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CMTM6 and PD-L1 coexpression is associated with an active immune microenvironment and a favorable prognosis in colorectal cancer

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ABSTRACT

Background CKLF-like MARVEL transmembrane domain-containing 6 (CMTM6), a programmed death-ligand 1 (PD-L1) regulator, is widely expressed in various tumors and regulates the immune microenvironment. However, its prognostic value remains controversial, and the roles of CMTM6 in colorectal cancer (CRC) are still unknown. In this study, we aimed to elaborate the expression patterns of CMTM6 and PD-L1 in CRC and investigate their relationship with the infiltration of T cells and the prognosis of patients with CRC.

Methods Analysis of CMTM6 mRNA levels, gene ontology enrichment analysis and single-sample gene set enrichment analysis were performed in a The Cancer Genome Atlas colon cancer cohort. The expression of CMTM6 and PD-L1 and the infiltration of T cells in tumor tissues from our cohort containing 156 patients with CRC receiving adjuvant chemotherapy and 77 patients with CRC without chemotherapy were examined by immunohistochemistryassay.

Results CMTM6 expression was upregulated in CRC compared with normal colon tissues, and CMTM6 levels were lower in advanced tumors than in early-stage tumors. High expression of CMTM6 correlated with lower pT stage and more $CD4^+/CD8^+$ tumor-infiltrating lymphocytes (TILs) and predicteda favorable prognosis in CRC. PD-L1 was expressed in CRC tissues at a low level, and PD-L1 positivity in tumor stroma (PD-L1(TS)), but not PD-L1 positivity in cancer cells (PD-L1(CC)), was associated with an increased density of $CD4^+$ TILs and a favorable prognosis. The coexpression status of CMTM6 and PD-L1(TS) divided patients with CRC into three groups with low, moderate and high risks of progression and death, and patients with $CMTM6^{High}/PD\text{-}L1(TS)^+$ status had the longest survival. Moreover, the prognostic value of CMTM6/PD-L1 expression was more significant in patients with CRC treated with adjuvant chemotherapy than in those not treated with chemotherapy.

Conclusion CMTM6 has a critical impact on the immune microenvironment and can be used as an independent prognostic factor for CRC. The coexpression status of CMTM6 and PD-L1 can be used as a new classification to stratify the risk of progression and death for patients with CRC, especially for patients receiving adjuvant chemotherapy. These findings may provide insights into improving responses to immunotherapy-included comprehensive treatment for CRC in the future.

BACKGROUND

CKLF-like MARVEL transmembrane domain-containing 6 (CMTM6), a member of the CMTM family, has been reported to be a regulator of programmed death-ligand 1 (PD-L1) that maintains the stability of PD-L1 on the cell membrane by inhibiting its ubiquitination-mediated degradation.[1 2] CMTM6 is widely expressed in various cells, including tumor and other types of cells, but its biological function is still unclear. It has been reported that CMTM6 activates the Wnt/β-catenin pathway to maintain the cancer stem cells of head and neck squamous cell carcinoma (HNSCC) and inhibits antitumor immunity, and CMTM6 overexpression may predict a poor prognosis for patients suffering from HNSCC.[3] However, high expression of CMTM6 may be related to a favorable prognosis in hepatocellular carcinoma[4] and lung adenocarcinoma,[5] and gene set enrichment analysis (GSEA) revealed that high expression of CMTM6 was associated with activated immune responses and inflammatory activities.[5]

Colorectal cancer (CRC) is a common lethal malignancy that ranks in the top three among all types of cancers in terms of incidence and mortality, accounting for 10% of tumor-related deaths each year.[6] CRC is a heterogeneous malignancy, as evidenced by significant variations in response to treatment and prognosis.[7] Despite great progress has been made in molecular biology technologies and therapeutic strategies, such as immunotherapy, which have substantially improved the outcome of patients,[8 9] it remains challenging to translate molecular observations

about genetic and epigenetic variations, the heterogeneity of tumors and tumor–host interactions, all of which will affect the clinical outcome of tumor patients, into clinical practice.[10–12]

