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Prognostic and predictive significance of GITR in metastatic renal cell carcinoma

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Abstract. – OBJECTIVE: Renal cell carcinoma (RCC) has gradually increased in recent years. There have been significant developments in metastatic RCC in recent years with the introduction of immune control point inhibitors. Glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein (GITR) is a co-stimulatory molecule and is seen in the highest amounts in activated CD4+ T lymphocytes and CD8+ T lymphocytes, forkhead box protein 3 (FOXP3) positive regulatory T cells (Treg). GITR leads to an increase in interleukin (IL)-2 and CD25 and Interferon Gamma. It shows an anti-tumoural effect by inhibiting the suppressive functions of FOXP3+ regulatory cells (Treg). Therefore, we aimed to evaluate the prognostic and predictive effect of GITR, tumor-infiltrating lymphocytes (CD4+CD8) (TIL), and FOXP3 in patients with metastatic RCC.

PATIENTS AND METHODS: Patients diagnosed with pathologically confirmed metastatic renal cancer between 2016 and 2021 were included in our study. Clinicopathological features and some laboratory tests were recorded. GITR, CD4, CD8, and FOXP3 were evaluated by immunohistochemistry (IHC) from biopsies or nephrectomy material and recorded.

RESULTS: The study included 41 patients. The median progression-free survival (PFS) was 10.5 months, and the median overall survival (OS) was 13.9 months. Median PFS was 7.9 months for the GITR-low group and 18.9 months for the GITR-high group. Median PFS was statistically significant and longer for the GITR-high group than the GITR-low group (p=0.003). When patients who received nivolumab in the 2nd line were evaluated, median PFS was found to be 5.7 months in the GITR-low group and 15.7 months in the GITR-high group. Median PFS was statistically significantly higher in the GITR-high group than in the GITR-low group (p=0.026).

CONCLUSIONS: In patients with metastatic RCC, higher GITR was associated with better PFS. At the same time, in patients using nivolumab, better PFS was seen in the GITR high group. If supported by prospective studies, GITR can be used as both a prognostic and predictive marker.

Key Words:
GITR, FOXP3, TIL, RCC, Treg.

Introduction

Renal cell carcinoma (RCC) has gradually increased in recent years and is the most common type of kidney tumor, with a frequency of up to 5% among all cancers[1]. Significant progress has been made in recent years with the introduction of immune checkpoint inhibitors in metastatic RCC[2]. Despite all these factors, survival in metastatic RCC remains limited to 20%. Because of these low survival results, immune-based markers and therapies have become the subject of research. In a meta-analysis[3] including metastatic RCC, the systemic immune-inflammation index was found to be a poor prognostic. Many studies are ongoing on this subject.

The tumor necrosis factor receptor (TNFR) superfamily is a transmembrane protein cluster of approximately 20 members, rich in cysteine and with additional variations. Glucocorticoid-induced TNF receptor-related protein (GITR), also known as TNFRSF18 and CD357, is one of the most important and first-found members of this family[4]. GITR is seen in the highest amounts in activated CD4+ T lymphocytes and CD8+ T lymphocytes, forkhead box protein 3 (FOXP3) positive regulatory T cells (Treg). GITR is seen at moderate levels in dendritic cells and macrophages from antigen-presenting cells and is expressed less frequently in unstimulated natural T

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cells and memory T cells[5,6]. GITR is a co-stimulatory molecule. GITR ligand (GITRL) is a molecule found on antigen-presenting cells (APC) and endothelial cells[7].

After antigen presentation by APC to naive T lymphocytes, the T cell receptor (TCR) is stimulated, and changes occur in activator and inhibitor cell surface receptors within 24-72 hours. After TCR stimulation, GITR and GITRL binding occurs. Then, activation of mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) pathways occurs through TNF receptor-associated factor 2 (TRAF-2) and TNF receptor-associated factor 5 (TRAF-5). This is followed by an increase in the T cell growth factor IL-2 and CD25 and Interferon Gamma. Thus, T cell proliferation and activation increase. The number of memory T cells increases by protecting T cells from activation-induced cell death[8,9]. As a result of the combination of GITR and GITRL, while activating CD8 T cells by increasing CD28 on one side, an anti-tumoral effect is shown by inhibiting the suppressive functions of Treg[10]. Inhibition of Tregs has also been shown to occur by inhibiting the Treg transcription factor. GITR also shows its effect through NF-kappa B (NF-κB), but since it has been determined[11] that NF-κB has no role in CD28 activation, it is thought that GITR acts not only through CD28 but also through other pathways. It has also been shown[12] in a mouse model that GITR agonism increases the Teff/Treg ratio. In conclusion, it has been revealed in Phase-1 studies[13,14] that GITR agonism plays a prognostic role in many cancers. Natural Treg cells are formed in the thymus by high-affinity binding of TCR and major histocompatibility complex (MHC) class II[15].

FOXP3 is a transcription factor expressed on the surface of Tregs and is more specific than CD25. FOXP3-positive Tregs inhibit the attack against self-antigens. They also inhibit anti-tumoral responses[16]. FOXP3 activation occurs after TCR and CD28 binding. FOXP3 interacts with the factors of activated T cells and increases the expression of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) while decreasing cytokines that aid lymphocyte growth such as IL-2 and IL-4. Therefore, it has an inhibitory effect on T-cell proliferation and differentiation[17,18].

GITR, which is found in excess in Tregs, eliminates the inhibitory functions of Tregs[19]. Tregs increase anti-inflammatory cytokines such as IL-10 and TGF-β, inhibit the activation of APC by CTLA-4 activation, and stimulate perforin and granzyme secretion. Tregs also contribute to self-tolerance by inhibiting IL-2 secretion[20]. FOXP3 expression, a transcription marker of Tregs, has been shown[21,22] to be a poor prognostic factor in some cancers, while, FOXP3 positivity has been shown[23] to be a favorable prognostic factor in some cancers. Since tumor cells are surrounded by infiltrating cells, tumors surrounded by immune cells are considered immunologically sensitive tumors. TILs are the most important determinants of the host immune response against tumors[24,25]. As the amount of CD4 T lymphocytes increases, the possibility of presentation to APCs increases, and they contribute to cytokine secretion and tumor death. The increased amount of CD8 T lymphocytes also increases tumor destruction[26]. Tumor-infiltrating lymphocytes (TIL) have been shown[27,28] to predict prognosis in many cancers.

