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Tumor-infiltrating PD1-Positive Lymphocytes and FoxP3-Positive Regulatory T Cells Predict Distant Metastatic Relapse and Survival of Clear Cell Renal Cell Carcinoma[1]

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Abstract

BACKGROUND: Clear cell renal cell carcinoma (CRCC) is the most common malignant tumor of the kidney, and the clinical outcome of CRCC is related with the metastatic potential of CRCC. A significant proportion of metastatic CRCC remains incurable. Recently, immunotherapy against specific targets such as programmed death 1 (PD1) has been adapted for fatal cases of CRCC. *MATERIALS AND METHODS:* In this study, we aimed to evaluate the potential of tumor-infiltrating PD1-positive lymphocytes or FoxP3-positive regulatory T cells (Tregs) as predictors of the metastatic potential or prognosis of CRCC and investigate possible correlations with Epstein-Barr virus (EBV) infection in 199 cases of CRCC. *RESULTS:* PD1 positivity, high Treg number, and EBV infection all predicted poor overall survival (OS) by univariate analysis. PD1 positivity and high Treg numbers were also significantly correlated with more distant metastatic relapse (DMR) and poor relapse-free survival (RFS) by univariate analysis. PD1 positivity and high Treg number were independent prognostic indicators for OS. In addition, PD1 positivity was an independent predictor of RFS and DMR. EBV infection was an independent predictor of OS of CRCC. *CONCLUSION:* This study demonstrates that intratumoral infiltration of PD1-positive or FoxP3-positive lymphocytes can be used as significant prognostic indicators of CRCC and PD1 positivity could be very helpful in the prediction of latent distant metastasis of CRCCs. Therefore, evaluation of the infiltration of PD-positive cells or Tregs in CRCC may be useful diagnostic tools for the selection of patients who could benefit from PD1- or Treg-based immunotherapy.

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Introduction

Renal cell carcinoma (RCC) is the most common type of malignant tumor of the kidney. RCC represents about 3% of human malignant tumors [1]. Most RCCs are treated by radical nephrectomy [2]. However, about 20% to 30% of localized RCCs develop latent metastatic progression after surgical treatment, which is closely correlated with a fatal course of RCC [3]. The 5-year survival for RCC with metastatic disease is less than 10% [1,4]. However, RCC is a very unpredictable tumor for distant metastasis. Therefore, it is important to find the factors that could predict metastasis of RCC and to explore new treatment modalities for these cases. For the treatment of fatal cases of RCC,

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immunotherapy with interleukin-2 and interferon-α has been adapted. However, despite intensive immunotherapy trials for advanced RCC, targeted agents rarely induced complete response [5]. Therefore, there is a great need for novel therapeutic approaches for advanced RCC; of these novel approaches, targeted immunotherapy shows great promise and is an area of active research and increased interest.

Programmed death 1 (PD1) is a member of the CD28 receptor family and negatively regulates T cell proliferation and function [6,7]. PD1 attenuates immune responses by decreasing induction of various cytokines [8,9]. Therefore, it is suggested that the presence of PD1-positive lymphocytes could be involved in the immune evasion of tumor cells. The infiltration of PD1-positive lymphocytes as a poor prognostic indicator of human malignant tumors has been suggested in follicular lymphoma [10] and clear cell RCC (CRCC) [11]. In CRCC, the infiltration of PD1-positive immune cell was seen in 29% (77 of 267) of CRCCs and significantly correlated with the progression of tumor and fatal outcome of CRCC [11]. On the basis of the prognostic impact of the infiltration of PD1-positive cells in human cancers, PD1 has been put forth as a novel target of immunotherapy of RCC [12,13].

Regulatory T cells (Tregs) are a population of immune cells related with immune suppression [14]. Principally, Tregs are immunosuppressive and can inhibit autoimmunity as well as antitumor responses [14,15]. Treg-mediated immune suppression has been used in the treatment of autoimmune disease. However, the immunosuppressive effects of Treg could result in adverse effects on tumor biology [16,17]. Because tumor cells also express self-antigen [18], Treg could be involved in the suppression of antitumor immune responses [19]. Possibly, Treg-related immunosuppressive function could be mediated by blocking the function of T cells and antigen-presenting cells [14,15]. The involvement of Treg in tumor biology has been suggested by high numbers of Treg in human malignant tumor. Peripheral blood from RCC patients showed higher numbers of Treg than healthy individuals [20]. High intratumoral Treg counts are related to poor prognosis of various human cancers, such as RCC [20], breast carcinoma [21], gastric carcinoma [17], and diffuse large B cell lymphoma [22].

Epstein-Barr virus (EBV) is a ubiquitous herpes virus in humans that is usually apathogenic, yet it is also associated with a number of malignant diseases, including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's lymphoma [23]. EBV infection in RCC is also highly variable between reports ranging from 6.8% to 89% in different studies [24,25]. Shimakage et al. reported that eight of nine cases of RCC show EBV expression [25]. In contrast, only 5 of 73 cases of RCCs showed EBV infection in another report [24]. Interestingly, the expansion of functional Treg has been reported in EBV-related human malignant tumors, such as nasopharyngeal carcinoma [26] and Hodgkin's lymphoma [27]. In EBV-infected Hodgkin's lymphoma, EBV could promote intratumoral migration of Treg by upregulating chemokine CCL20, resulting in tumor progression [28]. The possibility that Treg has an important role in the maintenance of EBV latent infection and that EBV infection induces recruitment of immunosuppressive Treg at tumor sites has been suggested [29].

In this study, we evaluated the impact of tumor-infiltrating PD1-positive lymphocytes for the prediction of distant metastasis and prognosis of CRCC. In addition, because the expression of PD1 in immune cells is related with the function or expansion of Treg, we also evaluated the impact of intratumoral Treg on the metastatic potential of CRCC and its association with EBV infection in CRCCs.