Tumor-infiltrating lymphocytes (TILs) play an important role in the development of CRC; however, whether they suppress or promote tumor development depends on TIL type and the immune microenvironment.[13–16] Immune infiltrate profiles are consistently associated with specific molecular features of CRC.[17] PD-L1 expression is reported to be related to T-cell subpopulations in various immune microenvironments,[12 18] and the programmed cell death-1 (PD-1)/PD-L1 axis has emerged as a highly clinically relevant mediator of tumor immune escape.[19 20] Although the genetic instability of tumor cells may result in immunogenicity, PD-L1 expression can enable tumor cells to evade immune elimination by negatively regulating T-cell immune responses.[21] Nonetheless, previous studies report conflicting results about the prognostic value of PD-L1 expression in CRC.[18 22–26] To date, we have not fully elucidated the complex and intricate relationships between PD-L1 expression, tumor molecular features and TILs. Furthermore, the effects of CMTM6, as a PD-L1 regulator, on PD-L1 expression and the immune microenvironment in CRC are still unknown. In this study, we investigated the CMTM6 expression pattern in CRC and the relationships between the CMTM6 level and clinicopathological characteristics, the immune microenvironment and the prognosis of patients with CRC. In addition, we considered the combined expression status of CMTM6 and PD-L1 in CRC tissues to establish a novel immunophenotyping system that may act as a predictive biomarker for immunotherapeutic strategies in patients with CRC.

MATERIALS AND METHODS

Public dataset acquisition and analyses of differentially expressed genes (DEGs) and immune infiltration

The RNA sequencing data (level 3) and clinical information of the NCI's Genomic Data Commons The Cancer Genome Atlas (TCGA) colon cancer cohort (TCGA cohort; 286 primary tumor, 26 paired and 15 unpaired normal colon specimens) were downloaded from the University of California Santa Cruz Xena browser (https://xenabrowser.net/datapages/). The levels of mRNA were shown as $\log_2(x+1)$ values (x: transformed RNA-Seq by Expectation Maximization normalized counts). Patients were defined as $CMTM6^{High}$ or $CMTM6^{Low}$ based on CMTM6 mRNA levels (the top 30% and the bottom 30%, respectively). The DEGs between the two groups were analyzed by the R package edger,[27] and significant differences were defined with fold change >1.5 and p value <0.05. Genes with upregulated expression in the $CMTM6^{High}$ group were subjected to gene ontology (GO) enrichment analysis via the online tool DAVID V.6.8 (https://david.ncifcrf.gov/).[28] False discovery rates <0.05 were considered significant. In addition, the infiltration of immune cells in the tumor microenvironment was analyzed using a single-sample GSEA (ssGSEA),[29] in which immune cell types were identified by specific gene markers,[30] and the enrichment score in the ssGSEA represented the relative abundance of each type of immune cell.

Patients and samples

A total of 233 patients were involved in this study and signed informed consent forms. The patients were pathologically and clinically diagnosed with CRC in Sun Yat-sen University Cancer Center (SYSUCC) from May 2007 to December 2015. The median age at surgery was 60 years (ranging from 28 to 86 years). All patients underwent surgery immediately without any neoadjuvant therapy, and 156 received adjuvant chemotherapy, while the other 77 did not (their clinicopathological parameters are shown in online supplemental table S1). Formalin-fixed, paraffin-embedded sections of tumor tissues were obtained from the pathology department of SYSUCC and re-evaluated by two pathologists according to the tumor-node-metastasis staging system of the eighth edition of the American Joint Committee on Cancer. The follow-up data of the patients were collected from the follow-up department of SYSUCC, and patients received regular follow-up (every 3 months for the first 2 years after surgery, every 6 months in the following 2 years and every year thereafter). Progression-free survival (PFS) was defined as the time span from the date of surgery to the date of cancer progression or death, and overall survival (OS) was defined as the time span from the date of surgery to the date of death. The follow-up was censored on December 31, 2019, and patients who did not experience progression or death during the follow-up period were censored at the last follow-up date. At the end of follow-up, 20.6% (48/233) of patients had progression, and 18.9% (44/233) of patients died from CRC. The median PFS and OS were 47.1 and 49.53 months, respectively (both ranging from 1.67 to 88.6 months).

