Original paper

Evaluation of PD-1, PD-L1, and cytotoxic T-lymphocyte-associated protein 4 expressions together with clinicopathological findings in clear cell, papillary, and chromophobe types of renal cell carcinoma

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Our study aimed to determine the expressions of programmed death protein 1 (PD-1), programmed death ligand protein 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to investigate and compare the differences between early and advanced cases in the 3 most common types of renal cell carcinoma (RCC) and reveal their correlations with prognosis and survival. A total of 166 RCC cases diagnosed between 2010 and 2019 in our hospital were included. PD-1, PD-L1, and CTLA-4 markers were applied to the paraffin blocks of the cases using an immunohistochemical method, and their expression status was evaluated by distinguishing subtypes in advanced- and early-stage RCCs. It was observed that PD-L1 positivity in the tumour cells, in clear cell RCC, was statistically significantly more frequent in advanced-stage cases compared to early-stage cases.

It was concluded that cases with PD-L1 positivity in tumour-infiltrating mononuclear cells (TIMC) in clear cell and chromophobe RCC had a shorter survival. The frequency of perinephritic fat invasion and necrosis was higher in cases with PD-L1 expression in TIMC.

We think that PD-1, PD-L1, and CTLA-4 must be considered together in advanced stage RCC for the treatment of both pathway inhibitors. Further large studies will shed light on the immunotherapy options at the advanced stage of all RCC types even in the absence of metastasis.

Key words: renal cell carcinoma, PD-1, PD-L1, CTLA-4.

Introduction

Current data indicate that renal cell carcinoma (RCC) is the 14th most common cancer (403,262 cases/year) worldwide. Renal cell carcinoma ranks 16th among cancer-related deaths, with 175,098 annual deaths [1]. Kidney cancer has several unique features for immune checkpoint inhibition (ICI) therapy. Renal cell carcinoma responds poorly to conventional chemotherapeutics, and although therapies targeting rapamycin (mTOR) and vascular endothelial growth factor (VEGF) increase the number of therapeutic options, almost all patients eventually become resistant to these molecularly targeted or antiangiogenic therapies [2]. Rarely, metastatic RCCs regress spontaneously without treatment, while control of metastatic disease is sometimes seen after cytoreductive nephrectomy [3]. These observations strongly suggest that a tumour immune response to the host may be present in some patients [4].

Current ICI treatments show promising effects in RCC patients by inhibiting 2 immune escape mechanisms that affect the differentiation and activity of effector T cells. The first and most targeted immune escape mechanism is reducing T cell activity by the interaction of programmed death protein 1/programmed death ligand 1 (PD-1/PD-L1) in the tumour microenvironment [5]. Retrospective analyses of the prognostic value of PD-1 expression in RCC are conflicting [6]. There are many publications showing that expression of PD-L1 in tumour cells in RCC is associated with a more advanced tumour stage, a worse response to tyrosine kinase inhibitor therapy, and a worse prognosis [7–9]. Expression of PD-L1 in tumour-infiltrating mononuclear cells (TIMC) in RCC is associated with worse prognosis in clear cell RCC, but the correlation of PD-L1 expression in TIMC is unknown in other histological subtypes. The second frequently targeted pathway is regulation of the initial priming of naive T cells in lymph nodes through cytotoxic T-lymphocyte-associated protein 4/B7 (CTLA-4/B7) signalling. Recent studies have shown that CTLA-4 expression may be a prognostic marker in RCC [5].

Our study aimed to determine the expression levels of PD-1 in tumour cells and PD-L1, PD-L1, and CTLA-4 in TIMC in the 3 most common types of RCC, namely clear cell RCC (CCRCC), papillary RCC (PRCC), and chromophobe RCC (ChRCC), to determine the differences among RCC types by distinguishing between early and advanced stages and to try to reveal their effects on prognosis and survival.