In conclusion, a high expression level of GITR, an activator marker, indicates a good prognosis. The high level of FOXP3, which is a surface marker of Tregs and a transcription factor, increases tumor growth. Therefore, we aimed to evaluate the effect of GITR, FOXP3, and TIL (CD4+CD8) on the prognosis in patients with metastatic RCC.

Patients and Methods

Patients and Tissue Collection

Between January 1, 2016, and January 1, 2021, the files of patients diagnosed with metastatic renal cancer who were admitted to Adnan Menderes University Medical Oncology Clinic were reviewed. Patients over the age of 18 who had sufficient follow-up data and consented to use their data were included in the study. Informed consent was obtained from all individual participants included in the study. At the same time, patients using steroids, patients with autoimmune diseases, patients with secondary cancer or hematological diseases, and patients with active infections were excluded because they could affect the lymphocyte population. The clinicopathological information such as age, gender, performance status at the time of diagnosis, comorbidities, as well as the year of diagnosis, progression times, recurrence times, metastasis locations, exitus date, hemogram, and biochemistry data at the time of diagnosis were recorded in IBM SPSS 25. We also aimed to show the effect of immunotherapy and other treatments on median survival and time to progression when renal cancer patients were classified according to the treatment received.

Estimation of GITR, FOXP3, and TIL

Hematoxylin-eosin-stained sections of the subjects included in the study were examined, and 4-micron-thick sections were prepared from paraffin blocks that best represented the tumor. The sections were then incubated for 1 hour in a 60°C oven. Tissue sections were subjected to immunohistochemical staining for the markers GITR, FOXP3, CD4, and CD8 using the Ventana BenchMark XT automated immunohistochemical stainer, (Basel, Switzerland) and the amounts of GITR, FOXP3, CD4, and CD8 were measured, as shown in Figure 1. Subsequently, we aimed to evaluate the response to treatment and identify biomarkers that would affect the treatment response.

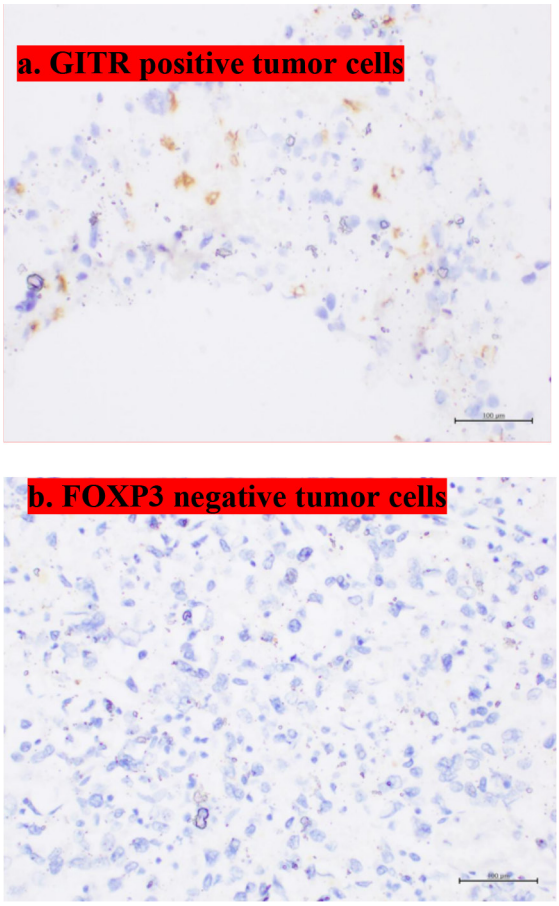

Figure 1. With the help of scale bars representing 100 µm, positive cytomembranous staining with GITR in tumor cells and negative cytomembranous staining with FOXP3 in tumor cells are shown in Figure 1. **a**, Positive cytomembranous staining with GITR in tumoral cells. **b**, Negative cytomembranous staining with FOXP3 in tumoural cells (Scale bars represent 100 µm).

Statistical Analysis

The normality of the data was evaluated by the Kolmogorov-Smirnov test. Parametric data were presented as mean ± standard deviation, and categorical data were presented as frequency (rate). The Chi-square or Fisher's Exact test was used to compare categorical data. The independent sample *t*-test was used to compare the continuous data of independent groups. The X-Tile program (IBM Corp., Armonk, NY, USA) as used to determine the optimal cut-off value of GITR and FOXP3 to predict survival. The log-rank test was used in univariate survival analyses. Median survival times were calculated using the Kaplan-Meier method. A Cox regression model was created with the parameters obtained ($p<0.05$) in univariate analysis, and dependent prognostic factors were determined. Patients were compared by grouping GITR and FOXP3 as high *vs.* low. SPSS 25 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. All statistical analyses were performed two-way, and $p<0.05$ was considered statistically significant.

This study was conducted and designed in accordance with the Declaration of Helsinki and Good Clinical Practice and was approved by the Ethics Committee of Aydın Adnan Menderes University (Acceptance Date-No.: 08.04.2021-2022/25929).

Results

In our study, we found that the GITR expression level measured in patients with metastatic RCC is a good prognostic marker. Although our study was retrospective and had a limited number of patients, the findings were significant. Additionally, in the patient population receiving nivolumab, progression-free survival (PFS) was higher in the GITR-high group than in the GITR-low group. This suggests that GITR may be a predictive marker for immunotherapy. If the prognostic and predictive importance of GITR is supported by prospective studies with a higher number of patients to be prognostic and predictive for immunotherapy, it can be used for predictive purposes, similar to Programmed cell Death Ligand-1 (PDL-1) in our daily practice.

Our study included 41 patients. The median age was 65 years (range 55.6-74.4), and 32 (78.0%) of the patients were males. 31 (75.6%) of the patients had a Karnofsky Performance Status (KPS) <70. About half of the patients, 21 (51.2%), were in the intermediate group, according to the Inter-

national Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk score.

In the GITR >5 (GITR-high) group, the number of FOXP3 >2 (FOXP3-high) patients was 7 (53.8%), while the number of FOXP3 <2 (FOXP3-low) patients was 6 (46.2%). In the GITR <5 (GITR-low) group, the number of FOXP3-high patients was 12 (29.3%), and the number of FOXP3-low patients was 29 (70.7%). The rate of FOXP3-high patients was higher in the GITR-high group compared to the GITR-low group (p=0.029). Otherwise, there was no significant difference between the GITR-low and GITR-high groups.

