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Analysis of CMTM6 and CMTM4 Expression as Potential Regulators of the PD-L1 Protein and its Association with Prognosis in Gliomas

Hui Xue[1#], Bin Qiu[1#], Hao Wang[1], Ping Jiang[1], Weiwei Zhang[1], Lixiang Xue[1,2] and Junjie Wang[1]**

[1]Department of Radiation Oncology, Peking University Third Hospital, China

[2]Medical Research Center, Peking University Third Hospital, China

[#]These authors contributed equally to this work

Abstract

Background: CMTM6 and CMTM4 have been described as PD-L1 regulators at the protein level that modulates stability *via* ubiquitination, but little is known proteins expression and modulation in human gliomas and tumor microenvironment. Clinical trials of targeting PD-1/PD-L1 in glioma have been initiated; however, the rate of objective response has been less than 30% while the rate of complete response has less than 16% due to inadequate T-cell infiltration and immunosuppressive microenvironment. The aim of this study is to investigate the relationship between CMTM6/CMTM4 and PD-L1 expression and its association with clinical prognosis in gliomas.

Materials and Methods: The expression of CMTM6/CMTM4 and PD-L1 in 177 glioma samples represented in tissue microarrays was determined by Multiplexed quantitative immunofluorescence. The assessment of the relationship between clinical factors and CMTM6/CMTM4 expression was analyzed by Chi-squared test. The assessment of the clinical factors associated with survival was analyzed by univariate and multivariate Cox proportional hazard regression analyses. The evaluation of the statistical significance was analyzed by Pearson's correlation assessment. The survival probability was analyzed by Kaplan-Meier survival analysis and log-rank test.

Results: In gliomas, CMTM6/CMTM4 and PD-L1 were colocalized in tumor (Ki67+) and microenvironment (CD68+ macrophages). CMTM6 was significantly related to PD-L1. Similarly, CMTM4 was significantly related to PD-L1. Meanwhile, CMTM6 was significantly related to CMTM4. CMTM6/CMTM4 showed a modest correlation with CD8+ T cell infiltration. Surprisingly, CMTM6 was associated with clinical factors related to recurrence and Ki67. CMTM4 expression showed a significant correlation with Ki67. CMTM6 was a risk factor for prognosis. Finally, we found that patients with high CMTM6/CMTM4/PD-L1 expression showed shorter OS than those with low expression. What is more, OS was significantly longer when CMTM6/CMTM4 and PD-L1 were high co-expression in macrophages, but OS did not obviously extended in patients whose tumors had high CMTM6/CMTM4 single expression in macrophages.

Conclusion: This study supports the role of CMTM6 and CMTM4 in stabilization of PD-L1 protein and suggests that high co-expression of CMTM6/CMTM4 and PD-L1 in macrophages might be prognosis indicators for anti-PD-L1 therapy in gliomas.

Keywords: CMTM6; CMTM4; PD-L1; Macrophages; Gliomas

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Correspondence:

Lixiang Xue, Department of Radiation Oncology, Peking University Third Hospital, Medical Research Center, 49 North Garden Road, Haidian District, Beijing, 100191, China, Tel: +86 13701076310;

E-mail: lixiangxue@bjmu.edu.cn

Junjie Wang, Department of Radiation Oncology, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing, 100191, China, Tel: +86 13701076310;

E-mail: junjiewang@pku.edu.cn

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Introduction

Gliomas are a deadly and immunosuppressive brain tumor [1]. Despite advances in comprehensive therapy, patients who suffer from gliomas still have a short median survival time due to the resistance to treatments and recurrence [2,3]. In the past few years, studies on anticancer immune therapies of other tumors have promoted improvements to the limited success of conventional therapies [4]. Antibodies is targeting the programmed death-1 (PD-1)/ligand 1 (PD-L1) represent promising immunotherapies. Clinical trials of drugs targeting PD-1/PD-L1 in glioma have been initiated [5]. However, the rate of objective response has been less than 30% in tumors treated with PD-1/PD-L1 inhibitors, while the rate of complete response has been less than 16% due to inadequate T-cell infiltration and immunosuppressive microenvironment [6-8]. Hence, new

immune-related therapeutic targets have to be further identified, and the more precise target patients regarding the anti-PD-L1 therapy have to be further exploited.