Material and methods

A total of 166 RCC cases, including 80 CCRCC, 50 PRCC, and 36 ChRCC, were diagnosed after specimens obtained with radical and partial nephrectomy in Cukurova University Faculty of Medicine (CUFM), Department of Pathology, between 2010 and 2019 were selected for the study. The age, gender, tumour diameter, and tumour spread of the cases were obtained from the histopathology reports. Distant metastasis and survival information of the cases were obtained using the CUFM hospital medical recording system. The most suitable paraffin blocks rich in tumour and microenvironment were selected for immunohistochemical staining. The appropriate dilution rate at which antibodies work was determined using sections from control groups and patients. Haematoxylin-eosin (H&E)-stained sections obtained from the tissues fixed in formaldehyde solution and embedded in paraffin were re-examined for WHO-ISUP nuclear grade, necrosis, microvascular invasion, perirenal adipose tissue invasion (PRATI), renal pelvis invasion (RPI), intravascular tumour thrombus, adrenal invasion, and lymph node metastasis. In all cases, the pathological T stage was re-evaluated according to the 2016 WHO classification [10]. In addition, the nuclear degrees determined according to the Fuhrman grade system in the old reports were re-determined according to the WHO-ISUP system of staging [10]. PD-L1 was applied to an entire single block selected from the paraffin blocks of the tumour. For PD-1 and CTLA-4, sections for immunohistochemistry were obtained with newly formed paraffin blocks by selecting and marking an area of 0.5 × 0.5 cm on the H&E-stained preparation of the tumour and cutting the tissue corresponding to this area from the paraffin block. For immunohistochemical examination, 5-micron-thick sections from the selected paraffin blocks were taken on positively charged slides so that the tissues would not be spilled. The sections were left in an oven at 60°C for one hour and then deparaffinized with xylol for 15 minutes. They were hydrated by passing through alcohol in gradually decreasing degrees and then washed in distilled water. PD-1 (Mouse Monoclonal, NAT105, 1 : 100 dilution; Roche-Ventana), PD-L1 (Rabbit Monoclonal, SP263; 1 : 100 dilution; Ventana), and CTLA-4 (Mouse Monoclonal, BSB-88, dilution 1 : 50, BioSB) antibodies were applied to the prepared sections. An OptiView dab detection kit, was used for PD-L1, an ultraView dab kit was used for PD-1 and CTLA-4, and the tissues were stained on a BenchMark XT immunohistochemistry device. Preparations stained in the automatic staining device were covered with a liquid-based sealant. As positive controls, tonsil tissue was used for PD-1 and CTLA-4, and placental tissue was used for PD-L1. The prepared sections were examined by 2 pathologists at different magnifications under an Olympus microscope. For PD-L1, membranous staining in tumour cells and membranous and cytoplasmic granular staining in TIMC were considered positive. Because no staining was observed in tumour cells with PD-1 and CTLA-4, membranous and cytoplasmic granular staining only in TIMC was considered positive. All 3 markers were scored according to the degree of expression. Mean values were obtained by counting at least 4 high-magnification fields (400×) for tumour cells and TIMC. The threshold value for expression degree was accepted as 1% in both tumour and TIMC; $< 1\%$ was considered negative, and $\geq 1\%$ was considered positive.

SPSS v.22 software was used for data analysis. Descriptive statistics were presented as mean ± standard deviation (SD) or median (minimum-maximum) for continuous variables, and frequency and percentage for categorical variables. The results of the analyses were presented as frequency and percentage for categorical variables and as mean ±SD or median (minimum-maximum) for continuous demographic data (e.g. gender, age). Pearson χ^2 test was performed

to compare the categorical variables with each other. Kaplan-Meier and Log Rank tests were used for survival analysis, and the results were also shown in graphs. *P*-values smaller than 0.05 were considered statistically significant. $P < 0.05$ was accepted as the statistical significance level.

Results

Clinicopathological findings

A total of 166 cases were included in the present study; radical nephrectomy was performed in 108 (65%), and partial nephrectomy was performed in 58 (35%) cases. Of the cases, 113 (68%) were males and 53 (32%) were females, with a mean age of 58.4 ±12.8 (16–95) years. Of the tumours, 92 (55.4%) were localized in the right kidney, and 74 (44.6%) were localized in the left kidney. Of 166 cases, 80 (48%) were diagnosed with clear cell RCC, 50 (30%) with papillary RCC, and 36 (22%) with chromophobe RCC. According to the WHO 2016 TNM staging system, 40 (50%) clear cell RCCs, 25 (50%) papillary RCCs, and 18 (50%) chromophobe RCCs had an advanced stage (≥ T2b), and the others had early-stage (T1a and T1b) tumours. The T2a group was not included in this study. The mean tumour diameter was 6.3 ±3.6 (1–18) cm.

Lymph node dissection was performed in 19 of the cases, and regional lymph node metastasis was detected in 9 (5.4%) cases. Distant metastasis developed in 20 (12%) of the cases, of which 8 (4.8%) were detected at the time of diagnosis (primary), and 12 (7.2%) were found during the follow-up period (secondary). Some cases had involvement of more than one organ. The most common organs with metastases were the lung in 8, the liver in 6, adrenal in 6, bone in 4, pleura in 2, pons in one, and scalp in one of the cases.

Sarcomatoid differentiation was observed in 5 (3%) cases, and rhabdoid differentiation was observed in 2 (1.2%) cases. There was necrosis in 36 (21.6%) cases, renal capsule invasion in 45 (27.1%) cases, RPI in 42 (25.3%) cases, PRATI in 42 (25.3%) cases, 28 (16.9%) cases had intravascular tumour thrombus, and 2 (1.2%) had adrenal gland invasion. According to WHO 2016 TNM classification [10], 42 (25.3%) cases were T1a, 41 (24.7%) were T1b, 10 (6%) were T2b, 61 (36.7%) were T3a, 1 (0.6%) was T3c, and 11 (6.6%) were T4.

Immunohistochemical findings

When the threshold value was accepted as 1%, PD-L1 in tumour (TPD-L1) was positive in 49 (29.5%) of the 166 subjects included in the present study, while PD-L1 was positive in 60 (36.1%), PD- L1 was positive in 34 (20.5%), and CTLA-4 was positive in 55 (33.1%) of them in TIMC. According to the results of the chi-square test and the cross-tables between the "TPD-L1, TIMC-PD-L1, PD-1, and CTLA-4 expression" variables and "stage" variable in all RCCs, no statistically significant differences were found. No statistically significant relationships were found among these variables at a $p < 0.05$ significance level.

