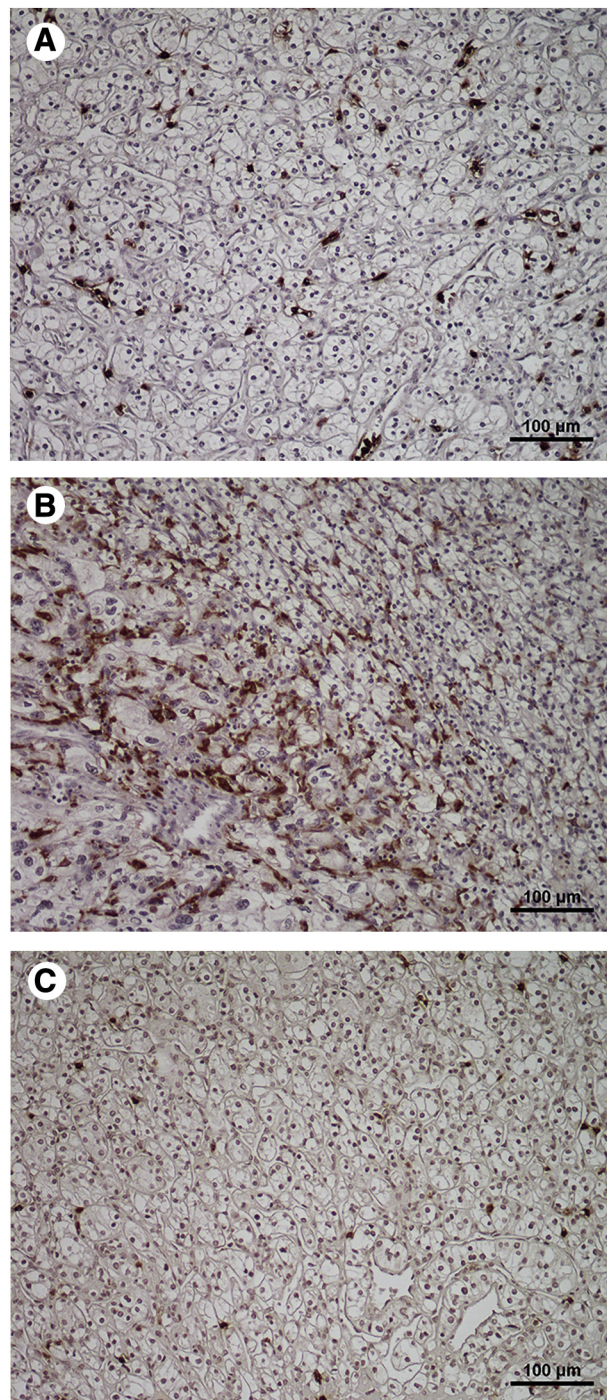


Fig. 1 Representative images of CD57+ cells (A), CD68+ cells (B), and mast cells (C) in human clear cell renal cell cancer tissues (original magnification $\times 200$). These cells were easily detected in the intratumoral area because they strongly and clearly stained in all tumor tissues.

cells. The immunohistochemical staining and terminal transferase dUTP nick end labeling (TUNEL) assays were performed according to our previous reports [17-19]. Briefly, 5- μ m-thick sections were deparaffinized stepwise, and antigen retrieval was performed at 95°C for 40 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0), except for the anti-Ki-67 antibody (121°C for 15 minutes in the same buffer). All sections were then immersed in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. The sections were then incubated overnight with primary antibodies at 4°C. The sections were then incubated with peroxidase using the labeled polymer method with Dako EnVision + Peroxidase (Dako Corp, Carpinteria, CA) for 60 minutes. The peroxidase reaction was visualized using the liquid 3,3'-diaminobenzidine tetrahydrochloride substrate kit. The

sections were then counterstained with hematoxylin. A variety of specimens that had been confirmed to be immunoreactive for the relevant antigens in preliminary studies were used as positive controls for Ki-67, CD57, CD68, and mast cell tryptase (tonsil), and CD31 (kidney). On the other hand, for the TUNEL method, the ApopTag In Situ Apoptosis Detection Kit (Intergen Company, Purchase, NY) was used, and positive and negative control sections were prepared as described by the manufacturer.

2.3. Evaluation

Expression of all molecules was assessed semiquantitatively, taking into account the percentage of positively stained cancer cells in 200 high-power fields (HPFs).