CMTM (CKLF-like MARVEL Transmembrane Domain-containing family), Chemokine-Like Factor Super Family (CKLFSF) is a gene family first reported in 2003. In humans, the family composed of nine members, namely: CKLF (Chemokine-like factor) and CMTM1-8 (CKLF-like MARVEL Transmembrane domain-containing member 1-8) (original named ckLFSF1-8). Genes in the CMTM family have different spliceosomes; at least one of the spliceosomes codes for MAL related proteins for vesicle trafficking and membrane link domain (MARVEL). Molecules containing this domain play an important physiological and pathological role in protein transport [9]. A series of studies have shown that CMTM family plays an important role in the immune system and tumorigenesis [10]. Recent reports have shown that CMTM6 and CMTM4 are new molecules that can be used to enhance the therapeutic benefits of immune checkpoint inhibitors [11-13]. CMTM6 and CMTM4 colocalize with PD-L1 at the plasma membrane and in recycling endosome, where they prevent PD-L1 from being targeted for lysosome-mediated degradation [13]. CMTM6 was identified as a major regulator of PD-L1, a key immunological checkpoint, and a potential target for immunotherapy. CMTM4, another CMTM family member that is closely related to CMTM6, was also identified as a positive regulator of PD-L1 in the absence of CMTM6.

CMTM6 and CMTM4 are prognostic biomarkers in several kinds of tumors, have attracted increasing attention from oncologists [14-21]. However, the potential role of CMTM6 and CMTM4 expression in samples of cancer patients in gliomas has not yet to be clarified. In this study, we evaluated the expression of CMTM6, CMTM4, and PD-L1 in 177 glioma samples to assess its association with prognosis in glioma patients.

Materials and Methods

Tissue Microarray (TMA) construction

TMAs were obtained from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). All tissue samples were approved from the human investigation committee protocol YB M-05-02. HBraG177Su01 contained 177 tumors resected from 162 patients between 2008 and 2011.

The clinical characteristics of the patients are also extracted from the database of Shanghai Outdo Biotech Co., Ltd. (Shanghai, China) and summarized in Table 1. A total of 134 (82.7%) patients were diagnosed at less than 60 years of age; 101 (62.3%) patients were male. 28 patients (14.2%) had tumors of pathological grade I; 30 patients (18.5%) had tumors of pathological grade I-II; 44 patients (27.2%) had tumors of pathological grade II; 28 patients (17.3%) had grade II-III, and 20 patients (12.3%) had tumors of pathological grade III, and 17 patients (10.5%) had tumors of pathological grade VI tumors according to the AJCC staging system.

Bioinformatics

The mRNA expression profile of CMTM6/CMTM4 in gliomas and normal or tissues was assessed *via* the UALCAN database (http://ualcan.path.uab.edu/), which is based on TCGA data.

Multiplexed immunofluorescence staining protocol

Multiplexed immunofluorescence staining was performed as previously described, with some modifications [14,15]. Briefly,

Table 1: The clinical characteristics of 177 gliomas cancer patients.

Variables	Number	%
Age (year)		
<60	26	14.70%
≥ 60	151	85.30%
Sex		
Male	65	36.80%
Female	112	63.20%
Grade (AJCC)		
I	23	13%
I+II	32	18%
II	48	27%
II–III	31	17.50%
III	20	11.30%
VI	23	13%
CMTM6		
Low	81	50%
High	81	50%
CMTM4		
Low	81	50%
High	81	50%
PD-L1		
Low	81	50%
High	81	50%

TMA were deparaffinized, and then we subjected TMA to antigen retrieval with EDTA pH 9.0 buffers at 100°C for 25 min. Next, we stained the same tissue with primary antibodies to detect tumor cells, macrophages, and CD8+ T cells, CMTM6, CMTM4, PD-L1 and Ki67. At last, we used secondary antibodies and amplification systems for signal detection. DAPI was used to highlight all nuclei.

Fluorescence signal quantification and cut-point selection

We used the inform to quantify the fluorescence signal. A representative image generated with the Inform software can be found in Figure 1. QIF scores were calculated by dividing the target pixel intensity by the area of the compartment of interest and then normalized to the exposure time and bit depth at which the images were captured. Patients with staining artifacts or the presence of less than 1% compartment area were systematically excluded after visual inspection. The median was used as the cut-off point to divide tumors into high and low expression groups.

Statistical analysis

The relationship between clinical factors and CMTM6/CMTM4 expression was analyzed by Chi-squared test. The clinical factors associated with survival were analyzed by univariate and multivariate Cox proportional hazard regression analyses. The linear correlation of two continuous variables was analyzed by Pearson's correlation assessment. The survival probability was analyzed by Kaplan-Meier survival analysis and log-rank test. To ensure the expression of CMTM6/CMTM4 and PD-L1 was truly colocalized in tumors and tumor microenvironment, we created a formula (satisfied cells of per sample % = one sum of all the cells that satisfy the condition in one sample/total cells in all samples) for inform analysis.

