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Investigation of the Status of Immune Checkpoint Molecules in Meningiomas by Immunohistochemistry

Ismail SAYGIN[1], Emel CAKIR[2], Seher Nazli KAZAZ[3], Ali Rıza GUVERCIN[4], Ilker EYUBOGLU[5], Muserref Muge USTAOGLU[1]

[1]Karadeniz Technical University, Faculty of Medicine, Department of Pathology, Trabzon, Türkiye
[2]Sancaktepe Sehit Prof. Dr. Ilhan Varank Training and Research Hospital, Department of Pathology, Istanbul, Türkiye
[3]Karadeniz Technical University, Department of Oncology, Trabzon, Türkiye
[4]Karadeniz Technical University, Department of Neurosurgery, Trabzon, Türkiye
[5]Karadeniz Technical University, Department of Radiology, Trabzon, Türkiye

Corresponding author: Ismail SAYGIN ✉ ismailsaygin@ktu.edu.tr

ABSTRACT

AIM: To investigate the status of immune checkpoint molecules (CTLA-4 and TIM-3) in meningiomas and thus contribute to the development of new personalized treatment strategies.

MATERIAL and METHODS: We utilized 402 cases of meningioma for this study. New blocks were prepared using the tissue microarray method, and sections obtained from these blocks were immunohistochemically stained with CTLA-4 and TIM-3 antibodies. Subsequently, statistical analysis were performed.

RESULTS: Our findings revealed that CTLA-4 expression were observed in 25.1% of meningiomas. CTLA-4 expression and the number of expressing lymphocytes were found to be significantly higher in high-grade tumors and in those with brain invasion. Meningiomas with staining of immune cells with TIM-3 are 3.5%, and the tumor grade was correlated with the number of immune cells expressing TIM-3.

CONCLUSION: Immune checkpoint molecules (CTLA-4 and TIM-3) with varying levels of expression can serve as prognostic and predictive biomarkers, as well as important targets for therapy. Drugs developed for CTLA-4 and TIM-3 molecules may prove to be more effective in treating meningiomas with high-grade, brain-invading, spontaneous necrosis, and macronucleolus.

KEYWORDS: CTLA-4, TIM-3, Meningioma, Immunotherapy, Immune checkpoint molecules

INTRODUCTION

Meningiomas stand as the most common central nervous system (CNS) tumors in adults, accounting for 36% of primary tumors (22). They are classified as grades 1, 2, and 3, according to World Health Organization (WHO). Two-thirds of meningiomas are benign and fall into grade 1, while approximately 20%–30% are grades 2 and 3 tumors, with a more aggressive clinical course. Grades 2 and 3 meningiomas recur at a higher rate after resection (16), and may metastasize to the lungs, liver, or bones (32).

The most effective method in treating meningiomas is total excision of the tumor. However, depending on the localization of the lesion, in cases of incomplete resection, adjuvant radiotherapy is added in addition to surgical treatment in order to reduce recurrence and mortality in grades 2 and 3 meningiomas. It is important to note that chemotherapy has no effect on meningiomas.

Incorrect grading and an inability to accurately determinate the risk of recurrence may also prevent optimal treatment (1).

Ismail SAYGIN : 0000-0002-6013-6378
Emel CAKIR : 0000-0002-9845-366X
Seher Nazli KAZAZ : 0000-0002-8181-000X
Ali Rıza GUVERCIN : 0000-0002-8689-0571
Ilker EYUBOGLU : 0000-0002-7732-1289
Muserref Muge USTAOGLU : 0000-0002-8414-5324

The most important cause of recurrence is subtotal excision, with reported rates of up to 40% even in cases of total excision (8,23). In cases where surgery is not feasible, and the side effects of radiotherapy are undesirable, there is currently no standard and effective medical treatment for meningiomas, regardless of tumor grade (12). The absence of an effective medical treatment, especially in high-grade meningiomas that cannot be operated on, negatively affects patient survival.