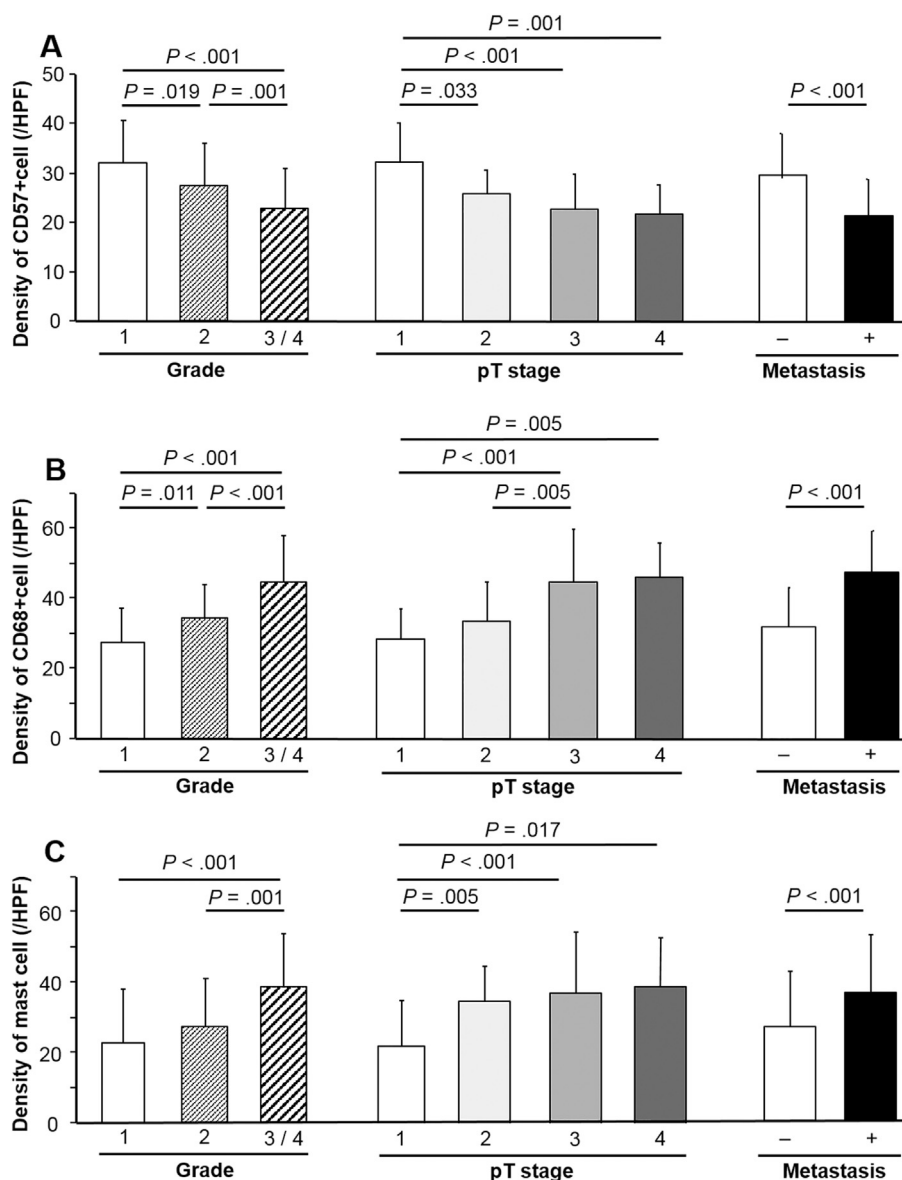


Fig. 2 A, The density of CD57+ cells was negatively associated with grade, pT stage, and status of metastasis. B and C, In contrast, densities of CD68+ cells and mast cells were positively associated with these clinicopathological features.

Similarly, the densities of the positively stained vessels, nuclear, and TICs were examined at 5-10 representative hot spot fields of view in HPFs within the tumor area for control of heterogeneity. The proliferative index (PI) represented the percentage of Ki-67-positive cells. The apoptotic index (AI) was estimated by the percentage of TUNEL-positive cells. The number of positively stained vessels per square millimeter defined the microvessel density (MVD). The number of TICs positively stained in the cytoplasm and cell membrane per HPF defined the densities of CD57, CD68, and mast cells in tryptase-stained cells. We used a computer-aided image analysis system (Win ROOF, version 5.0; MITANI Corp, Fukui, Japan) to calculate the statistical variables.