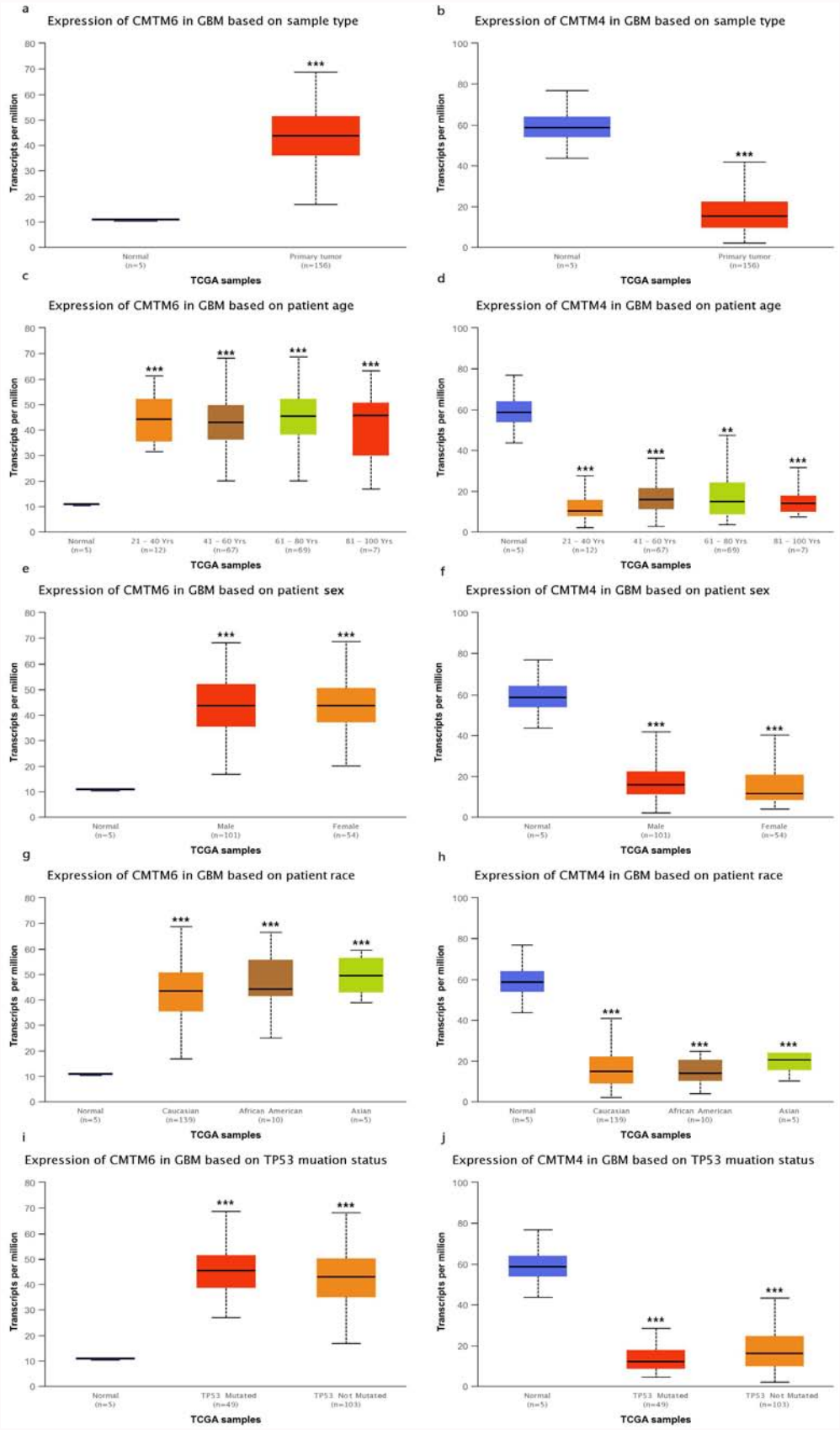

Figure 1: Bioinformatics analysis of the differential expression of CMTM6/CMTM4 and the prognosis of patients with gliomas. (a,b) The expression level of CMTM6/CMTM4 in gliomas was higher in cancer than in normal tissue according to the UALCAN database. (c,d) Expression of CMTM6/CMTM4 in gliomas based on age. (e,f) Expression of CMTM6/CMTM4 in gliomas based on patient sex. (g,h) Expression of CMTM6/CMTM4 in gliomas based on patient race. (I,j) Expression of CMTM6/CMTM4 in gliomas based on TP53 mutation status. *P<0.05; **P<0.01; ***P<0.001.

Results

The expression of CMTM6/CMTM4 in mRNA level in glioma patients

The upregulation/down regulation of CMTM6/CMTM4 in gliomas was supported by the UALCAN database (http://ualcan.path.uab.edu/; Figure 1a, 1b). As shown in Figure 1a, 1b, the expression level of CMTM6/CMTM4 in gliomas was higher in cancer than in normal tissue. As shown in Figure 1c, 1d, the CMTM6 expression on mRNA level increased in gliomas as the age increased while the CMTM4 expression on mRNA level decreased in gliomas as the age increased. Expression of CMTM6 on mRNA level in gliomas was higher in cancer than in normal tissue while expression of CMTM4 on mRNA level in gliomas was sharply lower in cancer than in normal tissue, but no significant statistical difference based on patient sex (Figure 1e, 1f) patient race. Figure 1g, 1h) and on TP53 mutation status (Figure 1i, 1j). Significant statistical difference of CMTM6/CMTM4 expression on mRNA level between cancer and normal tissue suggested that CMTM6 and CMTM4 likely play an important role in gliomas.