Materials and Methods

Patients and Samples

One hundred ninety-nine cases of CRCC patients diagnosed between July 1998 and August 2011, for whom initial diagnostic hematoxylin and eosin–stained slides and paraffin-embedded tissue blocks were available, were included in the present study. This study obtained Institutional Review Board approval. Informed consent was provided according to the Declaration of Helsinki. All of the cases were reviewed and reclassified according to the criteria of the World Health Organization Classification [30]. Pathologic staging was reviewed on the basis of the tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer [31]. The patients were grouped according to their sex, age (<55 *vs* ≥55), TNM stage (I *vs* II, III, and IV), presence of lymph node (LN) metastasis, histologic nuclear grade (1 *vs* 2 *vs* 3 and 4) [32], and histologic tumor necrosis. All of histologic findings were retrospectively reviewed by two pathologists without clinicopathologic information.

Immunohistochemical Staining and Scoring

Immunohistochemistry was performed using 3.0-mm tumor cores for tissue microarray. The tissue cores (one arrayed per case) were obtained from the most solid area of the tumor and were composed primarily of intact tumor cells. The tissue sections were treated with a microwave antigen retrieval procedure in sodium citrate buffer for 12 minutes. Antibodies for FoxP3 (1:50, clone 236A/E7; eBioscience, San Diego, CA) and PD1 (1:50, clone NAT; Abcam, Cambridge, United Kingdom) were used. Immunohistochemical analysis was performed without knowledge of the clinicopathologic information. Each case was evaluated to quantify the number of tumor-infiltrating FoxP3-positive Tregs and PD1-positive cells. The number of tumor-infiltrating FoxP3-positive Tregs and PD1-positive cells was counted at highest numbered five high-power fields. The counting was performed under a Nikon ECLIPSE 50i light microscope (Nikon, Tokyo, Japan) with a ×10 eyepiece with 22-mm field of view (Nikon) and ×40 objective lens (Plan Flour ×40/0.75; Nikon). The field size was 0.55 mm, and the area of one high power field (HPF) was 0.238 mm^2. Finally, the total area analyzed per case was 1.188 mm^2.

In Situ *Hybridization*

To determine the localization of EBV in RCCs, we performed EBV-encoded small RNA (EBER) *in situ* hybridization using a fluorescein-conjugated EBV probe for detection of EBER transcripts (NCL-EBV-K; Novocastra, Newcastle upon Tyne, United Kingdom). The negative control section was processed identically to the above except that the EBV probe solution was replaced by a negative control probe solution, which was provided in the kit. A specimen from a patient with known EBV-positive nasal NK/T cell lymphoma was used as a positive control. Because EBER could present in both nuclei and cytoplasm of infected cells [33], both nuclear and cytoplasmic staining for EBER were considered as positive staining. EBER *in situ* hybridization was considered positive if more than one cell was stained.

Statistical Analysis

After counting the number of FoxP3-positive Tregs and PD1-positive cells, we classified the patients into low *versus* high Treg subgroups and negative *versus* positive PD1 subgroups. The cutoff numbers for FoxP3-positive Tregs and PD1-positive cells were determined by receiver

operating characteristic curve analysis at the highest positive likelihood ratio point. The cutoff points were nine FoxP3-positive Tregs in five HPFs and one PD1-positive cell in five HPFs. More than nine Tregs in five HPFs were included in the high Treg subgroup and more than one PD1-positive cell in five HPFs were included in the PD1-positive subgroup. One hundred eleven cases of CRCC were placed in the high Treg subgroup, and 868 cases were placed in the PD1-positive subgroup. Pearson chi-squared test, two-sided *t* test, and one-way analysis of variance were used to analyze the association between the numbers of tumor-infiltrating FoxP3-positive Tregs and PD1-positive cells, EBV infection, and the variable clinicopathologic factors.

The end points of interest were distant metastatic relapse (DMR), relapse-free survival (RFS), and overall survival (OS). The follow-up end point was the date of last contact or death through December 2011. OS was measured from the date of diagnosis to the date of death or last contact. Patients who were alive at last contact were treated as censored for OS analysis. RFS was calculated as the time from diagnosis to the date of relapse, death, or last contact. Patients who were alive at last contact and who did not experience relapse were treated as censored for RFS analysis. DMR was calculated from the time of diagnosis to the date of relapse at a distant metastatic site. Patients who were alive at last contact or died were treated as censored for DMR analysis. Univariate and multivariate Cox proportional hazards regression analyses were performed to estimate the impact of clinicopathologic factors, EBV infection, Treg infiltration, and the number of PD1-positive cells on OS, RFS, and DMR. Kaplan-Meier survival curves were constructed to further illustrate the impact of OS and RFS when indicated. Statistical analysis was performed by using SPSS software (version 18.0). *P* values less than .05 were considered statistically significant.

Results

Association of PD1-Positive Cells, FoxP3-Positive Tregs, and EBV Infection with Clinicopathologic Characteristics of CRCC

Immunoreactivity for PD1 was found primarily in the cytoplasm of the cells (Figure 1, *A* and *B*) and FoxP3 was expressed in the nuclei of the cells (Figure 1, *C* and *D*). Positive signals for *in situ* hybridization