Immunohistochemistry

Immunohistochemistry (IHC) staining to detect CMTM6, PD-L1, cluster of differentiation (CD) 8, CD4 and FoxP3 was performed by a professional pathologist according to previous reports.[3 18 31] Briefly, after deparaffinization, rehydration, antigen retrieval, endogenous peroxidase inactivation and non-specific binding blockade, 4 µM-thick sections were incubated with primary antibodies (anti-CMTM6: Sigma–Aldrich, HPA026980; anti-PD-L1: Cell Signaling Technology (CST), #13684; anti-CD8: CST, #85336; anti-CD4: Abcam, ab252199 and anti-FoxP3: Abcam, ab20034) at 4°C overnight. Then, the slides were incubated with a corresponding secondary antibody for 30 min at 37°C and visualized with a DAKO EnVision Detection System (Dako). Finally, the slides were counterstained with hematoxylin (CST, #14166), dehydrated and cover-slipped.

All immunostained sections were evaluated independently and blindly by two professional pathologists

from SYSUCC, yielding reasonably consistent results. Five fields (more than 500 cells) in each specimen were selected randomly for analysis. The CMTM6 expression levels were scored as an IHC Score, which was calculated as the proportion score based on stained cell percentage (0, 0%; 1, 1%–25%; 2, 26%–50%; 3, 51%–75% and 4, 76%–100%) multiplied by the staining intensity score (0, negative; 1, weak; 2, moderate and 3, intense) (online supplemental figure S1A), and the median IHC Score was chosen as the cut-off value for defining high and low expression of CMTM6. The expression of PD-L1 was evaluated separately in cancer cells (CC), tumor stroma (TS) cells or whole tumor tissue (whole) on stained sections as previously described,[24] and was defined as 'positive' if PD-L1 staining was present on ≥1% of cells (online supplemental figure S1B,C).[18 32] The infiltration of $CD4^+$, $CD8^+$, and $FoxP3^+$ T cells was measured as the percentage of cells staining positive in the invasive margin (IM) or TS (online supplemental figure S1B,D).[18]

Co-immunoprecipitation (IP) and mass spectrometry

Co-IP and mass spectrometry were performed as previously described.[33] Briefly, 293 T cells (American Type Culture Collection (ATCC), Manassas, Virginia, USA) were transfected with pEnter-CMTM6-Flag, a plasmid expressing CMTM6 with a Flag-tag at the C-terminus (Vigene Biosciences, Jinan, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). Forty-eight hours later, cells were collected and lysed with lysis buffer (CST, Danvers, Massachusetts, USA) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitors (KeyGen Biotech, Nanjing, China). The cell lysates were centrifuged to obtain supernatants, which were incubated with M2 anti-Flag agarose beads (A2220, Sigma-Aldrich). After washing, the IP products were examined by mass spectrometry. Proteins detected in the IP product of CMTM6-Flag-expressing cells, but not in that of control cells, were subjected to GO enrichment analysis via DAVID V.6.8.[28]

Statistical analysis

Data were analyzed using SPSS V.23.0 (IBM Corporation) or GraphPad Prism 8.0 software (GraphPad Software, San Diego, California, USA).The correlation analysis was performed using the χ^2 test, while the survival analysis was performed by the Kaplan-Meier method to calculate the survival probability in terms of PFS and OS, and the log-rank test was used to examine intergroup differences. Univariate and multivariate analyses were executed via a Cox proportional hazard model. A p value <0.05 was considered to indicate statistical significance.