Expression of CMTM6/CMTM4 and PD-L1 in glioma tissues

To further explore, we detected the expression of CMTM6/CMTM4 and PD-L1 in 177 glioma cancer tissue samples using multiplexed immunofluorescence staining assay. As shown in Figure 2, CMTM6 and PD-L1 were located mainly in the cell membrane and cytoplasm, while CMTM4 was observed in the cell membrane, cytoplasm and nucleus. CD68 was located mainly in the cell membrane and cytoplasm of macrophages and CD8 was located mainly in the cell membrane and cytoplasm of T cells, while Ki67 was observed mainly in the cell nucleus of tumor cells. CMTM6 expression and CMTM4 expression were detected in approximately 88% and 89.5% of gliomas, respectively. CMTM6 and PD-L1 were located mainly in the cell membrane and cytoplasm, while CMTM4 was observed in the cell membrane, cytoplasm, and nucleus (Figure 2). PD-L1 expression was detected in approximately 76.5% of samples and was found in CMTM6/CMTM4-expressing samples. We showed that macrophages were major cell expressing CMTM6/CMTM4 and PD-L1, and the percentage of CD68/CMTM6 and CD68/CMTM4 double-positive cells in the tumor microenvironment was about 85.8% and 92.6% (Figure 5).

Relationship between CMTM6/CMTM4 and PD-L1 in gliomas

Patients with high PD-L1 expression had a significantly shorter OS than those with low PD-L1 expression (Figure 4c, p<0.0001). CMTM6 and CMTM4 expression was significantly related to PD-L1 positivity in glioma cancer tissues (Figure 3b, 3c; R=0.342, p=0.028 for CMTM6 and PD-L1; R=0.803, P<0.0001 for CMTM4 and PD-L1). Age, sex, TNM stage, grade, and CMTM6/CMTM4/PD-L1 expression were subjected into the univariate and multivariate logistic regression analyses. The results showed that CMTM6 expression was a risk factor for recurrence and Ki67 expression. CMTM4 expression was not significantly different between patients with other clinically related factors (Table 2). Visually, CMTM6/CMTM4 and PD-L1 were colocalized in tumors and macrophages. Surprisingly, colocalization was significantly higher in macrophages than in tumors (Figure 5). Combining all tumors together (n=177), results were statistically significant (p<0.0001) but modest correlation between CMTM6 and PD-L1 levels (R=0.342).

Table 2: CMTM6/CMTM4 expression and clinicopathological characteristics in gliomas.

Characteristic	CMTM6			CMTM4		
	Low	High	P Value	Low	High	P value
Gender						
Male	57	44	0.0513	52	49	0.7459
Female	24	37		29	32	
Age						
<60	72	62	0.0601	70	64	0.2988
≥ 60	9	18		11	16	
Histology						
I	12	11	0.3293	14	9	0.0819
I~II	15	15		10	20	
II	25	19		27	17	
II~III	15	13		14	14	
III	10	10		11	9	
IV	4	13		5	12	
Relapse						
Yes	35	49	0.0406	39	45	0.4318
No	46	32		42	36	
Ki-67						
High	30	51	0.0016	25	25	0.9999
Low	51	30		56	56	

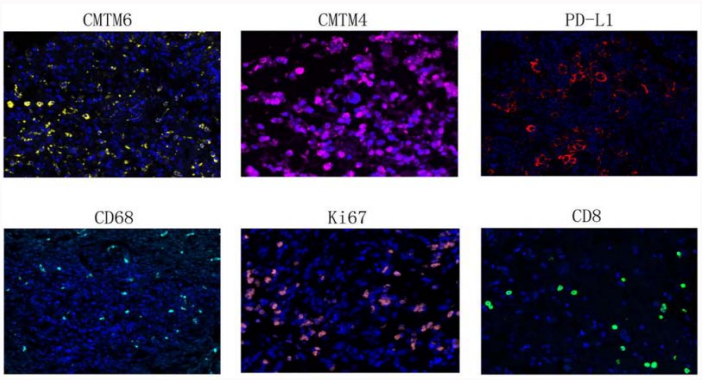

Figure 2: The localization of CMTM6/CMTM4/PD-L1/CD68/Ki67/CD8 staining in glioma tissues. CMTM6 and PD-L1 were located mainly in the cell membrane and cytoplasm, while CMTM4 was observed in the cell membrane, cytoplasm and nucleus. CD68 was located mainly in the cell membrane and cytoplasm of macrophages. CD8 was located mainly in the cell membrane and cytoplasm of T cells. Ki67 was observed mainly in the cell nucleus of tumor cells.