The number of patients with bone metastasis was 15 (51.7%) in the FOXP3-low group and 1 (8.3%) in the FOXP3-high group. The rate of bone metastatic patients was higher in the FOXP3-low group compared to the FOXP3-high group (p=0.013). Besides, other characteristics of the FOXP3-low and FOXP3-high groups were similar. The basic characteristics of all patients are shown in detail in Table I.

The median follow-up period was 26.6 months (95% CI: 18.9-34.2). Median PFS was 10.5 months (95% CI: 4.9-16.1), and median overall survival (OS) was 13.9 months (95% CI: 5.1-22.6). PFS was 7.9 months (95% CI: 5.7-10.1) for the GITR-low group and 18.9 months (95% CI: 13.1-24.8) for the GITR-high group. Median PFS was statistically significant and longer for the GITR-high group compared to the GITR-low group (p=0.003).

Table I. Baseline characteristics of the patients according to GITR and FOXP3.

	Total	GITR low n: 28	GITR high n: 13	p	FOXP3 low n: 29	FOXP3 high n: 12	p
Age	65±9.6	65±8.3	64±12.3	0.744	66±9.3	62±9.9	0.186
Gender							
Female	9 (22.0)	6 (21.4)	3 (23.1)	0.906	8 (27.6)	1 (8.3)	0.240
Male	32 (78.0)	22 (78.6)	10 (76.9)		21 (72.4)	11 (91.7)	
Karnofsky							
<70	31 (75.6)	19 (67.9)	12 (92.3)	0.129	21 (72.4)	10 (83.3)	0.694
>70	10 (24.4)	9 (32.1)	1 (7.7)		8 (27.6)	2 (16.7)	
IMDC							
Favorable	10 (24.4)	7 (25.0)	3 (23.1)	0.594	8 (27.6)	2 (16.7)	0.130
Intermediate	21 (51.2)	13 (46.4)	8 (61.5)		12 (41.4)	9 (75.0)	
Poor	10 (24.4)	8 (28.6)	2 (15.4)		9 (31.0)	1 (8.3)	
Lymph node metastasis							
No	27 (65.9)	19 (67.9)	8 (61.5)	0.734	21 (72.4)	6 (50.0)	0.278
Yes	14 (34.1)	9 (32.1)	5 (38.5)		8 (27.6)	6 (50.0)	
Liver metastasis							
No	33 (80.5)	21 (75.0)	12 (92.3)	0.398	22 (75.9)	11 (91.7)	0.398
Yes	8 (19.5)	7 (25.0)	1 (7.7)		7 (24.1)	1 (8.3)	
Bone metastasis							
No	25 (61.0)	17 (60.7)	8 (61.5)	0.960	14 (48.3)	11 (91.7)	0.013
Yes	16 (39.0)	11 (39.3)	5 (38.5)		15 (51.7)	1 (8.3)	
Brain metastasis							
No	38 (92.7)	26 (92.9)	12 (92.3)	1.000	27 (93.1)	11 (91.7)	1.000
Yes	3 (7.3)	2 (7.1)	1 (7.7)		2 (6.9)	1 (8.3)	
TIL							
≤18	21 (51.2)	16 (57.1)	5 (38.5)	0.265	15 (51.7)	6 (50.0)	0.920
>18	20 (48.8)	12 (42.9)	8 (61.5)		14 (48.3)	6 (50.0)	
FOXP3							
≤2	29 (70.7)	23 (82.1)	6 (46.2)	0.029	-	-	-
>2	12 (29.3)	5 (17.9)	7 (53.8)		-	-	

GITR: Glucocorticoid-induced TNF receptor-related protein, FOXP3: forkhead box protein3. TIL: Tumor infiltrating lymphocytes, IMDC: International Metastatic RCC Database Consortium.

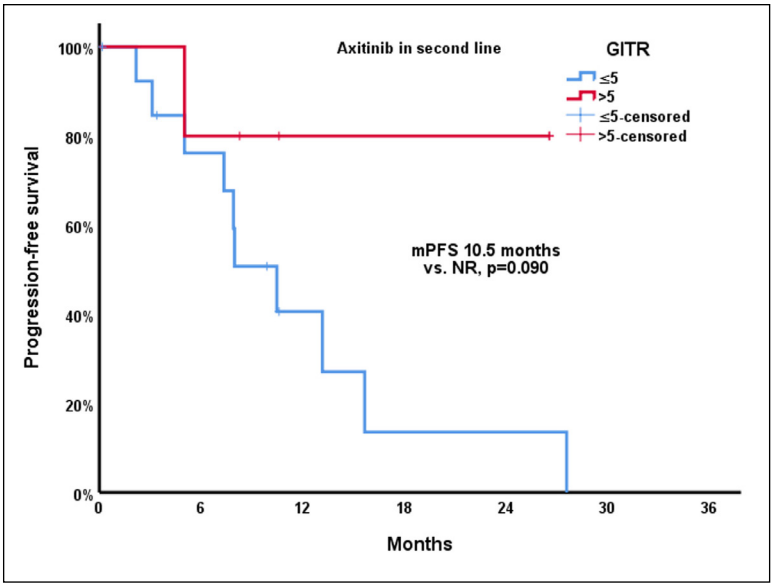

Figure 2. Progression-free survival by GITR level in patients receiving axitinib. When patients receiving axitinib in 2nd line were evaluated, the median PFS was 10.5 months in GITR-low group, while PFS data were not reached (NR) in GITR-high group. Median PFS was numerically higher in the GITR-high group than in the GITR-low group, but not statistically significant (p=0.090) as shown in Figure 2.

Median PFS was 8.0 months (95% CI: 5.9-10.1) for the FOXP3-low group and 16.3 months (95% CI: 14.7-17.8) for the FOXP3-high group. Median PFS was statistically significant and longer for the FOXP3-high group compared to the FOXP3-low group (p=0.028).

Median PFS was 10.0 months (95% CI: 4.6-15.5) for the TIL <18 (TIL-low) group and 13.2 months (95% CI: 5.5-20.8) for the TIL>18 (TIL-high) group. Median PFS was longer in the TIL-high group than in the TIL-low group but not statistically significant (p=0.683).