Considering the distribution of RCC types in relation to TPD-L1 expressions, 13 (7.8%) cases among CCRCCs, 21 (12.7%) cases among PRCCs, and 15 (9%) cases in ChRCCs showed positive staining. The group responsible for the difference was determined as CCRCC with a lower expression rate than the others ($p = 0.001$) (Table I). When the distribution of RCC types in relation to TIMC-PD-L1 expression was analysed, 34 (20.5%) cases among CCRCCs, 20 (12%) cases among PRCCs, and 6 (3.6%) cases among ChRCCs showed positive staining. The analysis result indicated that the group responsible for the difference was ChRCC, which had a lower expression rate than the others ($p = 0.022$) (Table I). Given the distribution of RCC types according to CTLA-4 expression, positive staining was found in 22 (13.3%) cases among CCRCCs, 27 (16.3%) cases among PRCCs, and 6 (3.6%) cases in ChRCCs; the rate of CTLA-4 in TIMC was higher in PRCC ($p = 0.001$) (**Table 1**).

Clear cell RCC cases were selectively included in the analysis, and the number of cases with advanced stage in CCRCC positive for TPD-L1 was statistically significantly higher than the number of cases with

Table I. Programmed death ligand 1 (PD-L1) expression in tumour cells, and PD-L1 and cytotoxic T-lymphocyte-associated protein 4 expression in tumour-infiltrating mononuclear cells in relation to renal cell carcinoma types

RCC type	TPD-L1		TIMC-PD-L1		CTLA-4	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Clear cell	13 (7.8)	67 (40.4)	34 (20.5)	46 (27.7)	22(13.3)	58 (34.9)
Papillary	21 (12.7)	29 (17.5)	20 (12.0)	30 (18.1)	27(16.3)	23 (13.9)
Chromophobe	15 (9)	21 (12.7)	6 (3.6)	30 (18.1)	6 (3.6)	30 (18.1)
p	0.001		0.022		0.001	

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4, PD-L1 – programmed death ligand 1, RCC – renal cell carcinoma, TIMC – tumour-infiltrating mononuclear cells, TPD-L1 – programmed death ligand 1 in tumour