2.4. Statistical analyses

Data are expressed as means and SD, unless otherwise stated. The Student *t* test was used to compare the continuous variables. The Scheffé method was used for multiple comparisons of the data. Survival analyses were performed using the Cox proportional-hazards model, and the results were described as hazard ratios (HRs) with the 95% confidence interval (CI) and *P*. Pearson correlation was used to evaluate the relationships between continuous variables and the correlation coefficient (*r*). Spearman rank correlation coefficient was calculated to confirm Pearson correlation. All statistical analyses were 2-sided, and the significance was defined as *P* < .05. Finally, all statistical analyses were performed by the statistical

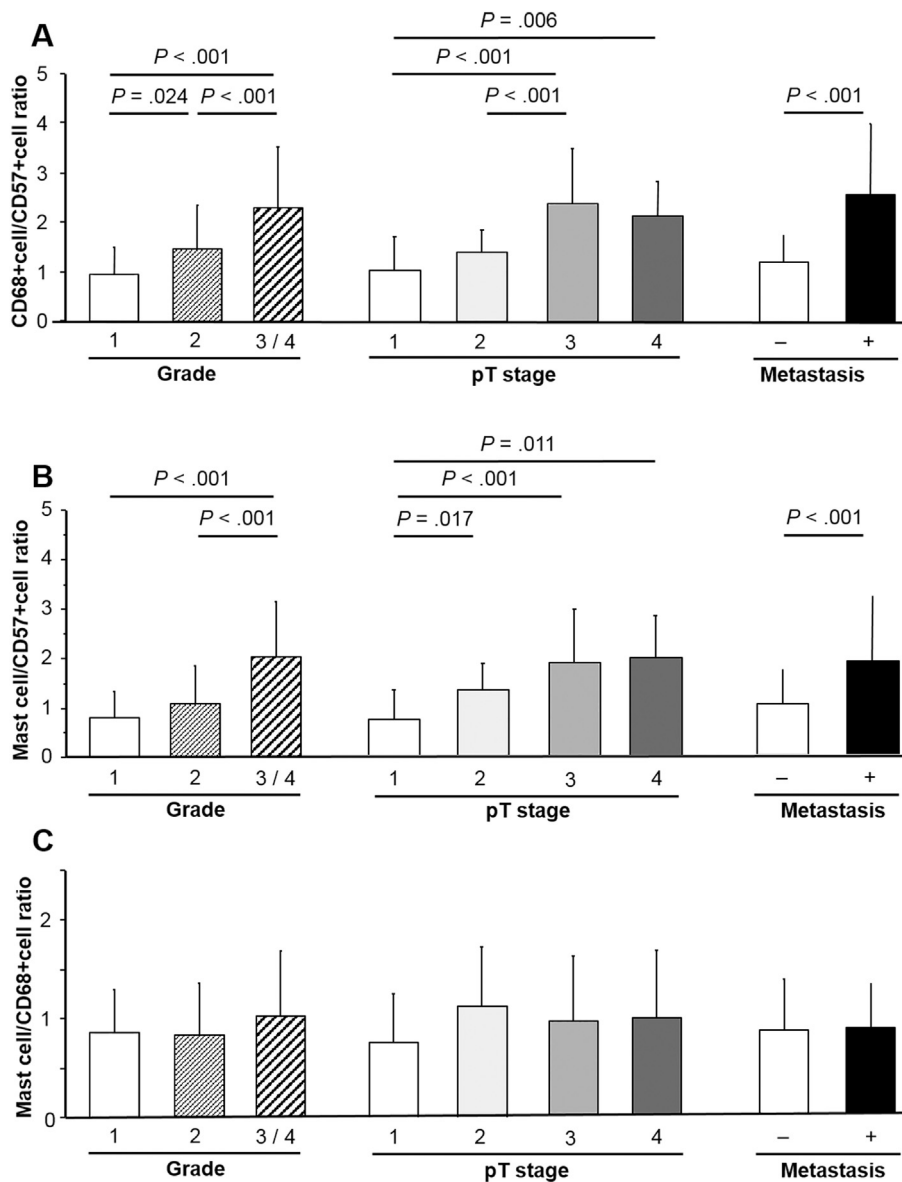


Fig. 3 A, The CD68+ cell/CD57+ cell ratio was positively associated with grade, pT stage, and status of metastasis. Similar results were detected in the mast cell/CD57+ cell ratio (B) but not in the mast cell/CD68+ cell ratio (C).

package StatView for Windows (version 5.0; Abacus Concept, Inc, Berkeley, CA).