Median OS was 11.9 months (95% CI: 5.7-10.1) for the GITR-low group and 28.2 months (95% CI: 12.0-44.3) for the GITR-high group. Median OS was longer for the GITR-high group than for the GITR-low group, but it was not statistically significant (p=0.070).

Median OS was 11.9 months (95% CI: 8.6-15.2) for the FOXP3-low group, and OS data were not reached (NR) for the FOXP3-high group. Median OS was longer for the FOXP3-high group than the FOXP3-low group and was statistically significant (p=0.034).

Median OS was 10.3 months (95% CI: 9.6-11.04) for the TIL-low group and 19.6 months (95% CI: 3.5-35.7) for the TIL-high group. Median OS was longer in the TIL-high group than in the TIL-low group, but it was not statistically significant (p=0.357).

Univariate analyses of PFS and OS are shown in detail in Table II.

When patients receiving axitinib in the 2nd line were evaluated, the median PFS was 10.5 months in the GITR-low group, while PFS data were not reached in the GITR-high group. Median PFS was numerically higher in the GITR-high group than in the GITR-low group, but not statistically significant (p=0.090), as shown in Figure 2.

When patients who received Nivolumab in the 2nd line were evaluated, the median PFS was 5.7 months in the GITR-low group and 15.7 months in the GITR-high group. Median PFS was statistically significantly higher in the GITR-high group than in the GITR-low group (p=0.026), as shown in Figure 3.

Multivariate Cox regression analysis was performed to determine the independent prognostic factors affecting PFS and is shown in Table III. GITR expression was the only independent prognostic factor affecting PFS in patients with metastatic RCC (HR: 5.892, 95% CI: 1.903-18.242, p=0.004).

Discussion

With the significant improvement in survival seen in inhibitors of immune checkpoint m olecules such as CTLA-4, PD-L1, and Programmed cell Death-1 (PD-1), research on immunotherapy has been increasing. Inhibition of these and other novel immune checkpoint activators has led to advancements in anti-tumoral therapy. Studies[29,30] exploring the stimulation of checkpoint activators such as inducible co-stimulator (ICOS), GITR, CD134, CD40, and CD137 are also being conducted rapidly.

Table II. Univariate analysis of PFS and OS.

	Median PFS (95% CI)	p	Median OS (95% CI)	p
Age				
≤65	13.2 (5.6-20.8)	0.420	28.2 (9.3-47.1)	0.983
>65	10.0 (7.0-13.1)		11.9 (5.9-17.9)	
Gender				
Female	15.7 (0-37.1)	0.636	16.1 (5.2-22.0)	0.891
Male	10.3 (5.2-15.4)		13.9 (3.7-24.1)	
Karnofsky				
<70	10.5 (6.2-14.7)	0.812	13.6 (9.5-17.8)	0.231
>70	15.7 (3.7-27.6)		29.1 (4.9-53.3)	
IMDC				
Favorable	13.2 (1.3-24.9)	0.271	13.3 (4.0-22.6)	0.948
Intermediate	10.5 (0.9-19.9)		13.9 (10.5-17.2)	
Poor	6.6 (4.5-8.7)		10.3 (0-26.3)	
Lymph node metastasis				
No	10.5 (4.8-16.2)	0.186	13.3 (8.9-17.6)	0.298
Yes	10.3 (0-25.3)		NR	
Liver metastasis				
No	14.9 (7.4-22.4)	<0.001	19.6 (1.9-37.2)	0.002
Yes	5.0 (4.7-5.4)		8.4 (7.5-9.3)	
Bone metastasis				
No	10.3 (4.3-16.3)	0.899	19.6 (4.6-34.5)	0.490
Yes	10.5 (1.3-19.6)		10.5 (7.1-13.9)	
Brain metastasis				
No	10.3 (7.1-13.5)	0.401	13.6 (5.2-22.0)	0.657
Yes	15.7 (NA)		53.3 (NA)	
Therapy				
Axitinib	13.2 (5.4-20.9)	0.689	16.2 (6.1-38.6)	0.266
Nivolumab	10.3 (2.2-18.5)		28.2 (4.3-52.1)	
FOXP3				
≤2	8.0 (5.9-10.1)	0.028	11.9 (8.6-15.2)	0.034
>2	16.3 (14.7-17.8)		NR	
GITR				
≤5	7.9 (5.7-10.1)	0.003	11.9 (8.1-15.7)	0.070
>5	18.9 (13.1-24.8)		28.2 (12.0-44.3)	
TIL				
≤18	10.0 (4.6-15.5)	0.683	10.3 (9.6-11.04)	0.357
>18	13.2 (5.5-20.8)		19.6 (3.5-35.7)	

GITR: Glucocorticoid-induced TNF receptor-related protein, FOXP: forkhead box protein3 TIL: Tumor infiltrating lymphocytes, PFS: Progression-free survival, OS: Overall Survival, CI: Confidence interval, NR: Not reached.

In a study by Zappasodi et al[13] involving seven different cancers, the GITR expression level in rat tumor models was evaluated by flow cytometry and immunohistochemistry. It was observed that RCC, non-squamous cell lung cancer (NSCLC) and malignant melanoma were the cancers with the highest GITR-expressing cell ratios in Tregs and the highest GITR expression intensity per cell.