Unfortunately, the probability of recurrence in different types of meningioma can vary greatly, and current algorithms for predicting disease progression may not be able to distinguish which patients are at the highest risk. However, advanced methods, such as cytogenetics, mutational profiling, and epigenetics, as well as new methods, such as immunotherapy, used in some other tumors, may increase our capacity for effective, patient-specific treatment of meningioma (1). Investigating immune cells within the tumor not only aids in diagnosis but also helps to identify biomarkers of progression to understand treatment response or the sensitivity of the targeted therapeutics (1).

Immune checkpoint molecules (ICMs) play a crucial role in regulating the immune response. Overexpression of ICM by tumor cells affects tumor-specific T-cell immunity within the cancer microenvironment. In this case, tumor cells escape from the immune system. The immune escape mechanisms of tumors using these checkpoints can be repaired with antibodies that block the inhibitory receptor–ligand interaction and thus inactivate the immune checkpoints (4).

CTLA-4 belongs to the CD28:B7 immunoglobulin superfamily and is homologous to CD28 (17). Both molecules bind to CD80 (B7-1) and CD86 (B7-2) on antigen presenting cells. CTLA-4 has a higher affinity for B7 than for CD28, and binding of CTLA-4 to CD80/86 is 500–2500 times more active than that of CD28 (4).

CTLA-4 is expressed by activated T cells and transmits an inhibitory signal to T cells (18,28,29). It also contributes to the inhibitory function of regulatory T cells (Treg). Initially, CTLA-4 regulates the severity of early-stage T cell activation (10). An anti-CTLA-4 drug, ipilimumab, received FDA approval in 2011 after demonstrating effective results in blocking CTLA-4 in melanoma (25).

T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3) belongs to the TIM family and was originally selectively expressed in terminally differentiated IFNγ producing CD4+ T helper type 1 cells and CD8+ cytotoxic T cells (19). Later, expression was detected in Th17 cells, regulatory T cells (Treg), and innate immune cells (dendritic cells, NK cells, and monocytes) (11-13).

The TIM3 receptor is an ICM that, along with other inhibitory receptors, mediates CD8+ T-cell depletion in terms of proliferation and secretion of cytokines, such as TNF (2,14). TIM3 is primarily activated by galectin-9 (27). As a result of activation, Th1 and Th17 responses are suppressed, and the immunosuppressive activity of Treg cells increases (30). Galectins are associated with poor prognosis and metastasis in many types of cancer (20).

Studies have shown that TIM-3 is a suitable target for the treatment of melanoma, such as CTLA4. Furthermore, it is considered a target for solid tumors, including lung, stomach, head and neck cancers, schwannoma, as well as hematological tumors, such as follicular B-cell non-Hodgkin lymphoma (7).

In this study, we aim to investigate the status of ICM in meningiomas and thus contribute to the development of new personalized treatment strategies.

MATERIAL and METHODS

The study is a retrospective study involving 402 meningioma cases. All cases were included after re-evaluation by two neuropathologists and after confirming their morphological findings.

The research protocol (2020/279) was approved by institutional review board (Decision no: KTU- 24237859-752/21.12.2020).

New blocks containing multiple tissues were prepared using appropriate paraffin blocks of the cases through the tissue microarray method. For this purpose, 3 mm tissues were removed from each block, and new blocks were prepared, each containing tumor tissue from nine cases. Subsequently, sections were taken from these blocks for immunohistochemical staining.

Immunohistochemical staining with CTLA-4 and TIM-3 antibodies was performed using Ventana's BenchMark Ultra automatic staining device. Anti-CTLA-4 100UL (Abcam) antibody prepared at 1/200 dilution, anti-TIM-3 100UL (Abcam) antibody prepared with 1/100 dilution. Slides stained with antibodies were evaluated by two neuropathologists with an Olympus BX51 light microscope.