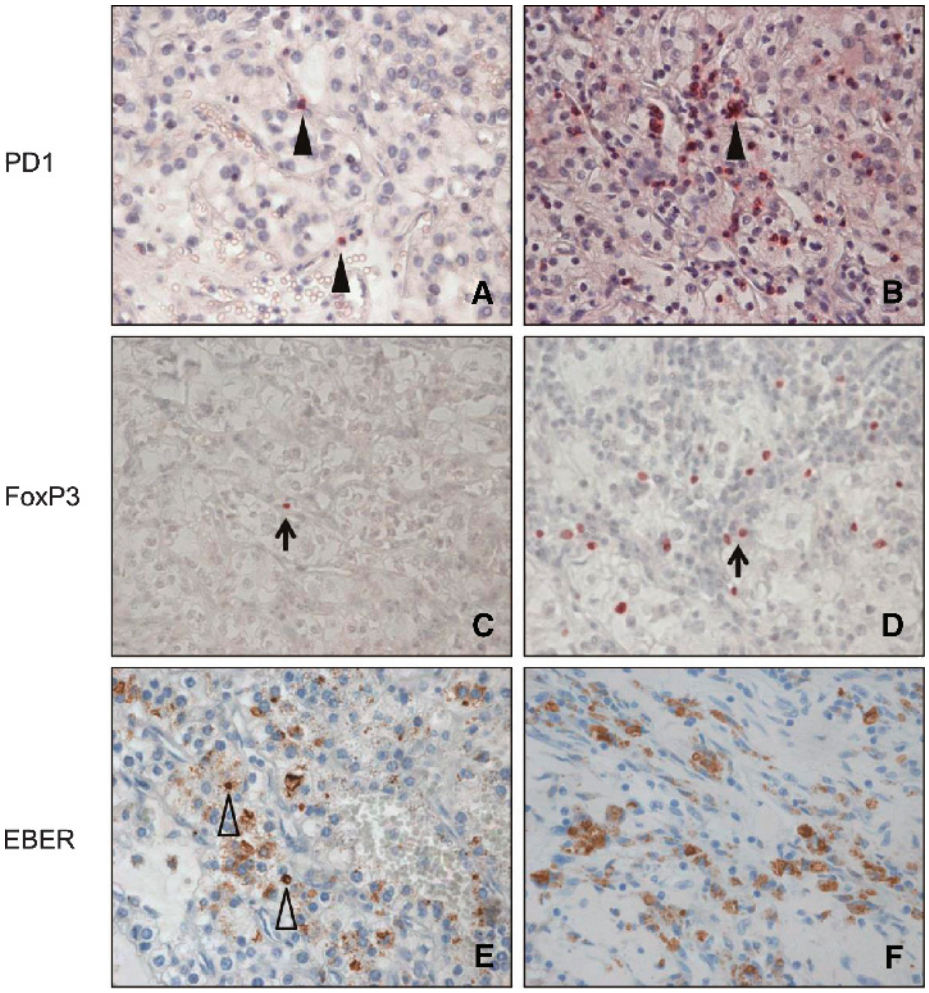

Figure 1. Immunohistochemical staining for FoxP3 and PD1 and *in situ* hybridization for EBER in CRCC. (A and B) PD1-positive cells (arrowheads) in the CRCC. (C and D) Cases with low numbers of FoxP3-positive Tregs (C) and high numbers of FoxP3-positive Tregs (D). Arrows indicate FoxP3-positive Tregs. (E) A positive signal for EBER appears in the nuclei (empty arrowheads) and cytoplasm of tumor cells. (F) Tumor-infiltrating inflammatory cells also stain positive for EBER. The representative field area of one microscopic image for PD1 and FoxP3 immunostaining is 0.086 mm^2. Original magnification, ×400.

Table 1. Clinicopathologic Variables, Intratumoral Infiltration of PD1-Positive Lymphocytes and FoxP3-Positive Tregs, and EBV Infection in CRCC.

Characteristics	*n*	PD1		Treg Score		EBV	
		Positive	*P*	High	*P*	Positive	*P*
Sex							
Male	140	67 (48%)	.042	87 (62%)	.005	51 (36%)	.204
Female	59	19 (32%)		24 (41%)		16 (27%)	
Age, years							
<55	76	31 (41%)	.587	43 (57%)	.858	31 (41%)	.095
≥55	123	55 (45%)		68 (55%)		36 (29%)	
TNM stage							
I	162	62 (38%)	.003	85 (52%)	.049	53 (33%)	.552
II to IV	37	24 (65%)		26 (70%)		14 (38%)	
LN metastasis							
Absence	197	84 (43%)	.103	109 (55%)	.206	66 (34%)	.623
Presence	2	2 (100%)		2 (100%)		1 (50%)	
DMR							
Absence	180	69 (38%)	<.001	96 (53%)	.009	60 (33%)	.758
Presence	19	17 (89%)		16 (84%)		7 (37%)	
Nuclear grade							
1	44	7 (16%)	<.001	22 (50%)	.198	13 (30%)	.545
2	117	53 (45%)		63 (54%)		43 (37%)	
3 and 4	38	26 (68%)		26 (68%)		11 (29%)	
Necrosis							
Absence	172	71 (41%)	.164	91 (53%)	.04	53 (31%)	.032
Presence	27	16 (56%)		20 (74%)		14 (52%)	
EBV							
Negative	132	56 (42%)	.752	71 (54%)	.427		
Positive	67	30 (45%)		40 (60%)			
Treg							
Low	88	16 (18%)	<.001				
High	111	70 (63%)					

Treg, FoxP3-positive regulatory T cells.

for EBER were detected in both tumor cells and infiltrating inflammatory cells (Figure 1, *E* and *F*). The mean number of PD1-positive cells in all CRCCs was 18 in 5 HPFs (mean ± SD, 18 ± 46; range, 0–293) and the mean number of Tregs in all CRCCs was 20 in 5 HPFs (mean ± SD, 20 ± 32; range, 0–255). EBER positivity was seen in 34% (67/199) of CRCC patients. The PD1-positive subgroup significantly correlated with the sex of patients ($P = .042$), higher clinical stage ($P = .003$), DMR ($P < .001$), higher histologic nuclear grade ($P < .001$), and the high Treg subgroup ($P < .001$). The high Treg subgroup significantly correlated with the sex of patients ($P = .005$), higher clinical stage ($P = .049$), DMR ($P = .009$), and presence of histologic tumor necrosis ($P = .040$). Presence of EBV infection significantly correlated only with the presence of histologic tumor necrosis ($P = .032$; Table 1).

The Infiltration of PD1-Positive Cells and FoxP3-Positive Tregs in CRCCs Correlates with Distant Metastatic Relapse, RFS, and OS according to Univariate Analysis

The factors significantly correlated with DMR, RFS, and OS by univariate analyses were TNM stage and intratumoral infiltration of FoxP3-positive Tregs or PD1-positive cells (Table 2). In CRCC patients, intratumoral infiltration of PD1-positive cells predicted shorter DMR [$P = .001$; hazard ratio (HR), 12.371; 95% confidence interval (CI), 2.855–53.608], RFS ($P < .001$; HR, 9.562; 95% CI, 3.729–24.518), and OS ($P < .001$; HR, 10.802; 95% CI, 3.259–35.806). High Treg number in CRCC also predicted shorter DMR ($P = .005$; HR, 5.776; 95% CI, 1.677–19.895), RFS ($P < .001$; HR, 5.708; 95% CI, 2.381–13.687), and OS ($P = .001$; HR, 6.218; 95% CI, 2.151–17.971). In addition to the prognostic impact of the infiltration of Tregs and PD1-positive cells, EBV infection was also significantly associated with OS ($P = .008$; HR, 2.792; 95% CI, 1.305–5.977). Kaplan-Meier survival curves for the impact on OS and RFS of CRCC patients are shown in Figure 2.