In a study[20] involving approximately 15 thousand patients and 31 different cancers, high GITR expression was found to be associated with a good prognosis. Many factors play a role in tumor formation in metastatic RCC. Long non-coding RNA (lncRNA) DNAJC3 antisense RNA 1 acts as an oncogene and plays an important role in tumorigenesis of metastatic RCC[31]. In another study[32], 4 ferroptosis- and immune-related differentially ex-

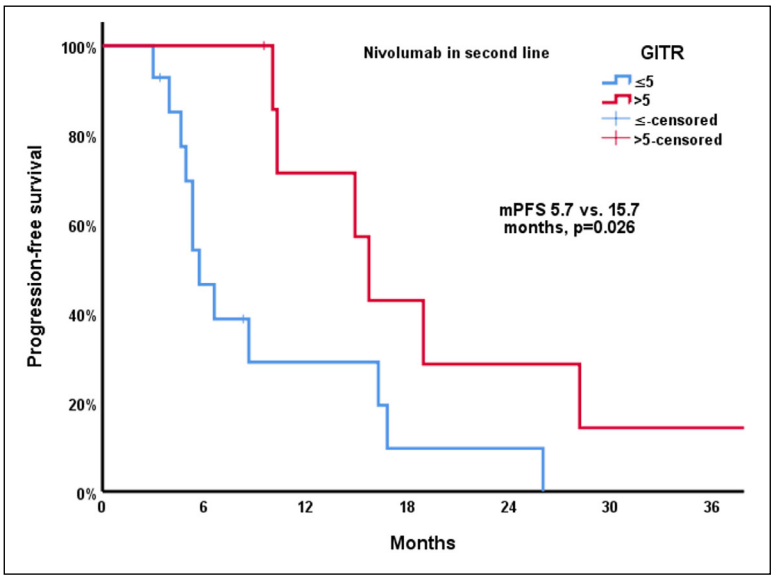

Figure 3. Progression-free survival by GITR level in patients receiving nivolumab. When patients who received Nivolumab in the 2nd line were evaluated, the median PFS was 5.7 months in the GITR-low group and 15.7 months in the GITR-high group. Median PFS was statistically significantly higher in the GITR-high group than in the GITR-low group (p=0.026) as shown in Figure 3.

pressed genes emerged as an important marker in diagnosis and prognosis in patients with metastatic RCC, but many studies are needed in this area.

We decided to perform this study to investigate the effect of GITR on prognosis and recurrence. As a primary hypothesis, we wanted to examine the effect of GITR, a T-cell stimulator marker, on survival in patients with metastatic RCC. In the study, which included 41 patients, the median progression-free survival (PFS) was 7.9 months for the GITR-low group and 18.9 months for the GITR-high group. Median PFS was statistically significant and longer for the GITR-high group than the GITR-low group (p=0.003). When patients receiving nivolumab were evaluated, the median PFS was 5.7 months in the GITR-low group and 15.7 months in the GITR-high group. Median PFS was statistically significantly higher in the GITR-high group than in the GITR-low group (p=0.026) in nivolumab recipients, as shown in Figure 2.

Our study was found to be consistent with the literature. In breast cancer, rectal adenocarcinoma, and cutaneous malignant melanoma, higher GITR expression levels were associated with increased PFS and OS[33]. In a study[34] in which human hepatocellular carcinoma cells were taken and evaluated *in vitro*, the use of GITR agonism in combination with CTLA-4 blockade was shown to increase the antitumoral effect by reducing Tregs.

Table III. Multivariate analysis of PFS and OS.

	PFS		OS	
	HR (95% CI)	p	HR (95% CI)	p
FOXP3				
≤2	Ref	0.091	Ref	0.158
>2	0.413 (0.148-1.153)		0.398 (0.111-1.428)	
GITR				
≤5	Ref	0.004	Ref	0.172
>5	0.218 (0.077-0.613)		0.489 (0.175-1.366)	
Liver metastasis				
No	Ref	0.056	Ref	0.052
Yes	2.977 (0.973-9.108)		3.139 (0.988-9.973)	

GITR: Glucocorticoid-induced TNF receptor-related protein, FOXP: forkhead box protein3. PFS: Progression-free survival, OS: Overall Survival, HR: Hazard ratio, Ref: Reference.

In a Phase-1 study[35] of 113 patients, the objective response rate (ORR) in the combination branch using MK-4166 (San Antonio, TX, USA), a GITR agonist, and pembrolizumab, was 62%, while the ORR when MK-4166 was used as a single agent was 2%. In a study[36] evaluating several different solid tumors, the combination of GITR agonist TRX518 (Cleveland, OH, USA) and anti-PD-1 decreased intratumoral Tregs and increased CD8 T cells. GWN323 (Houston, TX, USA), a GITR agonist, in combination with spartalizumab (anti-PD-1) showed[37] an increase in effector T cells and a decrease in Tregs. In a Phase-1 study[38] in patients with malignant melanoma, RCC, and colorectal cancer, MK-1248, a GITR agonist, was found to enhance tumor response when used in combination with pembrolizumab compared to the single pembrolizumab branch.

If we look at preclinical models, in a study by Bulliard et al[39], it was observed that the DTA-1 (NY, USA) molecule increased tumor destruction in rats with colon cancer and increased the CD8 T/Treg ratio. Again, in a study[12] using DTA-1 in rats with melanoma, it was shown that DTA-1 decreased Tregs and inhibited tumor growth. Combined anti-PD-1 and GITR agonist treatment in ovarian cancer rats enhanced tumor response by increasing the amount of IFN-gamma-producing effector T cells[40,41]. GITR agonism was found[42] to be synergistic with TIL and enhanced tumor response in an *in vitro* study of hepatocellular cancer tumors. In addition, the use of GITR agonism in combination with anti-PDL-1 and a peptide vaccine increases tumor response by increasing effector T cells and decreasing Tregs[43]. In a study[44] in rats with intracranial glioma, GITR agonist in combination with stereotaxic radiotherapy increased the survival benefit. MK-4166, a GITR agonistic agent, has been shown[45] to increase anti-tumoral response by reducing Tregs in rats with melanoma. GITRL and agonistic antibodies targeting the OX-40 molecule, a stimulatory molecule, have been shown[46] to have a synergistic effect when used together and to enhance tumor response by decreasing Tregs and increasing effector T cells.