CTLA-4 and TIM-3 were evaluated by counting the stained immune cells and interpreted with statistical analysis. The staining rate of CTLA-4 and TIM-3 in tumor-infiltrating immune cells was also performed as grouping immunohistochemical staining as <1% and ≥1%.

The data analysis was conducted using SPSS 26.0 statistical package program. Descriptive statistics were used to present the evaluation results, including numbers and percentages for categorical variables, and mean, standard deviation, minimum and maximum for numerical variables. Kolmogorov–Smirnov or Shapiro–Wilk tests were used to test the conformity of the measurement data to the normal distribution. T-test in independent groups was used for measurement variables that fit normally, and the Mann–Whitney *U* Test or Kruskall–Wallis analysis of variance was used for measurement variables that did not fit the normal distribution. Spearman correlation test was used for the data that did not conform to the normal distribution in the correlation analysis of the measurement data. The chi-square test was used for the analysis of categorical data. Throughout all statistical analyses, a significance value of $p<0.05$ was considered as statistically significant.

RESULTS

Out of the 402 cases included in the study, 289 were women, and 113 were men. Among these cases, 271 (67.4%) cases

were classified as WHO grade 1, 121 (30.1%) as grade 2, and 10 (2.5%) as grade 3.

In 301 (74.9%) cases, no staining with CTLA-4 was observed in lymphocytes. In 101 (25.1%) cases, staining was observed in at least one and at most 30 lymphocytic cells (Figure 1). Among the CTLA-4 immune cell positive cases, 7.5% exhibited transitional morphology and 7.0% displayed meningothelial morphology. The mean of lymphocytes stained in 101 CTLA-4 positive cases was 3.14 (± 4.352 standard deviation). CTLA-4 also showed varying degrees of cytoplasmic staining in meningothelial cells in 34 cases.

Comparison of CTLA-4 and immune cell positivity with tumor grade and morphological parameters is presented in Table I. Staining with CTLA-4 was 63/271 (23.2%) in grade 1 cases, 31/121 (25.6%) in grade 2 cases, and 7/10 (70.0%) in grade 3 cases (p=0.004). Staining with CTLA-4 was observed 88/374

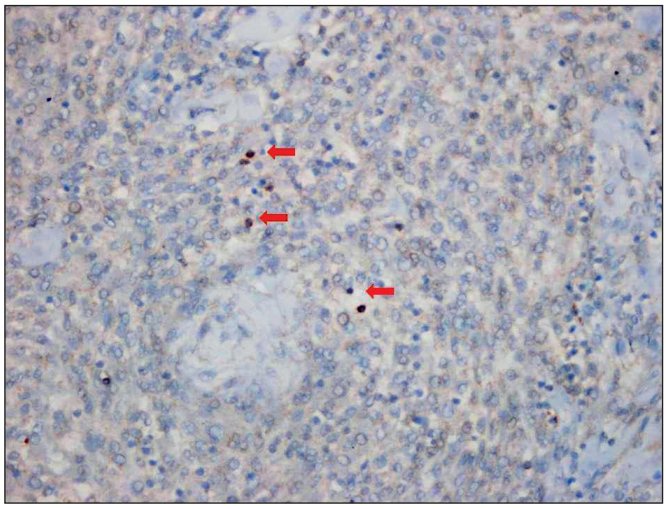

Figure 1: CTLA-4 x 400 (Red arrow: CTLA-4 positive lymphocytes).