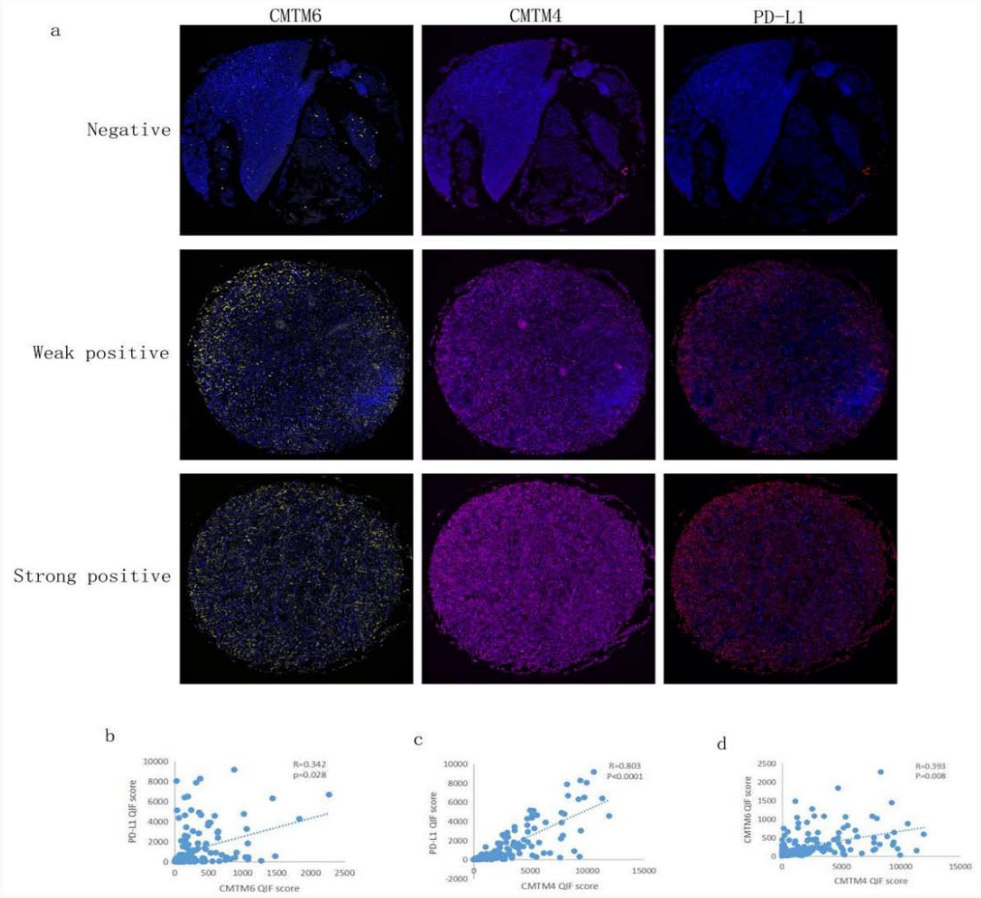

Figure 3: The expression of CMTM6 and PD-L1 in gliomas tissues. (a) Negative, weak positive, and strong positive expression of CMTM6, and negative, weak positive, and strong positive expression of PD-L1. (b-d) Pearson correlation analysis of CMTM6 and PD-L1 (b), CMTM4 and PD-L1 (c), and CMTM6 and CMTM4 (d) staining.

Relationship between CMTM6/CMTM4 expression and CD8+ T cells, CD68+ macrophages and Ki67 in gliomas

Consistent with its role in PD-L1 protein regulation, CMTM6/CMTM4 enhances the ability of PD-L1-expressing tumor cells to inhibit T cells. There was a weak correlation between CMTM6/CMTM4 expression and CD8+ T cells (R=0.298 for CMTM6 and CD8+ T cells, R=0.179 for CMTM4 and CD8+ T cells). CMTM6/CMTM4 expression in macrophages did not significantly predict OS (p=2.068e-1 and p=4.262e-3, respectively) (Figure 4f, 4g). CMTM6 expression showed no correlation with Ki67 on tumor cells (R=0.105), but CMTM4 expression showed a strong correlation with the presence of Ki67 on tumor cells (R=0.616).