Median OS was 11.9 months for the FOXP3-low group, and OS data for the FOXP3-high group were not reached (NR). Median OS was longer for the FOXP3-high group than the FOXP3-low group and was statistically significant (p=0.034). If we look at the literature, in a study conducted on metastatic tongue cancer, it was observed that FOXP3 increased tumor activity by increasing Tregs in the tumor microenvironment[47]. In three studies[21,48,49] in patients with metastatic RCC, the presence of increased FOXP3-positive Tregs was associated with poor prognosis. In two studies[50,51] conducted in patients with metastatic cervix, RCC, malignant melanoma, and breast cancer, FOXP3 increase caused short OS. In a study[52] with patients with bilateral breast cancer, FOXP3 positivity was associated with high TIL and poor prognosis. In a study in patients with NSCLC, the presence of FOXP3 was associated with poor prognosis[53]. In a study[54] of 20 healthy people and 20 breast cancer patients, FOXP3 positivity increased tumor growth. In a study[55] of 49 patients with metastatic RCC and 38 healthy volunteers, the presence of Tregs was found to be a predisposing factor for the development of RCC. In a meta-analysis by Shang et al[50], the prognostic value of FOXP3+ Tregs varies according to the type of carcinoma. High FOXP3+ Treg infiltration was associated with poor prognosis in most of the tumors examined, such as RCC, cervix, and breast. No prognostic effect of FOXP3+ Tregs was observed in pancreatic and ovarian cancer. FOXP3+ Tregs were associated with a favorable prognosis in head and neck, colorectal, and esophageal cancers. The literature data on the correlation between FOXP3 and prognosis are confusing, and the reason is unknown. New prospective studies with a higher number of patients are needed.

Median PFS was 10.0 months for the TIL-low group and 13.2 months for the TIL-high group. Median PFS was longer for the TIL-high group than the TIL-low group, but not statistically significant (p=0.683). When we look at the literature, in a meta-analysis[56] including 416 RCC patients, the presence of high TIL was found to be a favorable prognostic factor. In other studies[57,58] conducted on patients with RCC, high TIL was found to predict poor prognosis and high tumor grade. When we look at other cancers, in a study[59] conducted on patients with triple-negative breast cancer, the presence and rate of TIL correlated with disease response, while this correlation was not found in patients with both breast cancers. In a study[60] of 1,815 patients with metastatic ovarian cancer, low TIL was associated with poor survival. In a study[61] evaluating primary malignant melanoma cases, high TIL was found to be a favorable prognostic factor.

In a study[62] of 8,600 NSCLC patients, higher TIL was associated with better survival. This study has shown a positive correlation between

TIL and prognosis. In our study, although the correlation between TIL and the prognosis of patients with metastatic RCC was numerically significant, it was not statistically significant due to the small number of patients, the retrospective study design, and the inability to perform immunophenotyping at the same time.

The small number of patients and retrospective planning are among the limitations of our study. At the same time, due to the monovalent staining of markers such as GITR and FOXP3, it was not possible to determine how high the GITR positivity was in Tregs. Additionally, due to technical limitations, only CD4 and CD8-positive TILs were quantified, and natural killer cells (NK) could not be calculated. Since the FOXP3-negative Treg population is little known and CD25 is a Treg marker, we could not determine the actual regulatory T-cell population due to technical limitations. Patients with metastatic RCC who underwent nephrectomy were also included in our study. Considering that tumor immunogenicity is high in the primary tumor, the relatively low immune response in patients with nephrectomy stood out as one of the limitations in determining prognosis. If prospective studies with bivalent staining to determine immune cells for prognosis determination are designed, our study may become more meaningful and clinically applicable.

Conclusions

GITR is a useful and practical prognostic marker in patients with metastatic RCC. If supported by prospective studies with a higher number of patients, it holds promise as a marker that can be used in routine practice.

Ethics Approval

This study was conducted and designed in accordance with the Declaration of Helsinki and Good Clinical Practice and was approved by the Ethics Committee of Aydın Adnan Menderes University (Acceptance Date-No.: 08.04.2021-2022/25929).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Authors' Contributions

Design of the study, acquisition of data or analysis and interpretation of data: Esin OKTAY, Onur Yazdan BALÇIK, Ali AYTAÇ, Bilgin DEMİR. Drafting the article or making critical revisions related to the relevant intellectual content of the manuscript; supervision; Esin OKTAY, Onur Yazdan BALÇIK, Nil ÇULHACI, Gökçe Su CEYLAN, Dilara AKIN. Validation and final approval of the version of the article to be published: Esin OKTAY, Onur Yazdan BALÇIK, Ali AYTAÇ, Bilgin DEMİR, Nil ÇULHACI, Gökçe Su CEYLAN, Dilara AKIN.

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Conflict of Interest

The authors declare that they have no conflict of interest to declare.

References

1) Capitanio U, Bensalah K, Bex A, Boorjian SA, Bray F, Coleman J, Gore JL, Sun M, Wood C, Russo P. Epidemiology of renal cell carcinoma. Eur Urol 2019; 75: 74-84.
2) Rijnders M, de Wit R, Boormans JL, Lolkema MP, van der Veldt AA. Systematic review of immune checkpoint inhibition in urological cancers. Eur Urol 2017; 72: 411-423.
3) Wang Q, Zhu SR, Huang XP, Liu XQ, Liu JB, Tian G. Prognostic value of systemic immune-inflammation index in patients with urinary system cancers: a meta-analysis. Eur Rev Med Pharmacol Sci 2021; 25: 1302-1310.
4) Younes A, Cerutti A. Tumor Necrosis Factor Family of Ligands and Receptors in Cancer Therapy. The Oncogenomics Handbook 2005; 509-529.
5) Hanabuchi S, Watanabe N, Wang YH, Wang YH, Ito T, Shaw J, Cao W, Qin FX, Liu YJ. Human plasmacytoid predendritic cells activate NK cells through glucocorticoid-induced tumor necrosis factor receptor-ligand (GITRL). Blood 2006; 107: 3617-3623.

6) Clouthier DL, Watts TH. Cell-specific and context-dependent effects of GITR in cancer, autoimmunity, and infection. Cytokine Growth Factor Rev 2014; 25: 91-106.

7) Krausz LT, Bianchini R, Ronchetti S, Fettucciari K, Nocentini G, Riccardi C. GITR-GITRL system, a novel player in shock and inflammation. ScientificWorldJournal 2007; 7: 533-566.

8) Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, Ayroldi E, Riccardi C. Frontline: GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. Eur J Immunol 2004; 34: 613-622.

9) Schaer DA, Murphy JT, Wolchok JD. Modulation of GITR for cancer immunotherapy. Curr Opin Immunol 2012; 24: 217-224.

10) Snell LM, McPherson AJ, Lin GH, Sakaguchi S, Pandolfi PP, Riccardi C, Watts TH. CD8 T cell-intrinsic GITR is required for T cell clonal expansion and mouse survival following severe influenza infection. J Immunol 2010; 185: 7223-7234.