RESULTS

The association of CMTM6 expression with the clinicopathological characteristics and prognosis of patients with CRC

We first analyzed CMTM6 expression in 286 CRC and 41 normal colon specimens from the TCGA cohort and found that CMTM6 mRNA levels were dramatically higher in CRC tissues than in normal colorectal tissues (p<0.001; figure 1A). Next, we compared CMTM6 expression in CRC specimens with different consensus molecular subtypes (CMSs) or clinical stages. The results showed that CMTM6 expression in CMS1 tissues, characterized by immune infiltration and immune cell activation, was significantly higher than that in other tissues (p=0.002), especially that in CMS2 and CMS4 tissues (p<0.001 and p=0.024, respectively; figure 1B). In addition, CMTM6 mRNA levels were higher in early-stage CRC (stage I/II) than in advanced-stage CRC (stage III/IV; p=0.024; figure 1C).

Next, we examined CMTM6 expression in CRC tissues of 233 patients from SYSUCC by IHC assay. The results showed that CMTM6 protein levels tended to be higher in early-stage CRC (stage I/II) than in advanced-stage CRC (stage III/IV; p=0.052; figure 1D), similar to the findings in the TCGA cohort above. We classified these patients into two groups using the median CMTM6 IHC score as the cut-off value (<4, $CMTM6^{Low}$; ≥4, $CMTM6^{High}$) and found that CMTM6 expression levels were significantly correlated with tumor anatomic site (p=0.002) and pT classification (p=0.031; table 1). Kaplan-Meier analysis revealed that patients with CRC with $CMTM6^{High}$ status had a significantly longer PFS (p=0.004) and OS (p=0.002) than those with $CMTM6^{Low}$ status (figure 2E).

CMTM6 levels are positively correlated with the immune response in CRC tissues

To explore the biological roles of CMTM6 in CRC, DEGs were analyzed between the $CMTM6^{High}$ and $CMTM6^{Low}$ groups from the TCGA cohort, and the top 100 DEGs (50 upregulated and 50 downregulated genes) in the $CMTM6^{High}$ group compared with the $CMTM6^{Low}$ group were used to generate a heatmap (online supplemental figure S2A). GO enrichment analysis revealed that the top 50 upregulated genes in the $CMTM6^{High}$ group were mainly enriched in immune or immune-related pathways, such as the adaptive immune response, immune response and T cell receptor signaling pathways (online supplemental figure S2B).

We knocked down the expression of the CMTM6 gene in RKO colon cancer cells (ATCC) and performed RNA sequencing analysis but failed to identify DEGs clustered in immune-associated signaling pathways (data not shown). These findings were different from those in CRC tissues from the TCGA cohort. We presumed that the difference may be due to the different cell composition of two subjects: RKO cells contained tumor cells only, while CRC tissues contained tumor cells, fibroblasts, vessel cells and various immune cells; thus, the enrichment of immune-related pathways in DEGs of CRC tissues was likely contributed by those tumor infiltrating immune cells. Considering that CMTM6 has been reported to maintain PD-L1 stability via protein-protein interactions, we performed a co-IP-mass spectrometry assay and found that potential proteins interacting with CMTM6, which