Table I: Comparison of CTLA-4 and Immune Cell Positivity with Tumor Grade, Recurrence, and Morphological Parameters

		CTLA-4 positive			p-value
		No (<1%) n (%)	Yes (≥1%) n (%)	Total	
Grade	Grade 1	208 (76.8)	63 (23.2)	271	**0.004**
	Grade 2	90 (74.4)	31 (25.6)	121	
	Grade 3	3 (30.0)	7 (70.0)	10	
	Total	301 (74.9)	101 (25.1)	402	
Brain invasion	No	286 (76.5)	88 (23.5)	374	**0.007**
	Yes	15 (53.6)	13 (46.4)	28	
	Total	301 (74.9	101 (25.1)	402	
Spontaneous necrosis	No	282 (77.3)	83 (22.7)	365	**0.001**
	Yes	19 (51.4)	18 (48.6)	37	
	Total	301 (74.9)	101 (25.1)	402	
Macronucleolus	No	296 (75.7)	95 (24.3)	391	**0.023**
	Yes	5 (45.5)	6 (54.5)	11	
	Total	301 (74.9)	101 (25.1)	402	
Hypercellularity	No	269 (74.3)	93 (25.7)	362	0.431
	Yes	32 (80.0)	8 (20.0)	40	
	Total	301 (74.9)	101 (25.1)	402	
Small nucleus	No	261 (75.7)	84 (24.3)	345	0.377
	Yes	40 (70.2)	17 (29.8)	57	
	Total	301 (74.9)	101 (25.1)	402	
Pattern loss	Yes	282 (76.0)	89 (24.0)	371	0.069
	No	19 (61.3)	12 (38.7)	31	
	Total	301 (74.9)	101 (25.1)	402	
Recurrence	No	286 (75.1)	95 (24.9)	381	0.708
	Yes	15 (71.4)	6 (28.6)	21	
	Total	301 (74.9)	101 (25.1)	402	

(23.5%) in cases without brain invasion, and 13/28 (46.4%) with brain invasion. Significantly increased CTLA-4 positivity was noted in cases with brain invasion (p=0.007). CTLA-4 was positive in 83/365 (22.7%) cases without spontaneous necrosis and in 18/37 (48.6%) cases with spontaneous necrosis (p=0.001). CTLA-4 was positive in 95/391 (24.3%) of the cases without macronucleolus and 6/11 (54.5%) of the cases with macronucleolus (p=0.023). No statistically significant difference was found in the comparison of the cases with CTLA-4 positive lymphocytes in terms of gender (p=0.798). No statistically significant correlation was found between CTLA-4 positivity and tumor diameter and age (p=0.462 and 0.201, respectively).

The comparative results in terms of the number of lymphocytes stained with CTLA-4 and tumor grade, morphological parameters, recurrence, and gender are presented in Table II. The number of lymphocyte stained with CTLA-4 increases with tumor grade (p=0.016). The number of these cells was significantly higher in cases with brain invasion compared with those without (p=0.012). The number of lymphocyte stained with CTLA-4 was significantly higher in cases with macronucleolus compared with those without (p=0.016). The number of these cells increased significantly in cases with spontaneous necrosis (p=0.001). No statistically significant results were found between the number of CTLA-4 positive immune cells, gender, and recurrence (p=0.727 and 0.630, respectively). No statistically significant correlation was found between the number of CTLA-4 positive lymphocytes and tumor diameter and age (p=0.583 and 0.156, respectively).

The number of cases showing immune cell staining with TIM-3 was 14 (3.5%), while 388 (96.5%) cases did not show staining. In cases showing staining with TIM-3, at least one and at most 24 immune cells are stained (Figure 2). The comparison of TIM-3 immune cell positivity with tumor grade, morphological parameters, and recurrence is presented in Table III. However, for the comparison of TIM-3 immune cell positivity with tumor

Table II: Comparative Results in Terms of the Number of Lymphocytes Stained with CTLA-4 and Tumor Grade, Morphological Parameters, Recurrence, and Gender