The association with prognosis of CMTM6/CMTM4 and PD-L1 expression in patients with gliomas

Results showed that patients with high CMTM6/CMTM4/PD-L1 expression had shorter OS than those with low expression (Figure 4a-c, p=4.63e-10, p=4.63e-10, p=1.289e-10, p=2.972e-7). CMTM6 expression was a risk factor for prognosis (Table 3; HR: 1.763, 95% confidence interval [1.018-3.062], p=0.044). We found a significant association between CMTM6 expression and the clinicopathological factors recurrence and Ki67 expression using the chi-squared test (Figure 3; p=0.0406 for recurrence, p=0.0016 for Ki67). Similarly, CMTM6/CMTM4 expression in macrophages did not significantly predict OS (p=2.068e-1 and p=4.262e-3, respectively) (Figure 4f, 4g). OS was no significantly longer in patients whose tumors had high CMTM6 or CMTM4 single expression in macrophages in patients. Surprisingly, OS was significantly longer when CMTM6/CMTM4 and PD-L1 were high in the macrophages at the same time (Figure 4h, 4i, p<0.0001).

Discussion

The tumor microenvironment is a complex and continuously evolving system [16,17]. With the in-depth understanding of tumor knowledge, people increasingly realize the importance of tumor microenvironment in tumor genesis, development, treatment and prognosis [18-20]. Characteristic components of the tumor microenvironment include blood vessels, immune cells, stromal cells, and extracellular matrix. A dynamic interaction between tumor cells and components of the tumor microenvironment is formed, which supports tumor cell survival, local invasion, and distal metastasis. Why do patients with the same pathological type of tumor respond significantly differently to anti-PD-L1? This is largely due to differences in tumor microenvironments among patients with same tumors. Therefore, it is of great significance to study the influence and mechanism of each component in the tumor microenvironment in the process of tumor genesis and development to discover new therapeutic targets and improve the efficacy of chemoradiotherapy, targeted therapy and immunotherapy.

In this study, we detected CMTM6/CMTM4 expression, PD-L1 expression, CD68-positive macrophages, CD8-positive T cells, and