When we evaluated the prognostic significance of the combined infiltrative pattern of PD1-positive lymphocytes and FoxP3-positive Tregs (PD1/Treg pattern), the PD1/Treg pattern significantly correlated with DMR (log rank, $P < .001$), RFS (log rank, $P < .001$), and OS (log rank, $P < .001$) in overall CRCCs (Figure 3*A*). The $PD1^+$/high Treg subgroup showed shorter DMR, RFS, and OS duration and the $PD1^-$/low Treg subgroup had longest DMR, RFS, and OS duration. The 10-year survival rates of the $PD1^-$/low Treg, $PD1^+$/low Treg or $PD1^-$/high Treg, and $PD1^+$/high Treg groups were 98%, 75%, and 53%, respectively. The $PD1^-$/low Treg subgroup had only 1 of 72 patients (1%) with DMR. In contrast, 3 of 57 $PD1^+$/low Treg or $PD1^-$/high Treg subgroups (5%) and 15 of 70 of the $PD1^+$/high Treg subgroup (21%) showed DMR. When additional analysis was performed on the separation of CRCC patents according to TNM stage (stage I *versus* stages II, III, and IV), there were no patients experiencing DMR in the stage I $PD1^-$/low Treg subgroup and there were no dead patients in the high stage (stages II–IV) $PD1^-$/low Treg subgroup (Figure 3, *B* and *C*).

The Infiltration of PD1-Positive Cells and FoxP3-Positive Tregs in CRCCs Are Independent Prognostic Factors for Worse Survival Outcomes according to Multivariate Analysis

Multivariate analysis was performed using all patients with complete information for all variables. All of the variables significantly associated with DMR, RFS, or OS by univariate analyses were included

Table 2. Univariate Cox Regression Analysis for OS, RFS, and DMR in CRCC Patients.

Characteristics	*n*	DMR, HR (95% CI)	*P*	RFS, HR (95% CI)	*P*	OS, HR (95% CI)	*P*
Sex, male (*vs* female)	140	0.261 (0.060–1.131)	.073	0.416 (0.174–0.995)	.049	1.954 (0.743–5.141)	.175
Age, years, ≥55 (*vs* <55)	123	1.960 (0.705–5.453)	.197	2.309 (1.092–4.882)	.029	3.245 (1.233–8.540)	.017
TNM stage, II to IV (*vs* I)	37	10.938 (4.151–28.823)	<.001	5.591 (2.955–10.577)	<.001	3.750 (1.762–7.889)	<.001
LN metastasis, presence (*vs* absence)	2	25.896 (2.981–224.953)	.003	35.949 (7.375–175.220)	<.001	3.374 (0.454–25.095)	.235
Nuclear grade							
1	44	1	.092	1	.001	1	.013
2	117	30878 (0–2×10^{86})	.914	6.907 (0.928–51.407)	.059	4.617 (0.608–35.049)	.139
3 and 4	38	85638 (0–6×10^{86})	.906	17.767 (2.354–134.614)	.005	11.291 (1.453–87.734)	.020
Necrosis, presence (*vs* absence)	27	1.796 (0.594–5.435)	.300	1.853 (0.848–4.049)	.122	2.470 (1.048–5.820)	.039
EBV positive (*vs* negative)	67	1.059 (0.414–2.709)	.904	1.664 (0.877–3.156)	.119	2.792 (1.305–5.977)	.008
Treg, high (*vs* low)	111	5.776 (1.677–19.895)	.005	5.708 (2.381–13.687)	<.001	6.218 (2.151–17.971)	.001
PD1, positive (*vs* negative)	86	12.371 (2.855–53.608)	.001	9.562 (3.729–24.518)	<.001	10.802 (3.259–35.806)	<.001

Treg, FoxP3-positive regulatory T cell.