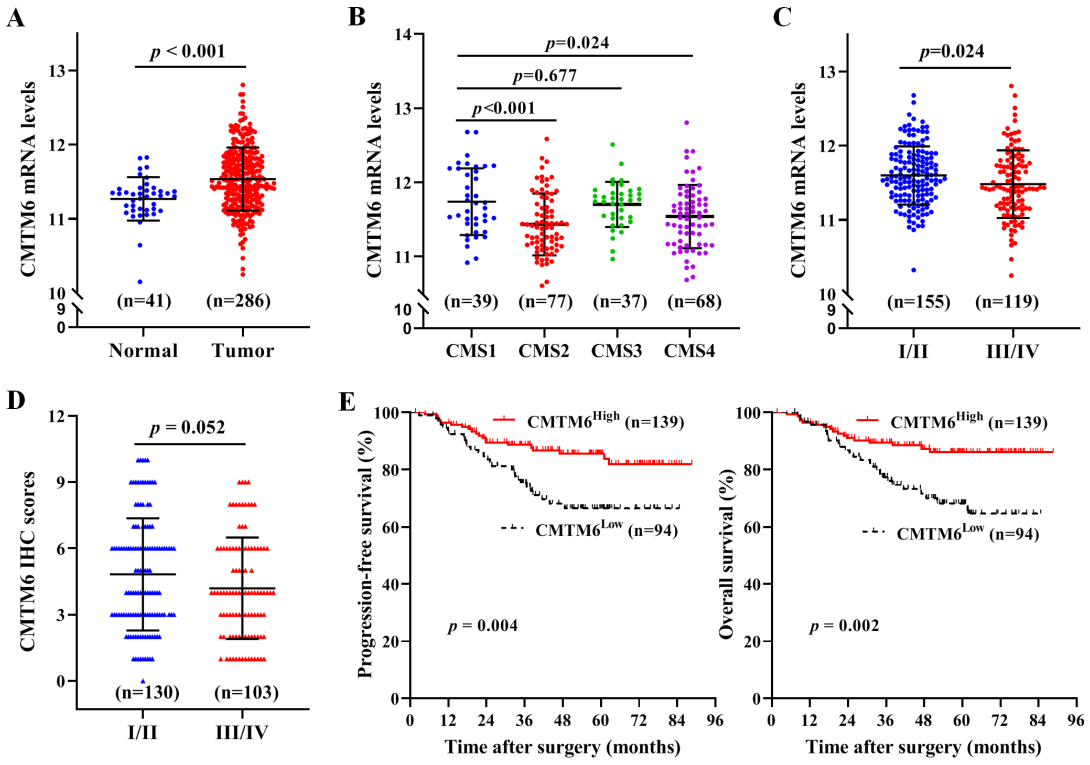

Figure 1 The expression pattern of CMTM6 in CRC and the association of CMTM6 expression with the prognosis of patients with CRC. (A–C) Relative CMTM6 mRNA levels (shown as log2(x+1) values) (A) in CRC tissues and adjacent normal tissues from the TCGA cohort or (B) in tumor tissues with various consensus molecular subtypes (CMS1–4) or (C) clinical stages (I/II and III/IV). (D) CMTM6 protein levels (IHC Scores) in CRC tissues at early (I/II) or advanced stages (III/IV). (E) Kaplan-Meier survival curves for progression-free survival (PFS) and overall survival (OS) of patients with CRC stratified by CMTM6 levels. CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; CRC, colorectal cancer; IHC, immunohistochemistry; TCGA, The Cancer Genome Atlas.

were pulled down by Flag (CMTM6) in CMTM6-Flag-expressing 293 T cells but not in blank vector-transfected 293 T cells, were related to immune-related pathways (online supplemental figure S2C). Based on these findings, how CMTM6 plays an immunomodulatory role, in addition to its interaction with PD-L1, should be investigated and clarified in future research.

To better understand the roles of CMTM6 in the immune response, ssGSEA was conducted to evaluate the immune cell composition of CRC samples from the TCGA cohort. As shown in figure 2A, there were many more tumor-infiltrating T cells, such as activated $CD4^+$ and $CD8^+$ T cells, effector memory $CD4^+$ and $CD8^+$ T cells, central memory $CD4^+$ T cells, gamma delta T cells, and Th1, Th2 and Th17 cells, but not regulatory T cells, in tumor tissues of the $CMTM6^{High}$ group than in those of the $CMTM6^{Low}$ group, indicating that CRC tissues with high levels of CMTM6 had a tumor microenvironment with an activated adaptive immune phenotype. However, there were no significant differences in natural killer (NK) cells, including $CD56^{bright}$ and $CD56^{dim}$ NK cells, eosinophils, macrophages, mast cells, neutrophils and plasmacytoid dendritic cells (DCs), between the $CMTM6^{High}$ and $CMTM6^{Low}$ groups; as an exception, there were differences in activated and immature DCs between the two groups. These data reflect that CMTM6 may play an important role in regulating the adaptive antitumor immune response in CRC.