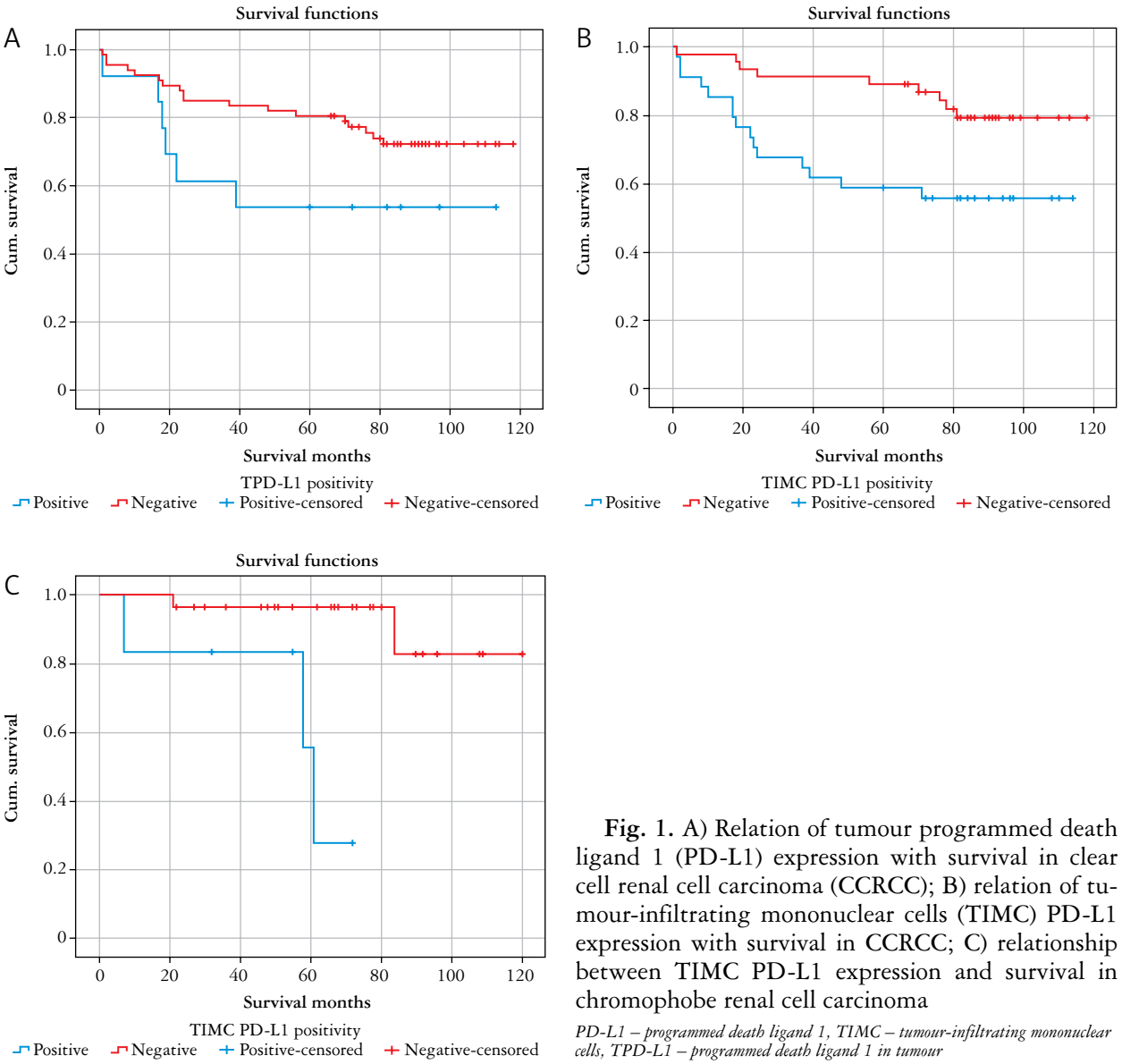

Fig. 1. A) Relation of tumour programmed death ligand 1 (PD-L1) expression with survival in clear cell renal cell carcinoma (CCRCC); B) relation of tumour-infiltrating mononuclear cells (TIMC) PD-L1 expression with survival in CCRCC; C) relationship between TIMC PD-L1 expression and survival in chromophobe renal cell carcinoma

PD-L1 – programmed death ligand 1, TIMC – tumour-infiltrating mononuclear cells, TPD-L1 – programmed death ligand 1 in tumour

an early stage ($p = 0.006$) (Fig. 1, Table II). The difference between the variables "TIMC-PD-L1, PD-1, and CTLA-4 expression" and the "stage" variable was not statistically significant.

In TPD-L1-positive patients, the number of metastatic cases was statistically significantly higher than that of non-metastatic cases ($p = 0.032$). In TIMC-PD-L1-positive patients, the number of cases with PRATI and necrosis was statistically significantly higher than the number of cases without those findings ($p = 0.009$ and $p = 0.019$) (Table II).

There was no statistically significant difference between the variables of perirenal adipose tissue invasion, renal capsule invasion, renal pelvis invasion, adrenal invasion, intravascular thrombus, metastasis and necrosis, and PD-1 and CTLA-4 expression variables (Table II).

The survival analysis comparing TPD-L1-positive (+) and TPD-L1-negative (–) groups in CCRCC revealed the mean survival to be 69.76 (±13.118) months (95% CI: 44.05–95.48) in the TPD-L1-positive (+) group, and 95.58 (±4.81) months (95% CI: 86.14–105.02) in the TPD-L1-negative (–) group. The difference between TPD-L1-positive (+) and TPD-L1-negative (–) groups was not statistically significant ($p = 0.082$) (**Table 3**). The survival analysis between TIMC-PD-L1-positive (+) and TIMC-PD-L1-negative (–) groups revealed the mean survival in the TIMC-PD-L1-positive (+) group as 73.61 (±8.07) months (95% CI: 57.79–89.42), and the mean survival in the TIMC-PD-L1-negative (–) group as 103.60 (±4.64) months (95% CI: 94.50–112.70). The TIMC-PD-L1-positive (+) group had a statistically significantly shorter survival time compared to the TIMC-PD-L1-negative (–) group ($p = 0.009$) (Fig. 2, Table III).

The survival analysis between TIMC-PD-L1-positive (+) and TIMC-PD-L1-negative (–) groups in ChRCC showed the mean survival in the TIMC-PD-L1-positive (+) group as 54.22 (±9.09) months

Table II. Investigation of the relationship of expressions with stage in clear cell renal cell carcinoma, metastasis, necrosis, and perirenal adipose tissue invasion in all renal cell carcinoma types

	Stage in CCRCC		Metastasis		Necrosis		Perirenal adipose tissue invasion	
	Early stage (%)	Advanced stage (%)	Present (%)	Absent (%)	Present (%)	Absent (%)	Present (%)	Absent (%)
TIMC PD-L1								
Positive (+)	14 (17.5)	20 (25.0)			19 (31.7)	41 (68.3)	22 (68.8)	10 (31.2)
Negative (–)	26 (32.5)	20 (25.0)			17 (16)	89 (84)	20 (39.2)	31 (60.8)
p	0.175				**0.019**		**0.009**	
TPD-L1								
Positive (+)	2 (2.5)	11 (13.8)	10(21.5)	39 (79.5)				
Negative (–)	38 (47.5)	29 (36.3)	10(8.6)	107(91.4)				
p	**0.006**		**0.032**					
PD-1								
Positive (+)	7 (8.8)	9 (11.3)						
Negative (–)	33 (41.3)	31 (38.8)						
p	0.576							
CTLA-4	8 (10.0)	14 (17.5)						
Positive (+)	32 (40.0)	26 (32.5)						
Negative (–)								
p	0.133							

CCRCC – clear cell renal cell carcinoma, CTLA-4 – cytotoxic T-lymphocyte-associated protein 4, PD-1 – programmed death protein 1, PD-L1 – programmed death ligand 1, TIMC – tumour-infiltrating mononuclear cells, TPD-L1 – programmed death ligand 1 in tumour

(95% CI: 36.39–72.04), and the mean survival in the TIMC-PD-L1-negative (–) group as 111.72 (±5.53) months (95% CI: 100.87–122.57). The TIMC-PD-L1-positive (+) group had a statistically significantly shorter survival time compared to the TIMC-PD-L1-negative (–) group ($p < 0.001$) (Fig. 2, Table III).