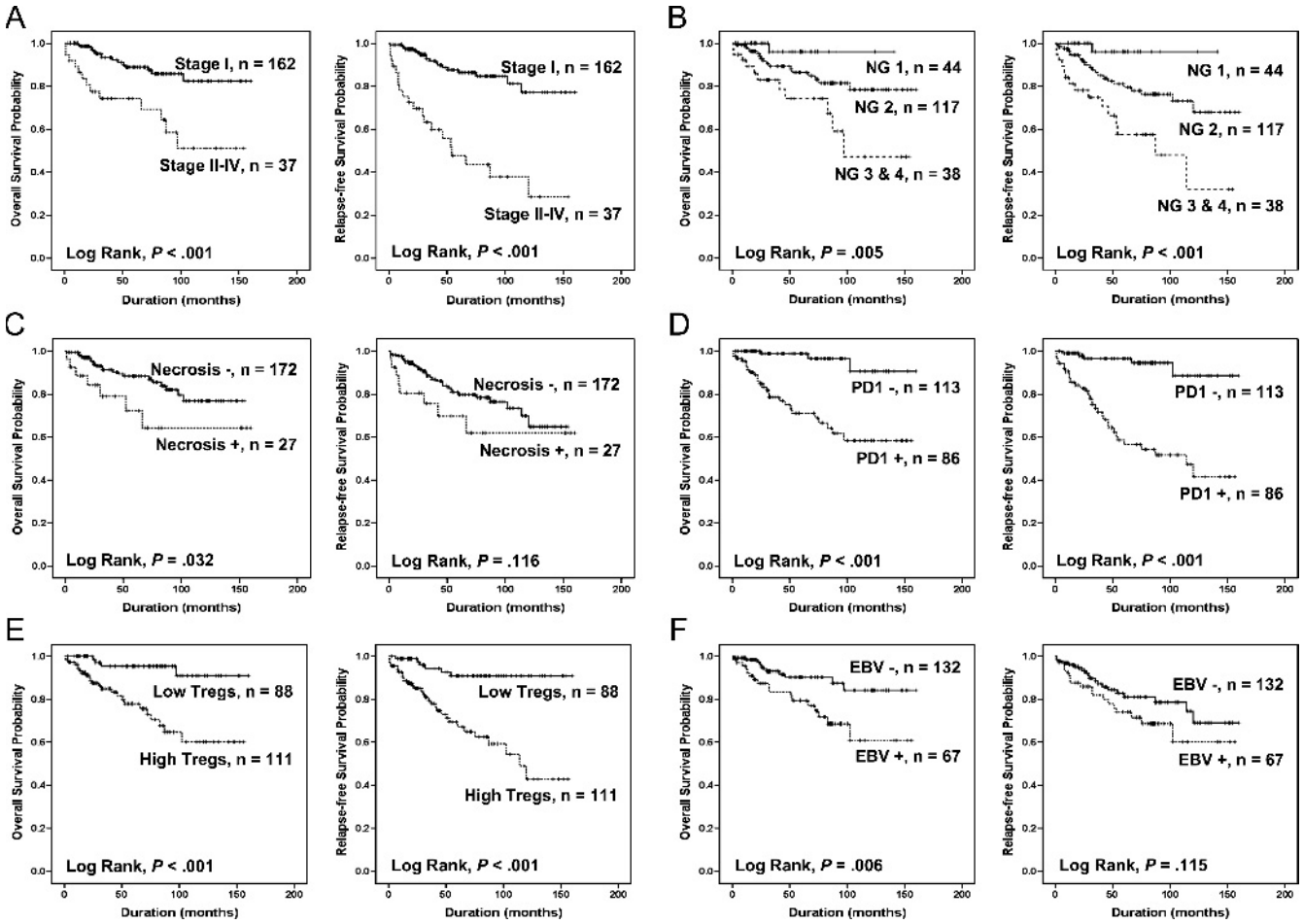

Figure 2. Kaplan-Meier survival analysis of RCC. (A–F) OS and RFS according to TNM stage (A), nuclear grade of tumor cells (B), histologic tumor necrosis (C), intratumoral infiltration of PD1-positive cells (D) and FoxP3-positive Tregs (E), and EBV infection (F) in CRCC patients.

in multivariate analysis. TNM stage and the PD1-positive subgroup were independent prognostic indicators of DMR and RFS for CRCC patients. EBV infection and intratumoral infiltration of Tregs or PD1-positive cells were all independent prognostic indicators of OS of CRCC patients (Table 3). The patients having tumors with infiltrations of PD1-positive cells had a 6.146-fold (95% CI, 1.249–30.238) greater risk of DMR ($P = .026$), a 5.618-fold (95% CI, 2.028–15.560) decrease in RFS ($P = .001$), and a 6.275-fold (95% CI, 1.813–21.715) decrease in OS ($P = .004$). The patients with high intratumoral Treg infiltration had a 3.195-fold (95% CI, 1.046–9.761) decrease in OS ($P = .042$). The HR for OS was 3.671-fold (95% CI, 1.691–7.966) greater in the EBER-positive patients than EBER-negative patients ($P = .001$; Table 3).

Discussion

In this study, we have examined the impact of tumor-infiltrating PD1-positive lymphocytes and Tregs and EBV infection for the prediction of metastatic potential and prognosis of CRCC. The infiltration of PD1-positive lymphocytes was an independent indicator of DMR, RFS, and OS of CRCC. This result suggests that intratumoral infiltration of PD1-positive lymphocytes could be used as a prognostic indicator for CRCC patients and helpful for the prediction of latent distant metastasis of CRCCs. Recent preliminary data from clinical trials with anti-PD1 antibody for the treatment refractory metastatic CRCC showed partial responses in 5 of 16 patients and stable disease state in 3 of 16 patients [13]. In another report, immunotherapy with anti-PD1 antibody showed response rates of 27% (9 of 33) in RCC, 18% (14 of 76) in non–small cell lung cancer, and 28% (26 of 94) in melanoma [12]. Therefore, PD1-targeted immunotherapy could potentially be applied to RCC patients, and there is a need to establish a reliable method for the selection of RCC patients who would be suitable candidates for PD1-targeted immunotherapy in the practical pathologic diagnostic field. Although we performed PD1 staining on paraffin-embedded tissue, our results are consistent with a previous report on the staining performed on frozen tissue [11]. The presence of the infiltration of PD1-positive lymphocytes significantly associated with high Treg numbers and poor prognostic indicators of CRCC such as higher TNM stage and histologic nuclear grade. Especially, PD1 positivity was helpful for the prediction of DMR, even for stage I CRCC. There was no DMR in 100 PD1-negative stage I patients; however, 6 of 62 PD1-positive stage I RCC patients showed DMR ($P = .002$). Therefore, in addition to the prognostic impact of PD1 positivity for CRCC, our findings suggest that PD1 immunostaining of paraffin-embedded tissue is also available for the evaluation of the infiltration of PD1-positive cells in tumors as possible criterion for selection of patients suitable for PD1-based immunotherapy.

In our study, the high Treg subgroup was also significantly associated with higher TNM stage, DMR, and tumor necrosis. Moreover,

high numbers of Tregs predicted shorter DMR, RFS, and OS for CRCC. In line with our study, high intratumoral Treg counts are related to poor prognosis of various human malignant tumors [17,20–22]. Peripheral blood of RCC patients showed higher numbers of Treg than that of healthy individuals [20]. Elevated Treg distribution in human malignant tumors was reduced to normal levels after curative resections of the tumor [34]. This suggests that some tumor-related factor could be involved in the expansion of Treg. However, exact mechanism(s) of how Tregs are involved in the modulation of the tumor microenvironment is not clear. Possibly, Treg-related immunosuppressive function could be mediated by blocking the function of T cells and antigen-presenting cells.