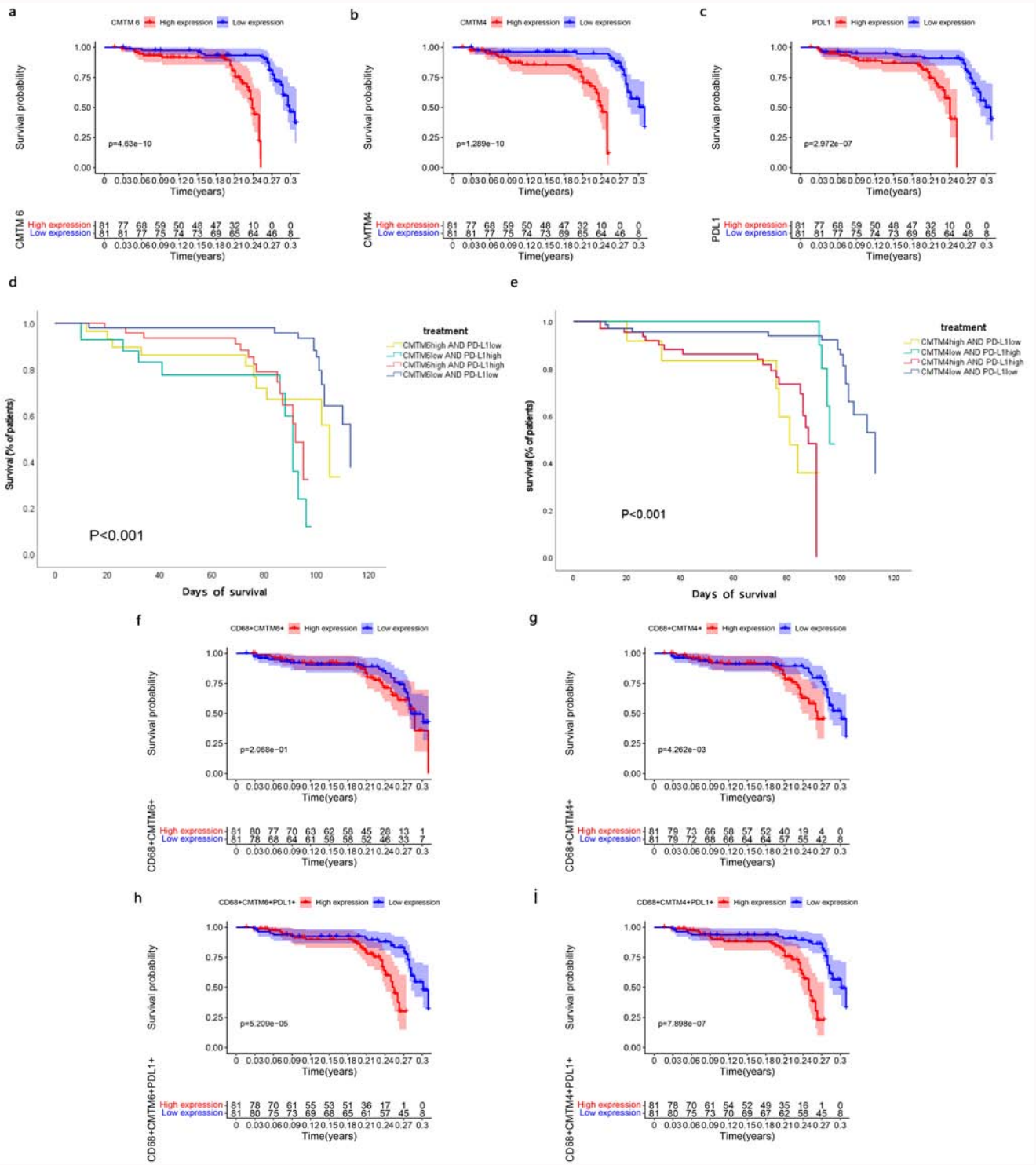

Figure 4: The overall survival of patients with gliomas. (a). Patients with high CMTM6 expression (n=81) showed a significantly lower overall survival than patients with low CMTM6 expression (n=81). (b). Patients with high CMTM4 expression (n=81) showed a significantly lower overall survival than patients with low CMTM4 expression (n=81). (c). Patients with high PD-L1 expression (n=81) showed a significantly lower overall survival than patients with low PD-L1 expression (n=81). (d). Patients with high CMTM6 and PD-L1 co expression showed a significantly lower overall survival than patients with low CMTM6 and PD-L1 co expression. (e). Patients with high CMTM4 and PD-L1 co expression showed a significantly lower overall survival than patients with low CMTM4 and PD-L1 co expression. (f,g). Overall survival did not obviously extended in patients with high CMTM6/CMTM4 single expression in macrophages. (h,i). Overall survival was significantly longer in patients with CMTM6/CMTM4 and PD-L1 high co-expression in macrophages. Red represents high CMTM6 expression, and blue represents low CMTM6 expression.*P<0.05; **P<0.01; ***P<0.001.

Ki67 tumor cells in the same tissue. We found that CMTM6/CMTM4 was broadly expressed in tumor and macrophage. We reported the association with prognosis of CMTM6/CMTM4 expression and its relationship with PD-L1 expression on macrophages in gliomas for the first time. High CMTM6/CMTM4/PD-L1 expression on protein level was associated with the lower OS than low expression.

Table 3: The univariate and multivariate Cox proportional hazard regression analyses between the clinical related factors and survival in gliomas patients.

Variables	Univariate analysis	Multivariate analysis	
		HR (95% CI)	P
gender	0.31	1.199 [0.624-2.302]	0.585491867
age	3.63E-05	1.015 [0.991-1.039]	0.224700446
grade	1.52E-21	4.211 [2.507-7.072]	5.48E-08
location	0.095	1.169 [0.700-1.950]	0.551224766
CMTM6	0.014	1.045 [0.556-1.950]	0.891705041
CMTM4	0.827	0.526 [0.168-1.644]	0.269241364
PD-L1	0.554	0.759 [0.249-2.314]	0.62739823

CMTM6/CMTM4 was significantly related to PD-L1 on protein level in glioma. Mechanistically, CMTM6/CMTM4 plays a key role in maintaining the stability of PD-L1 cell surface expression [21]. Therefore, preventing them from binding with PD-L1 may recover immunosuppression response and serves as a promising strategy for immunotherapy. Notably, some samples with high CMTM6 showed low PD-L1, suggesting that CMTM6 upregulation is not sufficient to mediate PD-L1 protein expression in glioma. Thus, CMTM6 likely is not the only PD-L1 regulator on protein level. On the other hand, CMTM4 has been shown to act as a backup PD-L1 protein stabilizer [11].

We found that CMTM6/CMTM4 and PD-L1 observed in tumors and macrophages and interestingly it was higher in macrophages. We targeted CD68-positive macrophages because reports have shown that CD68-positive macrophages are the predominant immune cell type expressing PD-L1 [22,23]. Current studies on tumor microenvironment focus on the effects of angiogenesis, macrophages, T cells and other immune-related cells. As we all know, TAMs are important parts of the tumor micro environment, involved in tumorigenesis, growth, invasion and metastasis, moreover, clinicopathological studies suggest that the accumulation of TAMs in tumors is correlated with poor clinical outcome [24-26]. At the same time, the plasticity of macrophages has also become the focus of tumor immunotherapy to change the tumor microenvironment and immune remodeling. More and more studies have reported the effects of tumor-associated macrophages on the tumor microenvironment and proposed tumor-associated macrophage-based immunotherapy strategies [27-31].