Discussion

Over the past decade, the role of immune checkpoint blockade has been better comprehended in cancer immunotherapy. It was discovered that PD-1, PD-L1, and CTLA-4 were negative regulators of the specific T-cell costimulatory molecule, CD28 [11]. The ability of the host to mount an immune response against cancer cells is limited by these signalling pathways. Given the observations that RCC can influence or silence T-cell responses and data collected specifically with PD-1, PD-L1, and CTLA-4, antibodies against these targets have been developed and studied in patients with metastatic RCC. It has been shown that immune checkpoint blockade with anti-PD-L1 antibodies can reduce tumour volume and prolong survival in RCCs [12]. In these studies, non-clear cell subtypes were grouped under a single heading due to their lower incidence than the CCRCC subtype. Although ICIs are included in the treatment protocols for CCRCC, studies investigating the effects of ICIs in the treatment of non-clear cell RCC are limited [13].

In our study, we determined the expression levels of PD-1/PD-L1 and CTLA-4, which are the most frequently targeted immune escape checkpoints, by distinguishing between advanced and early stages in the most common subtypes of RCC. We investigated their effects on survival and their relationship with clinicopathological parameters. When the findings of 166 cases in our study were evaluated, we determined PD-L1 in tumour cells in 49 (29.5%) cases, TIMC PD-L1 in 60 (36.1%), TIMC PD-1 in 34 (20.5%), and TIMC CTLA-4 expression in 55 (33.1%) cases, while none of these markers were expressed in 60 (36.1%) cases. When the expression differences between RCC types were examined, TPD-L1 was positive in 16.3% of CCRCC, 42% of PRCC, and 41.7% of ChRCC; TIMC-PD-L1 was positive in 42.5% of CCRCC, 40% of PRCC, and 16.7% of ChRCC; PD-1 was positive in 20% of CCRCC, 26% of PRCC, and 13.9% of ChRCC; and CTLA-4 was detected in 27.5% of CCRCC, 54% of PRCC, and 16.7% of ChRCC. The tumour PD-L1 expression rate was lower in CCRCC than in the other types, and the PD-L1 expression