		CTLA-4 positive lymphocyte				p-value
		Mean ± standard deviation	Number of cases	Minimum	Maximum	
Macronucleolus	No	0.77 ± 2.57	391	0	30	**0.016**
	Yes	1.55 ± 2.01	11	0	6	
	Total	0.79 ± 2.56	402	0	30	
Spontaneous necrosis	No	0.76 ± 2.65	365	0	30	**0.001**
	Yes	1.05 ± 1.39	37	0	6	
	Total	0.79 ± 2.56	402	0	30	
Brain invasion	No	0.78 ± 2.63	374	0	30	**0.012**
	Yes	0.96 ± 1.47	28	0	6	
	Total	0.79 ± 2.56	402	0	30	
Grade	Grade 1[a]	0.69 ± 2.34	271	0	30	**0.002**
	Grade 2[a]	0.82 ± 2.79	121	0	24	
	Grade 3[b]	3.00 ± 4.29	10	0	14	
	Total	0.79 ± 2.56	402	0	30	
Hypersellularity	No	0.83 ± 2.68	362	0	30	0.414
	Yes	0.38 ± 0.83	40	0	3	
	Total	0.79 ± 2.56	402	0	30	
Small nucleus	No	0.83 ± 2.74	345	0	30	0.500
	Yes	0.51 ± 0.88	57	0	3	
	Total	0.79 ± 2.56	402	0	30	
Pattern loss	No	0.77 ± 2.61	371	0	30	0.084
	Yes	0.97 ± 1.85	31	0	8	
	Total	0.79 ± 2.56	402	0	30	

[a,b]*There is a significant difference between the different letters for the tumor grade.*

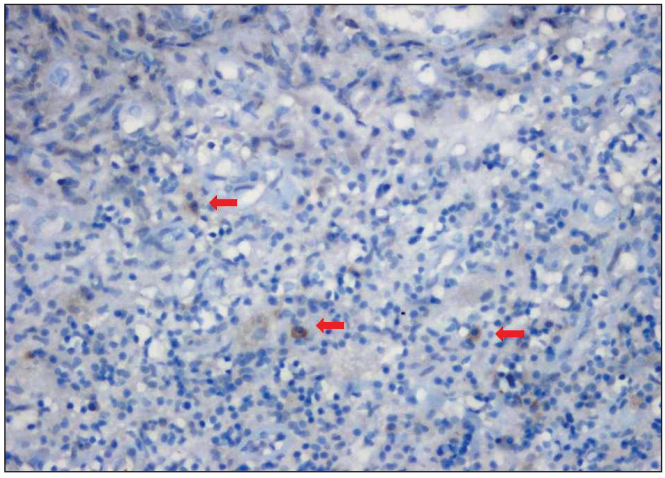

Figure 2: TIM-3 x 400 (Red arrow: TIM-3 positive lymphocytes).

grade, a *p* value could not be provided due to the small number of cases. No statistically significant relationship was found between TIM-3 immune cell positivity and tumor diameter. However, interestingly, a statistically significant relationship was observed between TIM-3 immune cell positivity and age. The mean ± standard deviation of TIM-3 immune cell positive patient age in 14 patients was 48.0 ± 8.69 (p=0.034). There was no statistically significant result in the comparison of TIM-3 immune cell positivity and gender (p=0.064).

No statistically significant results were obtained between the number of TIM-3 positive immune cells and morphological parameters, tumor diameter, age, recurrence, and gender in TIM-3 positive cases. Additionally, no statistically significant results were obtained when comparing the number of TIM-3 positive immune cells and tumor grade in TIM-3 positive cases (p=0.694). However, considering all cases, a statistically

Table III: Comparison of TIM-3 Immune Cell Positivity with Tumor Grade, Morphological Parameters, and Recurrence