The expression of PD1 has been observed on activated T cells, especially in the cytoplasm of Treg [35]. In addition, up-regulation of PD1 on tumor-infiltrating Treg was related with enhanced suppressive function of Treg [7]. Therefore, we also evaluated the impact of the intratumoral infiltration of Treg and their association with PD1 positivity. The infiltration of Treg was significantly correlated with the infiltration of PD1-positive cells. Eighty-one percent of patients with tumors with infiltration of PD1-positive lymphocytes were

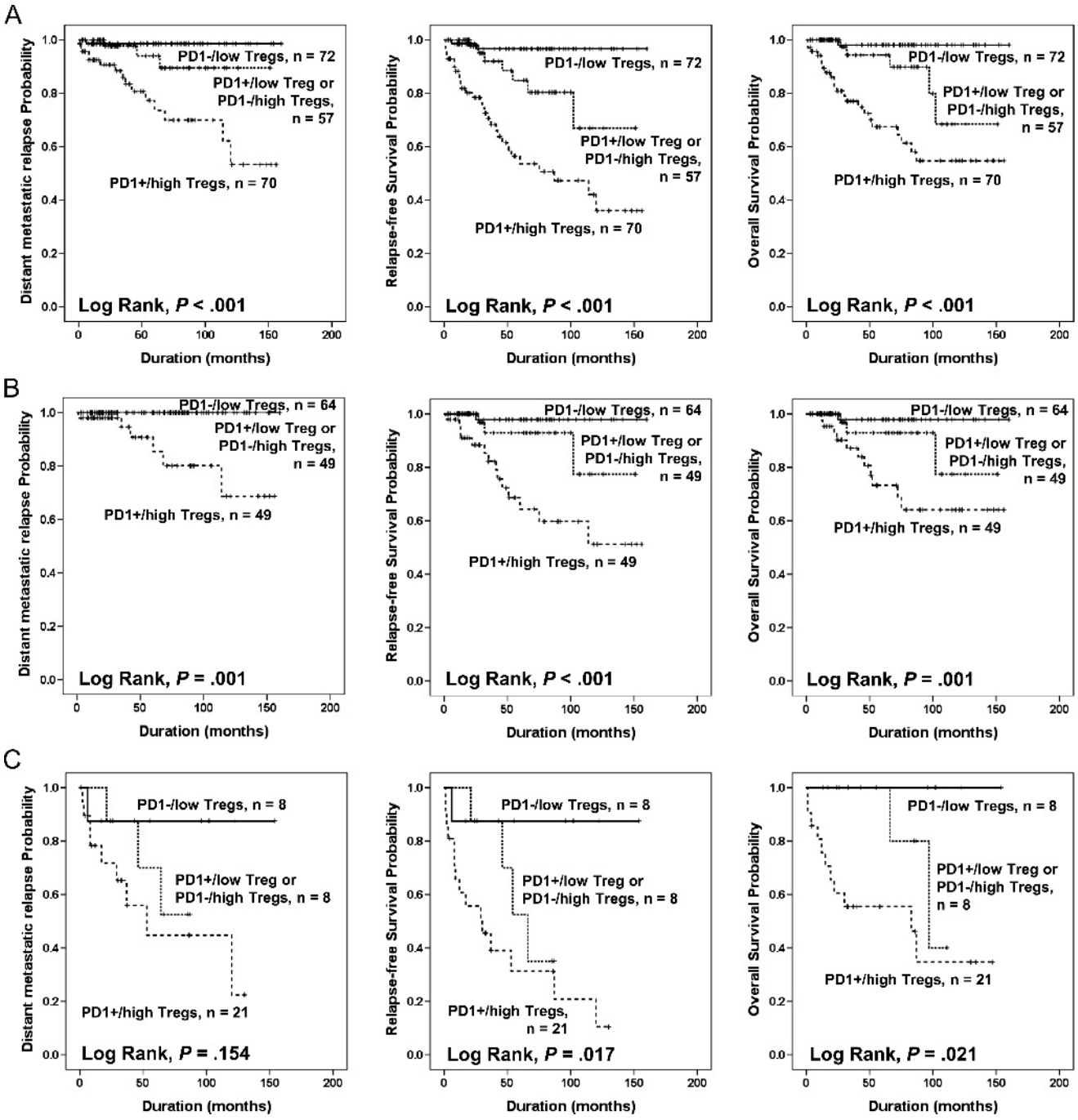

Figure 3. Kaplan-Meier analysis according to the combined intratumoral infiltration patterns of PD1-positive lymphocytes and FoxP3-positive Tregs in CRCC. (A–C) DMR, RFS, and OS in overall CRCC (A), stage I CRCC (B), and high stage (stages II–IV) CRCC (C).

Table 3. Multivariate Cox Regression Analysis for OS, RFS, and DMR in CRCC Patients.

Characteristics	DMR, HR (95% CI)	*P*	RFS, HR (95% CI)	*P*	OS, HR (95% CI)	*P*
TNM stage, II to IV (*vs* I)	6.811 (2.478–18.724)	<.001	3.883 (2.006–7.515)	<.001	2.147 (0.966–4.771)	.061
PD1, positive (*vs* negative)	6.146 (1.249–30.238)	.026	5.618 (2.028–15.560)	.001	6.275 (1.813–21.715)	.004
Treg, high (*vs* low)	NA	NA	2.149 (0.833–5.547)	.114	3.195 (1.046–9.761)	.042
EBV positive (*vs* negative)	NA	NA	NA	NA	3.671 (1.691–7.966)	.001
Age, years, ≥55 (*vs* <55)	NA	NA	NA	NA	2.767 (0.979–7.821)	.055
LN metastasis, presence (*vs* absence)	NA	NA	6.817 (1.357–34.236)	.020	NA	NA

Treg, FoxP3-positive regulatory T cell; NA, not applicable. Variables included in the multivariate analysis for DMR are TNM stage, LN metastasis, histologic nuclear grade, and intratumoral infiltration of FoxP3-positive Tregs or PD1-positive cells. Variables included in the multivariate analysis for RFS are sex, age of the patients, TNM stage, LN metastasis, histologic nuclear grade, and intratumoral infiltration of FoxP3-positive Tregs or PD1-positive cells. Variables included in the multivariate analysis OS are age of the patients, TNM stage, histologic nuclear grade, tumor necrosis, EBV infection, and intratumoral infiltration of FoxP3-positive Tregs or PD1-positive cells.

also in the high Treg subgroup. The number of PD1-positive lymphocytes in low Treg and high Treg subgroups were 2 ± 6 and 31 ± 59, respectively ($P < .001$). These results suggest that PD1 and Treg-mediated immunosuppression contribute to the aggressive progression CRCC. In line with our results, the high frequency of PD1-positive T cells in metastatic LNs of papillary thyroid carcinomas and the positive correlation with Treg infiltration have been reported recently [36]. In addition, during interleukin-2–based immunotherapy in patients with metastatic RCC, intratumoral Treg increased and was correlated with poor prognosis [16]. In this aspect, this result supports the hypothesis that PD1 functions as a negative regulator of antitumor immune responses and that blockage of PD1 and the function of Treg could be a good stratagem for the immune therapy of CRCC. Therefore, the evaluation of the infiltration of PD-positive cells or Tregs in CRCC may be useful for the discrimination of CRCC cases with poor prognoses, a group that could be candidate for PD1- or Treg-based additional immunotherapy.