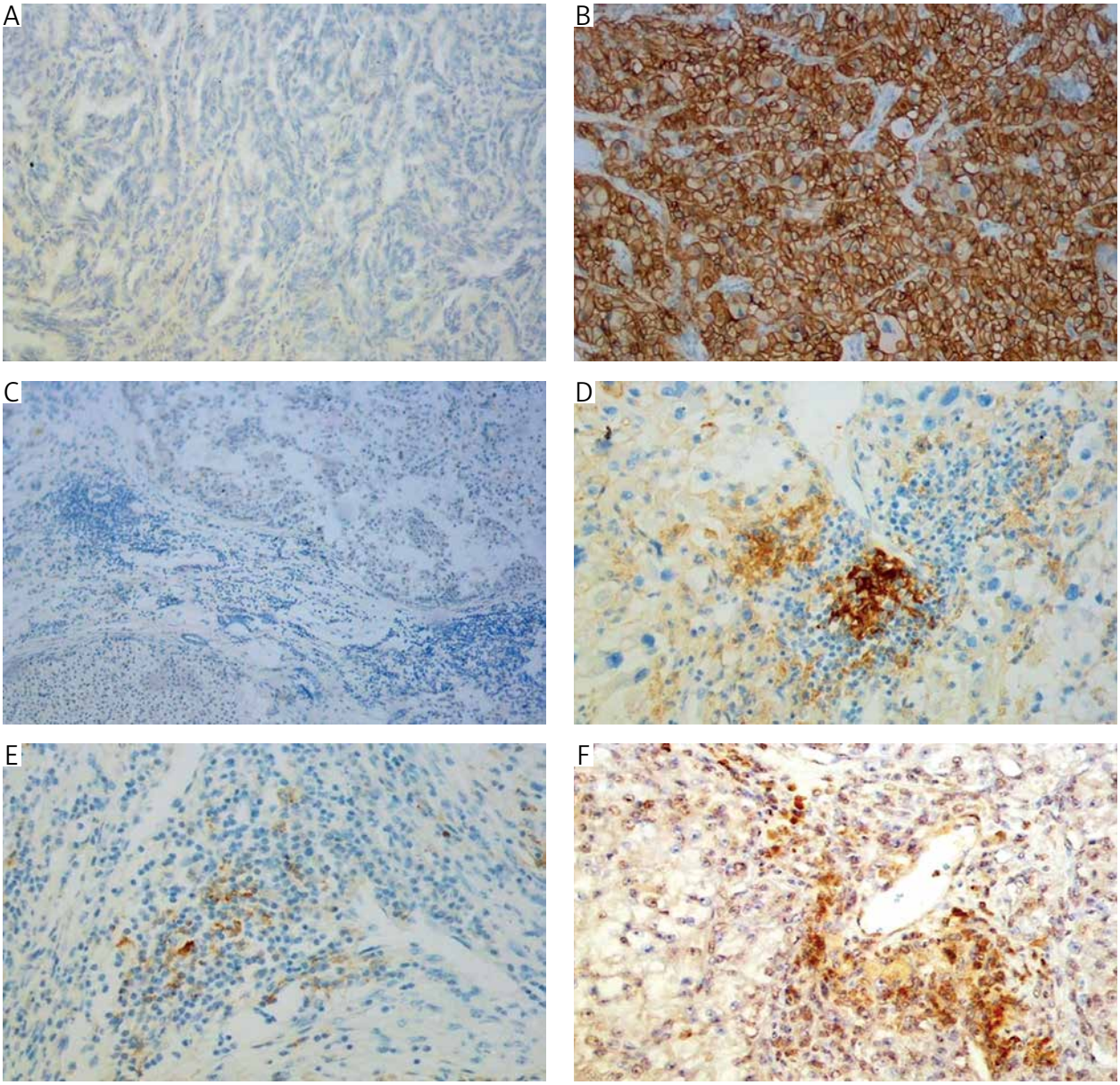

Fig. 2. A) Negative for programmed death ligand 1 (PD-L1) in tumour; B) positive PD-L1 expression in tumour cells; C) negative for tumour-infiltrating mononuclear cells (TIMC) PD-L1; D) positive TIMC PD-L1 expression; E) positive TIMC programmed death protein 1 expression; F) positive TIMC cytotoxic T-lymphocyte-associated protein 4 expression

Table III. The relationship between programmed death ligand 1 expression and survival in clear cell renal cell carcinoma

	MS (months)	SD (months)	95% CI		*p*
			Lowest	Highest	
CCRCC					
TPD-L1 (+)	69.76	13.11	44.05	95.48	0.082
TPD-L1 (–)	95.58	4.81	86.14	105.02	
TIMC-PD-L1 (+)	73.61	8.07	57.79	89.42	**0.009**
TIMC -PD-L1 (–)	103.6	4.64	94.5	112.7	
ChRCC					
Positive	54.22	9.09	36.39	72.04	**< 0.001**
Negative	111.72	5.53	100.87	122.57	
Total	104.27	6.41	91.69	116.85	

CCRCC – clear cell renal cell carcinoma, ChRCC – chromophobe renal cell carcinoma, PD-L1 – programmed death ligand 1, TIMC – tumour-infiltrating mononuclear cells, TPD-L1 – programmed death ligand 1 in tumour

rate in TIMC was lower in ChRCC than in the other types. CTLA-4 expression was more common in papillary type; however, no difference was found for PD-1 expression among the tumour types.

In a meta-analysis of 1863 RCC cases by Wang *et al*. in 2018, PD-L1 expression was evaluated in 1404 CCRCC and 459 non-clear cell RCC cases, and PD-L1 expression was found in 491 (26.4%) cases. PD-L1 expression was found in 414 (29.5%) CCRCC cases, while PD-L1 was expressed in 77 (16.8%) non-clear cell RCC cases. In that meta-analysis, PD-L1 expression was significantly correlated with primary tumour stage, lymph node involvement, distant metastasis, and nuclear grade. The meta-analysis also showed that PD-L1 expression was associated with poor overall survival (OS) in clear cell and non-clear cell RCCs [14]. Yeong *et al*. studied PD-L1 expression on the RCCs of Asian patients in 2019 and found that 83% of the cases had clear cell and 17% had non-clear cell RCCs. In that study, E1L3N and SP263 anti-PD-L1 clones were used, those ≥ 1 were considered positive, and it was observed that there was no significant difference for stage, nuclear grade, or survival in PD-L1 positive cases with the SP263 clone, which was also used in our study [15]. A study by Leite *et al*. performed on 148 CCRCC cases using "Abcam" polyclonal anti-PD-L1 found tumour cell PD-L1 expression to be positive in 56.5% of the cases. Univariate analysis showed a correlation of PD-L1 expression with nuclear Fuhrman grade and microvascular tumour embolization; however, there was no correlation with tumour stage and other clinicopathological parameters [16].

In our study, the PD-L1 SP263 clone was used. No statistically significant difference was found between advanced- and early-stage tumours for the expression of dyes when the subtypes were not considered. When RCC subtypes were analysed, statistically significantly higher TPD-L1 expression was observed in advanced CCRCC than in the early stage. There was no difference between advanced and early stages for TIMC-PD-L1, PD-1, or CTLA-4 expressions. This finding shows that TPD-L1 may be used as a prognostic parameter, especially in CCRCC. Various studies in the literature have found a relationship between TPD-L1 expression and advanced stage, lymph node involvement, high nuclear grade, necrosis, sarcomatoid differentiation, and metastasis [16–18]. Some other studies did not find a relationship between PD-L1 expression and any clinicopathological parameters.