Among members of the CMTM family, CKLF and CMTM1-4 located in a gene cluster at 16Q22.1 and CMTM6-8 located in a gene cluster at 3P22.3, and CMTM5 is independently located in 14Q11.2 [32]. CMTM family genes are silenced or down-regulated in a variety of tumors, and their abnormal expression is related to the occurrence, development and metastasis of tumors, which are potential tumor suppressor genes and provide new ideas for clinical treatment of tumors. In recent years, it has been found that restoring CMTM family genes expression in tumor cells can inhibit tumor cells proliferate migrate and even initiate apoptosis. It has been proven that CMTM3, CMTM5 and CMTM7 are new potential tumor suppressor genes which are involved in tumor genesis and development [33-42]. CMTM4 is widely and highly expressed in a variety of tissues, with the highest expression level in pancreas and testis. Using tissue microarrays and immunohistochemical detection of CMTM4 expression in esophageal and cardiac cancer, it showed that CMTM4 was positive in the paracancer and the positive rate in normal tissue was higher than that in tumor tissue [43]. CMTM4 is the most conservative member of the CMTM family [44]. Its tumor

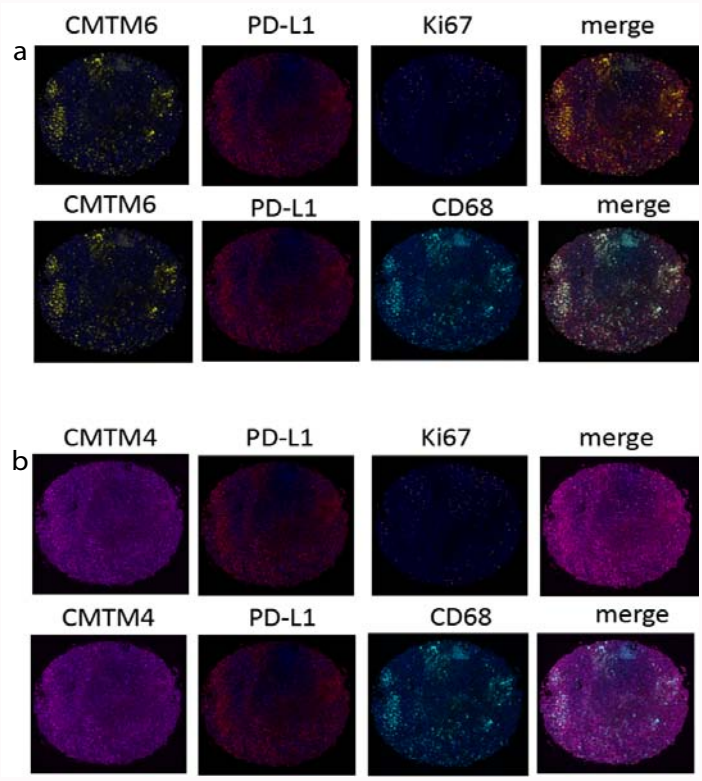

Figure 5: Expression of CMTM6/CMTM4 and PD-L1 in tumor cells (Ki67+) and macrophages (CD68+). (a). Expression of CMTM6 and PD-L1 in tumor cells (Ki67+) and in macrophages (CD68+); (b). Expression of CMTM4 and PD-L1 in tumor (Ki67+) cells and macrophages (CD68+).

suppressive effect has been demonstrated in HeLa cells and clear cell renal cell carcinoma cell lines [44-48]. There is no significant correlation between the type, pathological type, pathological differentiation degree and clinical stage. CMTM4 was overexpressed in HeLa cells, cell cycle was arrested in G2/M phase and cell growth was inhibited, but apoptosis was not induced [43,46]. Therefore, CMTM4 may be also a key molecule affecting cell growth and cycle regulation. In our study, we found that CMTM6 was associated with clinical factors, such as, recurrence and Ki67. According to a bioinformatics study, patients with high CMTM6 expression had a worse survival prognosis than those with low CMTM6 expression [49]. In addition, CMTM6 is expressed differently different WHO grades and different histopathology in tumors [49]. Therefore, on the other hand, CMTM6 may be involved in tumorigenesis in gliomas. Thus, the effect of CMTM6 in gliomas patients may depend on the balance of its function in both tumor and the immune system and we plan to further explore the mechanism of CMTM6 and CMTM4 on the tumorigenesis and progression in glioma in the future study. The expression level of CMTM family members in tumor tissue and the issue adjacent to carcinoma suggested that the CMTM may have potential application value in clinical diagnosis, individualized treatment and prognostic analysis of tumor.

To sum up, CMTM6/CMTM4 expression was significantly correlated with PD-L1in gliomas, which is agrees with that CMTM6/CMTM4 in the stabilization of PD-L1 in tumor cells. What is more, we found that the CMTM6/CMTM4 and PD-L1 colocolized in macrophages were associated with lower outcomes in glioma, which is suggesting that CMTM6/CMTM4 may be specific companion diagnostic biomarkers for immunotherapy in gliomas.

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