EBV infection occurs in early life for most of the human population and causes only mild disease. However, after remission, EBV remains in infected individuals for their lifetime [23,37,38]. When there are functional deficiencies in T-lymphocyte response, EBV infection could induce fatal lymphoproliferative disease and is involved in several types of human malignant tumors. However, there were very limited reports on the role and prevalence of EBV in RCCs [24,25]. In this study, we have demonstrated for the first time that EBV infection was significantly associated with poor survival of CRCC patients. EBV infection was an independent prognostic indicator of OS of CRCC patients. The prevalence of EBV infection in our CRCC group was 34% (67 of 199). This incidence of EBV infection in CRCC is relatively high. However, most of the EBER staining occurred in the cytoplasm of tumor cells (48 of 199, 24%) and inflammatory cells (39 of 199, 20%). Nuclear expression of EBER in tumor cells presented in only six cases of CRCC (3%). Even in these six cases, nuclear expression of EBER was seen in only one or two tumor cells in 3-mm tissue cores. We thought that the localization patterns of EBV may be explained by the fact that the kidney is a reservoir of EBV infection [39,40] and/or EBV has a possible tumorigenic role in CRCC. Therefore, we performed separate analyses according to the localization of EBV infection. When we did survival analysis in cases with EBER expression in tumor cells, regardless of whether EBER staining was nuclear or cytoplasmic, it did not predict OS (log rank, $P = .114$). In addition, there were no clinicopathologic factors associated with EBV infection. Therefore, we presumed that EBV infection itself did not contribute to the development of tumor cells in CRCC. However, when additional analysis was performed with RCC having EBER expression in inflammatory cells, the EBV infection of inflammatory cells predicted shorter OS (log rank, $P = .005$) and RFS (log rank, $P = .036$). In addition, the subgroup having EBV infection only in the inflammatory cells possibly correlated with intratumoral infiltration of Treg ($P = .059$). Although the relationship between EBV infection and Treg infiltration is still unclear, our findings suggest the possible relationship between EBV infection and a tumor-permissive immune environment involving Treg infiltration.

In conclusion, this study has shown that intratumoral infiltration of PD1-positive or FoxP3-positive lymphocytes was a poor prognostic indicator of CRCC and predicted poor survival. Especially, PD1 or FoxP3 positivity was very helpful for the prediction of latent distant metastasis of CRCCs. Even in stage I CRCCs, PD1 or FoxP3 positivity predicted DMR, RFS, and OS. Therefore, this study suggests that the evaluation of the infiltration of PD1-positive lymphocytes or FoxP3-positive Tregs in CRCC is helpful for the prediction of prognosis of CRCC patients and for the selection of the patients who could be suitable for PD1- or Treg-based immunotherapy in the practical pathologic diagnostic fields.

References

[1] Gupta K, Miller JD, Li JZ, Russell MW, and Charbonneau C (2008). Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review. *Cancer Treat Rev* **34**, 193–205.

[2] Scherr AJ, Lima JP, Sasse EC, Lima CS, and Sasse AD (2011). Adjuvant therapy for locally advanced renal cell cancer: a systematic review with meta-analysis. *BMC Cancer* **11**, 115.

[3] Webster WS, Lohse CM, Thompson RH, Dong H, Frigola X, Dicks DL, Sengupta S, Frank I, Leibovich BC, Blute ML, et al. (2006). Mononuclear cell infiltration in clear-cell renal cell carcinoma independently predicts patient survival. *Cancer* **107**, 46–53.

[4] Motzer RJ and Russo P (2000). Systemic therapy for renal cell carcinoma. *J Urol* **163**, 408–417.

[5] Escudier B (2012). Emerging immunotherapies for renal cell carcinoma. *Ann Oncol* **23**(suppl 8), viii35–viii40.

[6] Nishimura H and Honjo T (2001). PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol* **22**, 265–268.

[7] Park HJ, Kusnadi A, Lee EJ, Kim WW, Cho BC, Lee IJ, Seong J, and Ha SJ (2012). Tumor-infiltrating regulatory T cells delineated by upregulation of PD-1 and inhibitory receptors. *Cell Immunol* **278**, 76–83.

[8] Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, et al. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* **192**, 1027–1034.

[9] Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, and Nunes R (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* **2**, 261–268.

[10] Richendollar BG, Pohlman B, Elson P, and Hsi ED (2011). Follicular programmed death 1-positive lymphocytes in the tumor microenvironment are an independent prognostic factor in follicular lymphoma. *Hum Pathol* **42**, 552–557.

[11] Thompson RH, Dong H, Lohse CM, Leibovich BC, Blute ML, Cheville JC, and Kwon ED (2007). PD-1 is expressed by tumor-infiltrating immune cells

and is associated with poor outcome for patients with renal cell carcinoma. *Clin Cancer Res* **13**, 1757–1761.

[12] Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, et al. (2012). Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. *N Engl J Med* **366**, 2443–2454.

[13] McDermott D, Drake C, Sznol M, Sosman J, Smith D, Powderly J, Feltquate D, Kollia G, Gupta A, and Wigginton J (2011). A phase I study to evaluate safety and antitumor activity of biweekly BMS-936558 (anti-PD-1, MDX-1106/ONO-4538) in patients with RCC and other advanced refractory malignancies. *J Clin Oncol* **29**, 331.

[14] Byrne WL, Mills KH, Lederer JA, and O'Sullivan GC (2011). Targeting regulatory T cells in cancer. *Cancer Res* **71**, 6915–6920.

[15] Beyer M and Schultze JL (2006). Regulatory T cells in cancer. *Blood* **108**, 804–811.

[16] Jensen HK, Donskov F, Nordsmark M, Marcussen N, and von der Maase H (2009). Increased intratumoral FOXP3-positive regulatory immune cells during interleukin-2 treatment in metastatic renal cell carcinoma. *Clin Cancer Res* **15**, 1052–1058.