In our study, we examined the relationships of PD-1, PD-L1, and CTLA-4 expressions with clinicopathological parameters (nuclear grade, necrosis, renal capsule invasion, PRATI, RPI, adrenal invasion, intravascular thrombus, and metastasis). We found that metastasis was statistically significantly more frequent in TPD-L1-positive cases. The VHL/HIF pathway, which is the most frequently involved pathway in the pathogenesis of RCC, plays a role in increasing VEGF. There are some studies in the literature suggesting that TPD-L1 induces HIF [19]. The increased presence of metastases we detected in our study may be explained by this theory. It is thought that the increase in TPD-L1 leads to angiogenesis by increasing VEGF through HIF and that these newly formed vessels are used for metastasis. Another possibility is that TPD-L1 triggers the feature of metastasis by causing heterogeneity in the tumour. Data on this feature are limited in the literature and should be supported by further studies on large series. In our study, no statistically significant correlations were found between TPD-L1 and other clinicopathological data. However, we found a statistically significant positive correlation between TIMC PD-L1 expression and PRATI and necrosis. These data show the significance of analysing TPD-L1 and PD-L1 in TIMC as a prognostic parameter. Perirenal adipose tissue invasion is more frequent in cases with TIMC-PD-L1 positivity. This finding shows that the cases will progress locally aggressively and suggest that they may benefit from ICI treatment even if they do not have metastasis. Although a positive correlation was found between PD-L1 expression and high nuclear grade in most of the studies, in our study, cases with TIMC-PD-L1 expression had lower nuclear grades. Becaue studies have mostly focused on expression in tumour cells, the prognostic impact of PD-L1 expression in TIMC is unknown. Our results show that TIMC PD- L1 and TPD-L1 should be evaluated and considered when planning treatment in RCC.

Studies on RCC focused on CCRCC, the most frequent subtype, and the other subtypes are generally grouped under a single heading as non-clear cells because they are few in number. Choueiri *et al*. studied 101 non-clear cell RCC cases in 2014, and TPD-L1 was positive in 5.6% of ChRCC and 10% of PRCC cases, while TIMC-PD-L1 was positive in 36.1% of ChRCC and 60% of PRCC cases. TPD-L1-positive patients had higher tumour stage and nuclear grade, and it was concluded that PD-L1 positivity in TIMC was not associated with stage or survival [20]. A multicentre study by Erlmeier *et al*. on 374 PRHCs found that PD-1 and TPD-L1 expressions had no effect on prognosis. They did not detect PD-L1 expression in TIMC. In that study, NAT105 was used for PD-1, and Dako 22C3 was used for PD-L1 [21]. Motoshima *et al*. investigated PD-L1/2 expression in tumour cells in 102 PRHK cases in 2017. PD-L1 expression was found in 29 of the cases, but PD-L2 expression was not observed. PD-L1 expression was not significantly associated with any clinicopathological factors, including stage, progression-free survival (PFS), and OS [22]. In a study by Shin *et al*. in 2016 on

425 RCC cases, TPD-L1 was positive in 9.4% patients, and no difference in expression was observed among histopathological types. In that study, expression of PD-L1 and PD-1 in CCRCC was correlated with the high nuclear grade, necrosis, sarcomatoid differentiation, short PFS, and cancer-specific survival (CSS); however, it was not correlated with clinicopathological parameters or survival in PRCC and ChRCC [23]. Erlmeier *et al*. analysed the prevalence, distribution, and prognostic effect of PD-1 and PD-L1 expression in ChRCC in 2016. A total of 81 cases were selected; 25 of them (30.9%) had TIMC PD-1, and 11 (13.6%) had tumoural PD-L1 expression. However, those expressions had no effect on tumour stage, clinicopathological features, or 5–10-year overall survival. That study was the first study that analysed the prognostic effect of PD-1 and PD-L1 in ChRCC [24]. Our study is one of the rare series that examined the subtypes other than CCRCC. In our ChRCC series, TIMC-PD-L1 expression was statistically significantly lower than other subtypes, but its presence negatively affected survival. Our results indicate that it is necessary to study the prognostic effects of PD-L1 on ChRCC in larger series. Similarly to the literature data, we did not find any significant effect on survival in papillary RCC. The effects of PD-1, PD-L1, and CTLA-4 on survival, especially in CCRCC, were studied in detail. In their 2019 study investigating the clinical significance of PD-1 and PD-L1 expression in the tumour microenvironment of CCRCC, Mikami *et al*. found that positive PD-L1 expression in TIMC was associated with advanced stage, nuclear grade, and short OS. TPD-L1 expression, on the other hand, was associated with higher nuclear grade, and short OS and PFS, but not with stage. The findings showed that patients with CCRCC with high PD-1 positivity in TIMC had significantly shorter PFS and OS rates than negative ones [25]. Meta-analyses have been published showing the effects of PD-L1 expression on survival and prognosis in RCC. In the meta-analysis, which included 6 studies and 1323 cases, published by Iacovelli *et al*. in 2016, it was observed that PD-L1 expression increased the risk of death by 81% [9]. In the meta-analysis published by Lu *et al*. in 2020, 3389 RCC cases from 16 studies were included, of which 1631 cases and 8 studies reported OS. Six of those revealed a correlation between PD-L1 expression and OS. Thus, it was concluded that PD-L1 overexpression in RCC was a strong negative predictor of OS. The effects of PD-L1 on PFS were mentioned in 10 studies, including 2069 patients. Higher PD-L1 expression was shown to be significantly associated with poor PFS. Ten studies involving 1886 cases analysed the effects of PD-L1 on CSS and showed that higher PD-L1 expression was significantly associated with poor CSS [26]. When the 16 studies included in that last meta-analysis were examined, it was seen that PD-L1 was evaluated in both tumour and TIMC in only 2 studies, and only in tumour cells in the other 14 studies. The significance of PD-L1 expression in TIMC appears to be unclear because the focus of PD-L1 analyses is usually its expression by tumour cells. In our study, the OS was 95.5 months in cases without PD-L1 expression in tumour cells in CCRCCs, and 69.7 months in positive cases. Although OS was considerably shorter, a statistically significant result could not be obtained due to the wide confidence interval (95% CI: 44.058–95.481). PD-L1 expression in TIMC has been found to significantly reduce OS in CCRCC and ChRCC. Because the focus of studies in CCRCC is usually tumour cells, there are few studies showing that PD-L1 positivity in TIMC reduces survival [25]. Our study is one of the few studies showing that PD-L1 positivity in TIMC significantly shortens survival in the ChRCC group. Our findings show that the use of agents targeting PD-L1 may be beneficial in the treatment of ChRCC and CCRCC.