11) Ronchetti S, Nocentini G, Bianchini R, Krausz LT, Migliorati G, Riccardi C. Glucocorticoid-induced TNFR-related protein lowers the threshold of CD28 costimulation in CD8+ T cells. J Immunol 2007; 179: 5916-5926.

12) Schaer DA, Budhu S, Liu C, Bryson C, Malandro N, Cohen A, Zhong H, Yang X, Houghton AN, Merghoub T, Wolchok JD. GITR pathway activation abrogates tumor immune suppression through loss of regulatory T-cell lineage stability. Cancer Immunol Res 2013; 1: 320-331.

13) Zappasodi R, Sirard C, Li Y, Budhu S, Abu-Akeel M, Liu C, Yang X, Zhong H, Newman W, Qi J, Wong P. Rational design of anti-GITR-based combination immunotherapy. Nat Med 2019; 25: 759-766.

14) Knee DA, Hewes B, Brogdon JL. Rationale for anti-GITR cancer immunotherapy. Eur J Cancer 2016; 67: 1-10.

15) Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. Nat Immunol 2002; 3: 756-763.

16) Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008; 133: 775-787.

17) Piccirillo CA. Regulatory T cells in health and disease. Cytokine 2008; 43: 395-401.

18) Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006; 24: 209-226.

19) Munn DH, Mellor AL. Indoleamine 2, 3-dioxygenase and tumor-induced tolerance. J Clin Invest 2007; 117: 1147-1154.

20) Jafarzadeh A, Jamali M, Mahdavi R, Ebrahimi HA, Hajghani H, Khosravimashizi A, Nemati M, Najafipour H, Sheikhi A, Mohammadi MM, Daneshvar H. Circulating levels of interleukin-35 in patients with multiple sclerosis: evaluation of the influences of FOXP3 gene polymorphism and treatment program. J Mol Neurosci 2015; 55: 891-897.

21) Sell K, Barth PJ, Moll R, Thomas MA, Zimmer N, Oplesch E, Gudo M, Schrader M, Hofmann R, Schrader AJ. Localization of FOXP3-positive cells in renal cell carcinoma. Tumour Biol 2012; 33: 507-513.

22) Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NE, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res 2006; 12: 465-472.

23) Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 2009; 27: 1861-1892.

24) Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Pénault-Llorca F, Perez EA. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015; 26: 259-271.

25) Hodi FS, Dranoff G. The biologic importance of tumor-infiltrating lymphocytes. J Cutan Pathol 2010; 37: 48-53.

26) Zitvogel L, Galluzzi L, Kepp O, Smyth MJ, Kroemer G. Type I interferons in anticancer immunity. Nat Rev Immunol 2015; 15: 405-414.

27) Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, Rouas G, Francis P, Crown JP, Hitre E, De Azambuja E. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol 2013; 31: 860-867.

28) Maibach F, Sadozai H, Seyed Jafari SM, Hunger RE, Schenk M. Tumor-infiltrating lymphocytes and their prognostic value in cutaneous melanoma. Front Immunol 2020; 11: 2105-2119.

29) Wojtukiewicz MZ, Rek MM, Karpowicz K, Górska M, Polityńska B, Wojtukiewicz AM, Moniuszko M, Radziwon P, Tucker SC, Honn KV. Inhibitors of immune checkpoints—PD-1, PD-L1, CTLA-4—new opportunities for cancer patients and a new challenge for internists and general practitioners. Cancer Metastasis Rev 2021; 40: 949-982.

30) Marin-Acevedo JA, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. J Hematol Oncol 2018; 11: 1-20.

31) Na XY, Hu XQ, Zhao Y, Hu CH, Shang XS. LncRNA DNAJC3-AS1 functions as oncogene in renal cell carcinoma via regulation of the miR-27a-3p/PRDM14 axis. Eur Rev Med Pharmacol Sci 2021; 25: 1291-1301.

32) Xing XL, Liu Y, Liu JH, Zhou HF, Zhang HR, Zuo Q, Bu P, Duan T, Zhou Y, Xiao ZQ. A nomogram integrating ferroptosis-and immune-related biomarker for prediction of prognosis and diagnosis

in kidney renal clear cell carcinoma. Eur Rev Med Pharmacol Sci 2022; 26: 6176-6186.

33) Giampietri C, Scatozza F, Crecca E, Vigiano Benedetti V, Natali PG, Facchiano A. Analysis of gene expression levels and their impact on survival in 31 cancer-types patients identifies novel prognostic markers and suggests unexplored immunotherapy treatment options in a wide range of malignancies. J Transl Med 2022; 20: 467.481.

34) Pedroza-Gonzalez A, Zhou G, Singh SP, Boor PP, Pan Q, Grunhagen D, de Jonge J, Tran TK, Verhoef C, IJzermans JN, Janssen HL. GITR engagement in combination with CTLA-4 blockade completely abrogates immunosuppression mediated by human liver tumor-derived regulatory T cells ex vivo. Oncoimmunology 2015; 4: e1051297.

35) Papadopoulos KP, Autio K, Golan T, Dobrenkov K, Chartash E, Chen Q, Wnek R, Long GV. Phase I study of MK-4166, an anti-human glucocorticoid-induced TNF receptor antibody, alone or with pembrolizumab in advanced solid tumors. Clin Cancer Res 2021; 27: 1904-1911.

36) Davar D, Zappasodi R, Wang H, Naik GS, Sato T, Bauer T, Bajor D, Rixe O, Newman W, Qi J, Holland A. Phase Ib study of GITR agonist antibody TRX518 singly and in combination with gemcitabine, pembrolizumab, or nivolumab in patients with advanced solid tumors. Clin Cancer Res 2022; 28: 3990-4002.

37) Piha-Paul SA, Geva R, Tan TJ, Lim DW, Hierro C, Doi T, Rahma O, Lesokhin A, Luke JJ, Otero J, Nardi L. First-in-human phase I/Ib open-label dose-escalation study of GWN323 (anti-GITR) as a single agent and in combination with spartalizumab (anti-PD-1) in patients with advanced solid tumors and lymphomas. J Immunother Cancer 2021; 9: e002863.

38) Geva R, Voskoboynik M, Dobrenkov K, Mayawala K, Gwo J, Wnek R, Chartash E, Long GV. First-in-human phase 1 study of MK-1248, an anti–glucocorticoid-induced tumor necrosis factor receptor agonist monoclonal antibody, as monotherapy or with pembrolizumab in patients with advanced solid tumors. Cancer 2020; 126: 4926-4935.

39) Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, Wilson NS, Dranoff G, Brogdon JL. Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med 2013; 210: 1685-1693.

40) Lu L, Xu X, Zhang B, Zhang R, Ji H, Wang X. Combined PD-1 blockade and GITR triggering induce a potent antitumor immunity in murine cancer models and synergizes with chemotherapeutic drugs. J Transl Med 2014; 12: 1-11.

41) Wang B, Zhang W, Jankovic V, Golubov J, Poon P, Oswald EM, Gurer C, Wei J, Ramos I, Wu Q, Waite J. Combination cancer immunotherapy targeting PD-1 and GITR can rescue CD8+ T cell dysfunction and maintain memory phenotype. Sci Immunol 2018; 2: 3-16.

42) Van Beek AA, Zhou G, Doukas M, Boor PP, Noordam L, Mancham S, Campos Carrascosa L, van der Heide-Mulder M, Polak WG, Ijzermans JN, Pan Q. GITR ligation enhances functionality of tumor-infiltrating T cells in hepatocellular carcinoma. Int J Cancer 2019; 145: 1111-1124.

43) Villarreal DO, Chin D, Smith MA, Luistro LL, Snyder LA. Combination GITR targeting/PD-1 blockade with vaccination drives robust antigen-specific antitumor immunity. Oncotarget 2017; 8: 39117-39130.

44) Patel MA, Kim JE, Theodros D, Tam A, Velarde E, Kochel CM, Francica B, Nirschl TR, Ghasemzadeh A, Mathios D, Harris-Bookman S. Agonist anti-GITR monoclonal antibody and stereotactic radiation induce immune-mediated survival advantage in murine intracranial glioma. J Immunother Cancer 2016; 4: 1-3.

45) Sukumar S, Wilson DC, Yu Y, Wong J, Naravula S, Ermakov G, Riener R, Bhagwat B, Necheva AS, Grein J, Churakova T. Characterization of MK-4166, a clinical agonistic antibody that targets human GITR and inhibits the generation and suppressive effects of T regulatory cells. Cancer Res 2017; 77: 4378-4388.

46) Leyland R, Watkins A, Mulgrew KA, Holoweckyj N, Bamber L, Tigue NJ, Offer E, Andrews J, Yan L, Mullins S, Oberst MD. A novel murine GITR ligand fusion protein induces antitumor activity as a monotherapy that is further enhanced in combination with an OX40 agonist. Clin Cancer Res 2017; 23: 3416-3427.

47) Li K, Huang SH, Lao XM, Yang L, Liao GQ, Liang YJ. Interaction of cancer cell-derived Foxp3 and tumor microenvironment in human tongue squamous cell carcinoma. Exp Cell Res 2018; 370: 643-652.

48) Siddiqui SA, Frigola X, Bonne-Annee S, Mercader M, Kuntz SM, Krambeck AE, Sengupta S, Dong H, Cheville JC, Lohse CM, Krco CJ. Tumor-infiltrating Foxp3– CD4+ CD25+ T cells predict poor survival in renal cell carcinoma. Clin Cancer Res 2007; 13: 2075-2081.

49) Zhan HL, Gao X, Zhou XF, Pu XY, Wang DJ. Presence of tumour-infiltrating FOXP3+ lymphocytes correlates with immature tumour angiogenesis in renal cell carcinomas. Asian Pac J Cancer Prev 2012; 13: 867-872.

50) Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep 2015; 5: 15179.

51) Luo Q, Zhang S, Wei H, Pang X, Zhang H. Roles of Foxp3 in the occurrence and development of cervical cancer. Int J Clin Exp Pathol 2015; 8: 8717.

52) Goto R, Hirota Y, Aruga T, Horiguchi S, Miura S, Nakamura S, Takimoto M. The number of FoxP3-positive tumor-infiltrating lymphocytes in patients with synchronous bilateral breast cancer. Breast Cancer 2020; 27: 586-593.

53) Zhu J, Li Z, Chen J, Li W, Wang H, Jiang T, Ma Y. A comprehensive bioinformatics analysis of

FOXP3 in nonsmall cell lung cancer. Medicine 2022; 101: e32102.

54) Khalife E, Khodadadi A, Talaeizadeh A, Rahimian L, Nemati M, Jafarzadeh A. Overexpression of regulatory T cell-related markers (FOXP3, CTLA-4 and GITR) by peripheral blood mononuclear cells from patients with breast cancer. Asian Pac J Cancer Prev 2018; 19: 3019-3025.

55) Griffiths RW, Elkord E, Gilham DE, Ramani V, Clarke N, Stern PL, Hawkins RE. Frequency of regulatory T cells in renal cell carcinoma patients and investigation of correlation with survival. Cancer Immunol Immunother 2007: 11; 1743-1753.

56) Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 2011; 105: 93-103.

57) Xu WH, Xu Y, Wang J, Wan FN, Wang HK, Cao DL, Shi GH, Qu YY, Zhang HL, Ye DW. Prognostic value and immune infiltration of novel signatures in clear cell renal cell carcinoma microenvironment. Aging (Albany NY) 2019; 11: 6999-7020.

58) Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC, Riquet M, Crozet L, Ouakrim H, Goc J, Cazes A, Flejou JF. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. Clin Cancer Res 2013; 19: 4079-4091.

59) Stanton SE, Disis ML. Clinical significance of tumor-infiltrating lymphocytes in breast cancer. J Immunother Cancer 2016; 4: 1-7.

60) Hwang WT, Adams SF, Tahirovic E, Hagemann IS, Coukos G. Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. Gynecol Oncol 2012; 124: 192-198.

61) Weiss SA, Han SW, Lui K, Tchack J, Shapiro R, Berman R, Zhong J, Krogsgaard M, Osman I, Darvishian F. Immunologic heterogeneity of tumor-infiltrating lymphocyte composition in primary melanoma. Hum Pathol 2016; 57: 116-125.

62) Geng Y, Shao Y, He W, Hu W, Xu Y, Chen J, Wu C, Jiang J. Prognostic role of tumor-infiltrating lymphocytes in lung cancer: a meta-analysis. Cell Physiol Biochem 2015; 37: 1560-1571.