3. Results

3.1. Densities of CD57+ cells, CD68+ cells, and mast cells

Densities of CD57+ cells, CD68+ cells, and mast cells were 28.1 (9.5), 34.5 (13.6), and 28.4 (16.8), respectively. The representative images of these TICs are shown in Fig. 1. With regard to clinicopathological features, the density of CD57+ cells was negatively associated with grade, pT stage, and presence of metastasis (Fig. 2A). In contrast, densities of CD68+ cells and mast cells were positively associated with these clinicopathological features (Fig. 2B and C). Next, upon analysis, we found that the ratios of CD68+ cells/57+ cells and mast cells/CD57+ cells were significantly associated with grade, pT stage, and status of metastasis (Fig. 3A and B). However, such a significant relationship was not found between the pathological features and mast cell/CD68+ cell ratio (Fig. 3C).

3.2. Survival analyses

As shown in Table 1, univariate analyses showed that the CD57+ cell density was negatively associated with shorter cause-specific survival (CSS) periods (HR = .93, $P < .001$). In contrast, densities of CD68+ cells and mast cells were identified as worse predictors for survival (HR = 1.06, $P < .001$ and HR = 1.02, $P = .007$, respectively; Table 1). However, these densities were not identified as independent predictors for CSS in a multivariate analysis model including grade, pT stage, and metastatic status (Table 1).

On the other hand, the ratios of CD68+ cells/CD57+ cells and mast cells/CD57+ cells were significantly associated with CSS in univariate analysis (HR = 1.90, $P < .001$ and HR = 1.83, $P < .001$, respectively), whereas the mast cell/CD68+

Table 2 Correlation with cell proliferation-, apoptosis-, and angiogenesis-related parameters

	PI		AI		MVD	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Density						
CD57+ cell	-0.27	<.001	0.29	<.001	-0.24	.001
CD68+ cell	0.46	<.001	-0.20	.007	0.31	<.001
Mast cell	0.11	.141	-0.07	.372	0.18	.015
Ratio						
CD68+/CD57+	0.47	<.001	-0.31	<.001	0.40	<.001
Mast cell/CD57+ cell	0.25	<.001	-0.20	.008	0.30	<.001
Mast cell/CD68+ cell	-0.16	.060	0.07	.358	0.02	.788

cell ratio was not (Table 1). In addition, multivariate analysis demonstrated that the CD68+ cell/CD57+ cell ratio was a significant and independent predictor (Table 1; HR = 1.41, $P = .031$).

3.3. Correlation with cell survival and angiogenesis

Correlations between TIC-based parameters and cancer cell proliferation, apoptosis, and angiogenesis are shown in Table 2. PI was significantly correlated with densities of CD57+ cells ($P < .001$) and CD68+ cells ($P < .001$) and ratios of CD57+ cells/CD68+ cells ($P < .001$) (Table 2). However, *r* values of the CD57+ cell density and mast cell/CD57+ cell ratio were relatively low (<3.0). Similar results were obtained with regard to AI, and *r* values for the CD57+ cell density, CD68+ cell density, and the mast cell/CD57+ cell ratios were 3.0 or less (Table 2). On the other hand, except for the mast cell/CD68+ cell ratio, all parameters were significantly associated with MVD. However, *r* values for CD57+ cell density and mast cell density were less than 0.3. Thus, only the CD68+ cell/CD57+ cell ratio was significantly correlated with PI, AI, and MVD, with a relatively high *r* value (>3.0).

Table 1 Survival analyses for CSS

	Univariate analyses			Multivariate analyses ^a		
	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Density						
CD57+ cells	0.93	0.90-0.96	<.001	0.96	0.92-1.00	.063
CD68+ cells	1.06	1.04-1.08	<.001	1.00	0.98-1.02	.913
Mast cells	1.02	1.01-1.04	.007	0.98	0.96-1.01	.187
Ratio						
CD68+ cell/CD57+ cell	1.90	1.55-2.30	<.001	1.41	1.03-1.93	.031
Mast cell/CD57+ cell	1.83	1.44-2.34	<.001	1.10	0.75-1.60	.638
Mast cell/CD68+ cell	1.05	0.65-1.69	.836	0.90	0.54-1.49	.694

^a Adjusted by grade, pT stage, and metastasis.