		TIM-3 positive			p-value
		No (<1%) n (%)	Yes (≥1%.) n (%)	Total	
	Grade 1	263 (97.0)	8 (3.0)	271	p value cannot be given
	Grade 2	117 (96.7)	4 (3.3)	121	
	Grade 3	8 (80.0)	2 (20.0)	10	
	Total	388 (96.5)	14 (3.5)	402	
Spontaneous necrosis	No	353 (96.7)	12 (3.3)	365	0.375
	Yes	35 (94.6)	2 (5.4)	37	
	Total	388 (96.5)	14 (3.5)	402	
Hypercellularity	No	348 (96.1)	14 (3.9)	362	0.378
	Yes	40 (100.0)	0 (0.0)	40	
	Total	388 (96.5)	14 (3.5)	402	
Small nucleus	No	331 (95.9)	14 (4.1)	345	0.235
	Yes	57 (100.0)	0 (0.0)	57	
	Total	388 (96.5)	14 (3.5)	402	
Macronucleolus	No	379 (96.9)	12 (3.1)	391	0.052
	Yes	9 (81.8)	2 (18.2)	11	
	Total	388 (96.5)	14 (3.5)	402	
Pattern loss	No	359 (96.8)	12 (3.2)	371	0.294
	Yes	29 (93.5)	2 (6.5)	31	
	Total	388 (96.5)	14 (3.5)	402	
Brain invasion	No	361 (96.5)	13 (3.5)	374	1.000
	Yes	27 (96.4)	1 (3.6)	28	
	Total	388 (96.5)	14 (3.5)	402	
Recurrence	No	367 (96.3)	14 (3.7)	381	1.000
	Yes	21 (100.0)	0 (0.0)	21	
	Total	386 (96.5)	14 (3.5)	402	

Table IV: Comparative Results in Terms of the Number of Lymphocytes Stained with TIM-3 and Tumor Grade, Morphological Parameters, Recurrence, and Gender

		TIM-3 positive immune cell				p-value
		Mean ± standard deviation	Number of cases	Minimum	Maximum	
Grade	Grade 1	0.10 ± 0.65	271	0	8	**0.015**
	Grade 2	0.16 ± 0.94	121	0	7	
	Grade 3	2.60 ± 7.54	10	0	24	
	Total	0.18 ± 1.41	402	0	24	
Macronukleolus	No	0.12 ± 0.76	391	0	8	**0.007**
	Yes	2.27 ± 7.21	11	0	24	
	Total	0.18 ± 1.41	402	0	24	
Pattern loss	No	0.13 ± 0.78	371	0	8	0.349
	Yes	0.81 ± 4.30	31	0	24	
	Total	0.18 ± 1.41	402	0	24	
Spontaneous necrosis	No	0.13 ± 0.79	365	0	8	0.504
	Yes	0.68 ± 3.94	37	0	24	
	Total	0.18 ± 1.41	402	0	24	
Brain invasion	No	0.13 ± 0.78	374	0	8	0.951
	Yes	0.86 ± 4.53	28	0	24	
	Total	0.18 ± 1.41	402	0	24	
Hypercellularity	No	0.20 ± 1.48	362	0	24	0.206
	Yes	0.00 ± 0.00	40	0	0	
	Total	0.18 ± 1.41	402	0	24	
Small nucleus	No	0.21 ± 1.52	345	0	24	0.122
	Yes	0.00 ± 0.00	57	0	0	
	Total	0.18 ± 1.41	402	0	24	
Recurrence	No	0.19 ± 1.44	381	0	24	0.372
	Yes	0.0 ± 0.00	21	0	0	
	Total	0.10 ± 1.41	402	0	24	

significant result was obtained in the comparison of TIM-3 positive immune cell count and tumor grade (p=0.015) (Table IV). Furthermore, when all cases are considered, a statistically significant relationship was found between the number of immune cells expressing TIM-3 and the presence of macronucleolus in TIM-3 positive cases. TIM-3 expressing lymphocytes are increased in cases with macronucleolus.

DISCUSSION

Meningiomas are the most common adult primary tumor of the CNS and have a relatively high recurrence rate. About 12% of grade 1 meningiomas recur within 5 years after total resection; 19% recur in 10 years (15). Even after total resection, the overall recurrence rate for grade 2 tumors in all localizations is 29%–40% within 5 years (15).

Meningiomas are generally considered “benign” tumors and are typically treated with surgery and radiation. However, treatment options are very limited in relapsed or unresectable cases because they are resistant to chemotherapy. Recent advances in cancer treatment with immunotherapy have focused on immune checkpoint blockade. Some recent studies have supported the use of immunotherapy as a potentially effective treatment strategy for meningiomas (9).