Kahlmeyer *et al*., in a study of 342 cases in 2019, showed that PD-1 and CTLA-4 expressions were associated with poorer survival, and the patients with both CTLA-4 expression and PD-1 expression had a higher risk for survival. This, to our knowledge, is the first description of CTLA-4 expression as a prognostic marker in RCC [5]. In a recent study by Liu *et al*., it was shown that CTLA-4 was overexpressed in CCRCC and was closely associated with disease progression and poor prognosis. Mutation analysis showed that CTLA-4 was significantly associated with multiple immune checkpoints, suggesting that CCRCC subjects with highly expressed CTLA-4 may benefit more from combined therapy with ICIs [27]. In our study, however, CTLA-4 was expressed at a higher rate in papillary RCC compared to other types. However, it was shown that PD-1 and CTLA-4 did not show any difference between advanced and early stages, did not affect survival in RCC subtypes, and were not correlated with any clinicopathological parameters. This finding, which shows that treatments targeting the inhibition of the CTLA-4 pathway will be more effective than treatment targeting PD-L1 in PRCC, needs to be supported by further studies. Another finding that we have not seen in the literature is the positive correlation between PD-L1 expressed in the tumour and CTLA-4 expressed in TIMC. It has been shown in some studies that PD-L1 expressed in the tumour increases inflammatory cytokines, and the induction of CTLA-4 with these cytokines can be shown as a factor. The clones used for PD-L1 differ in many studies.

Several recent studies have shown that there may be expression differences among clones. In the study published by Carlsson *et al*. in March 2020, two different PD-L1 clones (SP142 and 28.8) were used comparatively for the first time. A significant-

ly higher rate of positivity was found with clone 28.8 compared to SP142 [28]. Lee *et al*. used the 22C3, SP142, and E1L3N tests for PD-L1 in a retrospective cohort of 591 cases in 2020. When these 3 tests were compared, PD-L1 expression scores showed moderate to high positive correlation. Staining in TIMCs was statistically similar, although relatively less frequent with E1L3N [29]. Sommer *et al*. compared PD-L1 expression with 4 different immunohistochemical methods in advanced RCC and found that PD-L1 positivity in tumours with Ventana SP142 was significantly lower than the others (Ventana SP263, Dako 28-8, Dako 22C3). In that study, there were small statistically insignificant differences among the 4 tests for PD-L1 positivity in TIMC [30]. In the most recent Food and Drug Administration-approved inter-clone study, the lower PD-L1 positivity with the SP142 clone, which is frequently used in RCC, compared to the other 3 clones, including SP263, showed that the SP263 clone used in our study might be more suitable for RCC.

Conclusions

In several studies in the literature, different result have been obtained on these immune checkpoints. The reasons for these differences are the different clones used, intra-tumour heterogeneity (ITH), and the variability of the determined threshold values (such as 1% and 5%). Because the ITH is quite high in RCC, single-site sampling from the tumour may overlook some RCC patients who could benefit from immunotherapy. Based on the data of our study, it can be predicted that ICI therapy can be used as effectively not only in CCRCC but also in PRCC and ChRCC. It may be appropriate to evaluate PD-L1, PD-1, and CTLA-4 altogether in advanced RCC, to apply a treatment that affects both pathways if necessary, and to use an agent that specifically addresses the CTLA-4 pathway in PRCC. In addition, our findings showed that PD-L1 might also be used as a prognostic marker, particularly in CCRCC and ChRCC. The positive correlation of high TIMC-PD-L1 with locally aggressive behaviour in all types indicated the significance of examining the tumour microenvironment. Even in the absence of metastasis, further studies with large series will shed light on the importance of immunotherapy in all types of locally advanced RCC.

The authors declare no conflicts of interest.

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