4. Discussion

The present study showed that CD57+ cell density acts as an antitumor factor for tumor progression and prognosis in patients with clear cell RCC. In contrast, high densities of CD68+ cells and mast cells were associated with high grade, high stage, and worse survival in these patients. As mentioned above, in various cancer tissues, CD57 and CD68 were recognized as markers of NK cells and macrophages, respectively [7-15]. Many reports showed that NK cell density was negatively associated with malignant aggressiveness and that elevated CD57+ NK cell levels predicted favorable prognosis in patients with various malignancies [8,10,11]. In contrast, CD68+ macrophages were reported to be positively associated with grade, tumor growth, and progression, and the high density of CD68+ macrophages was recognized as a predictor of worse prognosis in several cancers [20,21]. Similarly, mast cells have also been reported to participate in tumor development and lead to an unfavorable outcome in a variety of cancers [22,23]. In patients with metastatic RCC, a similar negative relationship was detected in between CD57+ cell and grade [9], and the low density of intratumoral CD57+ NK cells was identified as a predictor of worse overall survival by multivariate analyses [7]. Several reports have shown that the increased density of CD68+ macrophages is associated with tumor progression and poor survival of RCC patients [3,24]. Thus, these previous reports support our results. Currently, the pathological significance and prognostic roles of mast cells in patients with RCC are controversial. In contrast to our results, several investigators have reported that mast cell density was negatively correlated with grade and stage [4,25]. In addition, several reports have shown that mast cell density was not correlated with these pathological features [26,27]. Furthermore, in survival analyses, the density of mast cells is reported to be negatively associated with recurrence-free survival and CSS in RCC patients [4]; however, other investigators have shown that the mast cell number was not associated with survival [26]. Thus, more detailed studies are necessary to elucidate the pathological significance and prognostic role of mast cells in patients with RCC.

In our study population, CD68+ cells had procarcinogenic activities and CD57+ cells acted as antitumor factors. Therefore, the CD68+ cell/CD57+ cell ratio was speculated to reflect more accurate pathological functions compared to the density of CD57+ cells or CD68+ cells alone. In fact, a significant relationship between the combination pattern of CD68+ cells and CD57+ cells and outcome was reported in colorectal cancer [28]. In addition, the pathological significance of the ratio of various immune cells, such as macrophages and lymphocytes, was studied in various cancer tissues [9,29]. Therefore, we emphasize that more detailed analyses of the ratio of immune cells in RCC tissues are important to discuss the pathological characteristics in these patients.

Our results showed that the densities of CD57+ and CD68+ cell were significantly associated with cancer cell proliferation, apoptosis, and angiogenesis. In addition, mast cell density was

positively associated with angiogenesis. However, unfortunately, their correlation coefficients were relatively low, except for the correlation between PI and CD68+ cell density ($r = .46$). On the other hand, the CD68+ cell/CD57+ cell ratio was significantly correlated with all cancer-related parameters with relatively high r values (with PI = .47, AI = -.31, MVD = .40). CD68+ macrophages are reported to exert tumor-promoting functions by the stimulation of cancer cell proliferation in many types of cancers, including RCC [3,16,17]. In addition to cell proliferation, the density of CD68+ macrophages was positively correlated with MVD in various types of cancers, including RCC [12-15]. Furthermore, CD57+ NK cells were negatively correlated with tumor proliferation and positively correlated with apoptosis in patients with hepatocellular carcinoma [30]. These findings supported that the CD68+ cell/CD57+ cell ratio could reflect such malignant aggressiveness. However, there is no general agreement regarding the relationship between apoptosis and the density of CD68+ cells in cancer tissues. In short, although there is a report that CD68+ macrophages promoted the apoptosis of colon cancer cells [31], significant proapoptotic activity was not found in several cancers [12,13]. Furthermore, the relationship between CD57+ cells and angiogenesis in malignancies is not fully understood.

The major limitation of this study is that CD68 was used for the detection of macrophages. In general, CD68 is recognized as a general macrophage marker. On the other hand, CD163, CD204, and CD206 (macrophage mannose receptor) were suggested as a marker of type 2 macrophages, which are widely considered to be the class of activated macrophages [32,33]. Thus, there is a possibility that the marker used affects the detection of macrophages. On the other hand, it has been reported that the number of CD68+ tumor-associated macrophages (TAMs) was significantly correlated to the number of CD163+ and CD204+ TAMs in clear cell RCC tissues [33]. In addition, other investigators reported that TAMs isolated from RCC tissues showed the expression of CD68 and CD163, but not CD206 [34]. Another limitation is that therapeutic strategy such as drugs and courses is not consistent.

In conclusion, CD57+ cells, CD68+ cells, and mast cells were speculated to play important roles in tumor growth, progression, and malignant aggressiveness in RCC tissues. In particular, the CD68+ cell/CD57+ cell ratio is closely associated with these malignant behaviors in patients with RCC. We propose stimulated cancer cell proliferation and angiogenesis and suppressed apoptosis as a mechanism for these pathological roles. Although our results may currently not be practical to adopt in clinical practice, we believe that they can be useful to understand the comprehensive immune system within cancer tissues.

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