The literature on immunocheckpoint molecules in meningiomas are very limited. Some studies have shown that ICMs, such as NY-ESO-1, PD-L1, PD-L2, B7-H3, and CTLA-4, are expressed in meningiomas and may be responsible for suppressing the anti-tumor immune response (9).

Proctor et al. observed the presence of lymphocytes expressing CTLA-4 in grades 2 and 3 meningiomas (24). In

our study, it was observed that the number of CTLA-4 positive lymphocytes was higher in high-grade meningiomas. In our study, CTLA-4 positive lymphocyte count was also found to be higher in cases with brain invasion, spontaneous necrosis, and macronucleolus.

Fang et al. reported in their study that PD-1 and TIM-3 expression increased in immune cells in meningiomas. However, they could not find a relationship between them (6). Erdogan et al. compared TIM-3 expression with tumor grade and some parameters in a limited number of meningiomas, but they also did not find statistically significant results (5). In our study, we observed immune cell positivity with TIM-3 in 14 cases (3.5%). There was no statistically significant difference between TIM-3 immune cell positivity and morphological parameters. Unfortunately, TIM-3 immune cell positivity and tumor grade could not be compared because the numbers were small. Interestingly, there is a statistically significant correlation between TIM-3 immune cell positivity and age. As age increases, the number of TIM-3 positive lymphocytes decreases. In addition, considering all cases in our study, the number of TIM-3 positive lymphocytes increases as the tumor grade increases. TIM-3-expressing lymphocytes are also increased in cases with macronucleolus.

In the study by Yu et al. on colorectal carcinomas, TIM-3 expression was found to be correlated with tumor size and TNM stage (31). In our study, however, no relationship was found between TIM-3 expression and tumor diameter. In a separate study by Liu et al. on gliomas, TIM-3 expression was found to be increased in high-grade tumors (21). Similarly, in our study, TIM-3 expression is increased in high-grade tumors. Contrary to our study, in the study by Takamatsu et al. on renal cell carcinomas, TIM-3 expression was found to be statistically significantly decreased in tumors with high nuclear grade and pathological tumor grade (26).

There are recent studies supporting the use of immunotherapy as a potentially effective treatment strategy for meningiomas. Some studies were ongoing on immunotherapy in meningiomas (9).

Studies show that using combination therapy was more useful instead of using a single type of antibody. Although better responses are expected from combination therapy, the data obtained according to tumor types may vary (3).

CONCLUSION

CTLA-4 expressing lymphocytes were observed in 25.1% of meningiomas. CTLA-4 expression and the number of expressing lymphocytes were found to be significantly higher in high-grade tumors and those with brain invasion. Additionally, CTLA-4 expression and the number of expressing lymphocytes were higher in cases with spontaneous necrosis and macronucleol.

Meningiomas with staining of immune cells with TIM-3 accounted for 3.5%. The tumor grade showed a correlation with the number of immune cells expressing TIM-3. Furthermore, the number of immune cells with TIM-3 expression increases with the presence of macronucleolus.

These results indicate that ICMs with varying levels of expression can be used as a prognostic and predictive biomarker, as well as an important target for therapy. Considering the finidings from the literature and the results of our study, it can be predicted that the drugs developed for CTLA-4 and TIM-3 molecules may be more effective in meningiomas with high-grade, brain-invading, spontaneous necrosis, and macronucleolus.

AUTHORSHIP CONTRIBUTION

Study conception and design: IS, EC
Data collection: IS, SNK
Analysis and interpretation of results: IS, EC, SNK, MMU
Draft manuscript preparation: ARG, IE, MMU
Critical revision of the article: IS, EC, SNK, MMU, ARG, IE
All authors (IS, EC, SNK, MMU, ARG, IE) reviewed the results and approved the final version of the manuscript.

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