New Phenomenon

Analysis of CMTM6 and CMTM4 expression as potential regulators of the PD-L1 protein and its association with prognosis in glioma cancer

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Chemokine-like factor super family (CKLFSF) is a gene family reported in 2003. In humans, the family is composed of nine members, namely Chemokine-like factor (CKLF) and CKLF-like MARVEL transmembrane domain-containing member 10B (CMTM1–8) [1]. Genes in the CMTM family have different spliceosomes, which code for MAL-related proteins for vesicle trafficking and membrane link domain (MARVEL). Studies have shown that molecules containing this domain play important physiological and pathological roles in protein transport [2]. CMTM family plays an important role in the immune system and occurrence of tumors. Among them, CMTM3 and CMTM7 have functional characteristics of tumor suppressors, which are co-located with early endosomal marker Rab5, regulating the traffic and stability of membrane molecules, such as EGFR, VE-cadherin and BCR. Australian and Dutch researchers found that CMTM6 is the only gene that can regulate PD-L1 expression in the absence of IFNγ. CMTM6 inhibits PD-L1 ubiquitination and extends its half-life, avoiding lysosome-mediated degradation. Inhibition of CMTM6 expression could significantly promote T cell activation. In CMTM6-knockout cells, CMTM4 also has the function of stabilizing PD-L1 and is the standby regulatory molecule of PD-L1 [3,4]. These studies indicate that CMTM family plays an important role in the regulation of key membrane molecules involved in tumor genesis and development. CMTM6 and CMTM4 are new molecules that can be used to enhance the therapeutic benefits of immune checkpoint inhibitors. CMTM6 and CMTM4 are prognostic biomarkers in several kinds of tumors, which have attracted increasing attention.

Gliomas are a deadly and immunosuppressive brain tumor. 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 [5]. In the past few years, studies on anticancer immune therapies have promoted improvements to the limited success of conventional therapies. Antibodies targeting PD-L1 represent promising immunotherapies. Clinical trials of anti-PD-L1 drugs in glioma have been initiated. However, the rate of objective response and the rate of complete response are very low due to inadequate T-cell infiltration and immunosuppressive microenvironment [6]. Hence, new immune-related therapeutic targets have to be further exploited. Thus, in this study we evaluated the expressions of CMTM6 and CMTM4 in human glioma samples to assess its association with prognosis.

The upregulation/downregulation of CMTM6/CMTM4 in gliomas is supported by the TCGA (http://ualcan.path.uab.edu) and CGGA (http://www.cgga.org.cn) databases. CMTM6 mRNA level is significant higher while CMTM4 mRNA level is sharply lower in glioma than in normal tissue (Supplementary Figure S1A,D). CMTM6 mRNA level is increased while CMTM4 mRNA level is decreased as pathological grades increase (Supplementary Figure S1B,C), which indicates glioma prognosis. In addition, CMTM6/ CMTM4 mRNA levels are significantly different in different molecular phenotype of IDH (Supplementary Figure S1E,F), which is closely related to the clinical prognosis and clinical treatment selection. These bioinformatics data suggest that CMTM6 and CMTM4 likely play an important role in gliomas.

We further detected the expressions of CMTM6/CMTM4 and PD-L1 in the same glioma tissue samples by multiplexed immunofluorescence staining assay. TMAs were obtained from Shanghai Outdo Biotech (Shanghai, China), which contained 177 tumors, and the clinical characteristics of the patients are summarized in Supplementary Table S1. All experimental procedures followed the Human Ethical Committee protocol (YB M-05-02). The results showed that CMTM6 and PD-L1 are located mainly on the cell membrane and in the cytoplasm, while CMTM4 is observed in the cell membrane, in the cytoplasm and nucleus (Figure 1A). CD68 is located mainly on the cell membrane and in cytoplasm of macrophages, CD8 is located mainly on the cell membrane and in the cytoplasm of T cells, while Ki67 is observed mainly in the nucleus of tumor cells. CMTM6 and CMTM4 are detected in about 88% and 89.5% of samples, respectively. PD-L1 is detected in about 76.5% of samples.

To verify the results of bioinformatics analysis, the expression of CMTM4/CMTM6/PD-L1 among different pathological grades of
glioma was calculated after scoring the QIF in each sample. CMTM6, CMTM4 and PD-L1 protein levels are all increased as pathological grades increase (Supplementary Figure S1G,I), which is corresponding to the bioinformatics analysis for CMTM6 and PD-L1 at the mRNA level. However, the CMTM4 protein level is not consistent with results of mRNA level from bioinformatics analysis, which needs more exploration. Furthermore, macrophages are major cells expressing CMTM6/CMTM4 and PD-L1, and co-localization of CMTM6/CMTM4 and PD-L1 is significantly higher in CD68+ macrophages than in Ki67+ tumors (Figure 1F,G).

To confirm that CMTM6 and CMTM4 play a key role in maintaining the stability of PD-L1 [3], we analyzed the linear correlation of CMTM6/CMTM4 and PD-L1 by Pearson’s correlation assessment. Results showed that CMTM6 and CMTM4 expressions are significantly related to PD-L1 positivity (Figure 1B–D). Thus, preventing CMTM6/CMTM4 from binding with PD-L1 may recover...
immunosuppression response and serve as a promising strategy for immunotherapy in glioma cancer.

We further analyzed whether the survival probability is related to CMTM6/CMTM4/PD-L1 protein level by Kaplan-Meier survival analysis and log-rank test. The median was used as the cut-off point to divide patients into high and low expression groups. Results showed that patients with high CMTM6/CMTM4/PD-L1 expression (Figure 2A–C), high CMTM6/CMTM4 and PD-L1 co-expression (Figure 2D–E), and high CMTM4 and PD-L1 co-expression showed a significantly lower overall survival than patients with low expression (Figure 2F–H). Overall survival was not obviously extended in patients with high CMTM6/CMTM4/PD-L1 single expression in macrophages. (I–J). Overall survival is significantly higher 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.

Figure 2. 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 high PD-L1 co-expression showed a significantly lower overall survival than patients with low CMTM4 and low PD-L1 co-expression. (E) Patients with high CMTM4 and high PD-L1 co-expression showed a significantly lower overall survival than patients with low CMTM4 and low PD-L1 co-expression. (F–H) Overall survival is not obviously extended in patients with high CMTM6/CMTM4/PD-L1 single expression in macrophages. (I–J). Overall survival is significantly higher 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.
suggest that CMTM family may have potential application value in clinical diagnosis, individualized treatment and prognostic analysis of tumor.

In summary, we showed that CMTM6/CMTM4 expression is significantly correlated with PD-L1 in gliomas, which corresponds to the role of CMTM6/CMTM4 in the stabilization of PD-L1 in tumor cells. What is more, we found that macrophages are major cells expressing CMTM6/CMTM4 and PD-L1 in gliomas, which are associated with lower OS. This study suggests that CMTM6/CMTM4 may be specific companion diagnostic biomarkers for further guiding the immunological intervention in gliomas.

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