[17] Noh SJ, Park SY, Kim KR, Kim CY, Kwon KS, Park HS, Lee H, Chung MJ, Moon WS, and Jang KY (2010). The prognostic significance of the tumor-infiltrating FoxP3-positive regulatory T cells in gastric carcinoma. *Korean J Pathol* **44**, 9–15.

[18] Nishikawa H, Kato T, Tanida K, Hiasa A, Tawara I, Ikeda H, Ikarashi Y, Wakasugi H, Kronenberg M, Nakayama T, et al. (2003). $CD4^+$ $CD25^+$ T cells responding to serologically defined autoantigens suppress antitumor immune responses. *Proc Natl Acad Sci USA* **100**, 10902–10906.

[19] Baecher-Allan C and Anderson DE (2006). Immune regulation in tumor-bearing hosts. *Curr Opin Immunol* **18**, 214–219.

[20] Liotta F, Gacci M, Frosali F, Querci V, Vittori G, Lapini A, Santarlasci V, Serni S, Cosmi L, Maggi L, et al. (2011). Frequency of regulatory T cells in peripheral blood and in tumour-infiltrating lymphocytes correlates with poor prognosis in renal cell carcinoma. *BJU Int* **107**, 1500–1506.

[21] Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, and Banham AH (2006). Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* **24**, 5373–5380.

[22] Lee NR, Song EK, Jang KY, Choi HN, Moon WS, Kwon K, Lee JH, Yim CY, and Kwak JY (2008). Prognostic impact of tumor infiltrating FOXP3 positive regulatory T cells in diffuse large B-cell lymphoma at diagnosis. *Leuk Lymphoma* **49**, 247–256.

[23] Boyle P and Levin B (2008). *International Agency for Research on Cancer, and World Health Organization, "World Cancer Report 2008"*. WHO Press, Geneva, Switzerland.

[24] Kim KH, Han EM, Lee ES, Park HS, Kim I, and Kim YS (2005). Epstein-Barr virus infection in sarcomatoid renal cell carcinoma tissues. *BJU Int* **96**, 547–552.

[25] Shimakage M, Kawahara K, Harada S, Sasagawa T, Shinka T, and Oka T (2007). Expression of Epstein-Barr virus in renal cell carcinoma. *Oncol Rep* **18**, 41–46.

[26] Lau KM, Cheng SH, Lo KW, Lee SA, Woo JK, van Hasselt CA, Lee SP, Rickinson AB, and Ng MH (2007). Increase in circulating Foxp3+CD4+ $CD25^{high}$ regulatory T cells in nasopharyngeal carcinoma patients. *Br J Cancer* **96**, 617–622.

[27] Barath S, Aleksza M, Keresztes K, Toth J, Sipka S, Szegedi G, and Illes A (2006). Immunoregulatory T cells in the peripheral blood of patients with Hodgkin's lymphoma. *Acta Haematol* **116**, 181–185.

[28] Baumforth KR, Birgersdotter A, Reynolds GM, Wei W, Kapatai G, Flavell JR, Kalk E, Piper K, Lee S, Machado L, et al. (2008). Expression of the Epstein-Barr virus-encoded Epstein-Barr virus nuclear antigen 1 in Hodgkin's lymphoma cells mediates up-regulation of CCL20 and the migration of regulatory T cells. *Am J Pathol* **173**, 195–204.

[29] Li J, Qian CN, and Zeng YX (2009). Regulatory T cells and EBV associated malignancies. *Int Immunopharmacol* **9**, 590–592.

[30] Eble J, Sauter G, and Epstein J (2004). *World Health Organization Classification of Tumors: Tumors of the Urinary System and Male Genital Organs. Pathology and Genetics*. IARC Press, Lyon, France.

[31] Edge SB, Byrd DR, Compton C, Fritz A, Greene F, and Trotti A (2010). *AJCC Cancer Staging Handbook: From the AJCC Cancer Staging Manual*. Springer, New York, NY.

[32] Fuhrman SA, Lasky LC, and Limas C (1982). Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* **6**, 655–663.

[33] Schwemmle M, Clemens MJ, Hilse K, Pfeifer K, Troster H, Muller WE, and Bachmann M (1992). Localization of Epstein-Barr virus-encoded RNAs EBER-1 and EBER-2 in interphase and mitotic Burkitt lymphoma cells. *Proc Natl Acad Sci USA* **89**, 10292–10296.

[34] Tokuno K, Hazama S, Yoshino S, Yoshida S, and Oka M (2009). Increased prevalence of regulatory T-cells in the peripheral blood of patients with gastrointestinal cancer. *Anticancer Res* **29**, 1527–1532.

[35] Raimondi G, Shufesky WJ, Tokita D, Morelli AE, and Thomson AW (2006). Regulated compartmentalization of programmed cell death-1 discriminates $CD4^+CD25^+$ resting regulatory T cells from activated T cells. *J Immunol* **176**, 2808–2816.

[36] French JD, Kotnis GR, Said S, Raeburn CD, McIntyre RC Jr, Klopper JP, and Haugen BR (2012). Programmed death-1^+ T cells and regulatory T cells are enriched in tumor-involved lymph nodes and associated with aggressive features in papillary thyroid cancer. *J Clin Endocrinol Metab* **97**, E934–E943.

[37] Gratama JW, Oosterveer MA, Zwaan FE, Lepoutre J, Klein G, and Ernberg I (1988). Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. *Proc Natl Acad Sci USA* **85**, 8693–8696.

[38] Yao QY, Ogan P, Rowe M, Wood M, and Rickinson AB (1989). Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. *Int J Cancer* **43**, 67–71.

[39] Becker JL, Miller F, Nuovo GJ, Josepovitz C, Schubach WH, and Nord EP (1999). Epstein-Barr virus infection of renal proximal tubule cells: possible role in chronic interstitial nephritis. *J Clin Invest* **104**, 1673–1681.

[40] Iwama H, Horikoshi S, Shirato I, and Tomino Y (1998). Epstein-Barr virus detection in kidney biopsy specimens correlates with glomerular mesangial injury. *Am J Kidney Dis* **32**, 785–793.