Furthermore, we investigated the infiltration of $CD4^+$, $CD8^+$ and regulatory ($FoxP3^+$) T cells in CRC tissues from the SYSUCC cohort by IHC assay. The results showed that there were significantly more $CD4^+$ and $CD8^+$ T cells in both the TS and IM of $CMTM6^{High}$ tissues than in those of $CMTM6^{Low}$ tissues (figure 2B,C), but there were no differences in $FoxP3^+$ regulatory T (Treg) cells between the two groups (figure 2D), similar to the findings in the TCGA cohort.

The relationship between PD-L1 expression and CMTM6 levels in CRC

Because CMTM6 has been reported as a PD-L1 regulator, we next investigated the relationship between PD-L1 expression and CMTM6 levels in CRC. CMTM6 expression was much higher (59.7% of CRC tissues with IHC score >4) than PD-L1 expression in CRC. There was a low frequency of PD-L1 expression in whole CRC tissues (PD-L1(whole)), and only 30% of tissues showed PD-L1 staining on ≥1% of cells ($PD\text{-}L1^+$) (online supplemental table S2). CMTM6 expression levels (IHC scores) were significantly higher in $PD\text{-}L1(whole)^+$ than in $PD\text{-}L1(whole)^-$ CRC tissues (Student's t-test, p<0.05; online supplemental figure S3); the χ^2 test showed that

Table 1 CMTM6 expression and clinicopathological features

Variables	Number of cases (%)	CMTM6 expression		P value*
		Low (n=94)	High (n=139)	
Gender				
Male	131 (56.2)	58 (24.9)	73 (31.3)	0.180
Female	102 (43.8)	36 (15.5)	66 (28.3)	
Age (years)				
<60	120 (51.5)	49 (21.0)	71 (30.5)	0.894
≥60	113 (48.5)	45 (19.3)	68 (29.2)	
Pathology				
AC	133 (57.1)	48 (20.6)	85 (36.5)	0.139
MAC	100 (42.9)	46 (19.7)	54 (23.2)	
Anatomy				
Colon	158 (67.8)	75 (32.2)	83 (35.6)	**0.002**
Rectum	75 (32.2)	19 (8.2)	56 (24.0)	
Location				
Left	68 (29.2)	30 (12.9)	38 (16.3)	0.466
Right	165 (70.8)	64 (27.5)	101 (43.3)	
pT classification				
T1–3	133 (57.1)	62 (26.6)	71 (30.5)	**0.031**
T4	100 (42.9)	32 (13.7)	68 (29.2)	
pN classification				
N0	134 (57.5)	55 (23.6)	79 (33.9)	0.893
N1–2	99 (42.5)	39 (16.7)	60 (25.8)	
pM classification				
M0	212 (91.0)	83 (35.6)	129 (55.4)	0.252
M1	21 (9.0)	11 (4.7)	10 (4.3)	
Clinical stage				
I–II	130 (55.8)	51 (21.9)	79 (33.9)	0.788
II–IV	103 (44.2)	43 (18.5)	60 (25.8)	
CEA (ng/μL)				
<5	107 (45.9)	48 (20.6)	59 (25.3)	0.228
≥5	126 (54.1)	46 (19.7)	80 (34.3)	
CA19-9 (kU/L)				
<35	169 (72.5)	63 (27.0)	106 (45.5)	0.136
≥35	64 (27.5)	31 (13.3)	33 (14.2)	
PD-L1(TS)				
Negative	141 (60.5)	63 (27.0)	78 (33.5)	0.103
Positive	92 (39.5)	31 (13.3)	61 (26.2)	
PD-L1(CC)				
Negative	178 (76.4)	74 (31.8)	104 (44.6)	0.532
Positive	55 (23.6)	20 (8.6)	35 (15.0)	
PD-L1 (whole)				
Negative	163 (70.0)	71 (30.5)	92 (39.5)	0.146
Positive	70 (30.0)	23 (9.9)	47 (20.2)	

*P values were calculated using a two-sided Wald χ^2 test. P value <0.05 in bold is statistically significant.
AC, adenocarcinoma; CA19-9, carbohydrate antigen 19-9; CC, cancer cells; CEA, carcinoembryonic antigen; CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; MAC, mucinous adenocarcinoma; PD-L1, programmed death-ligand 1; TS, tumor stroma.

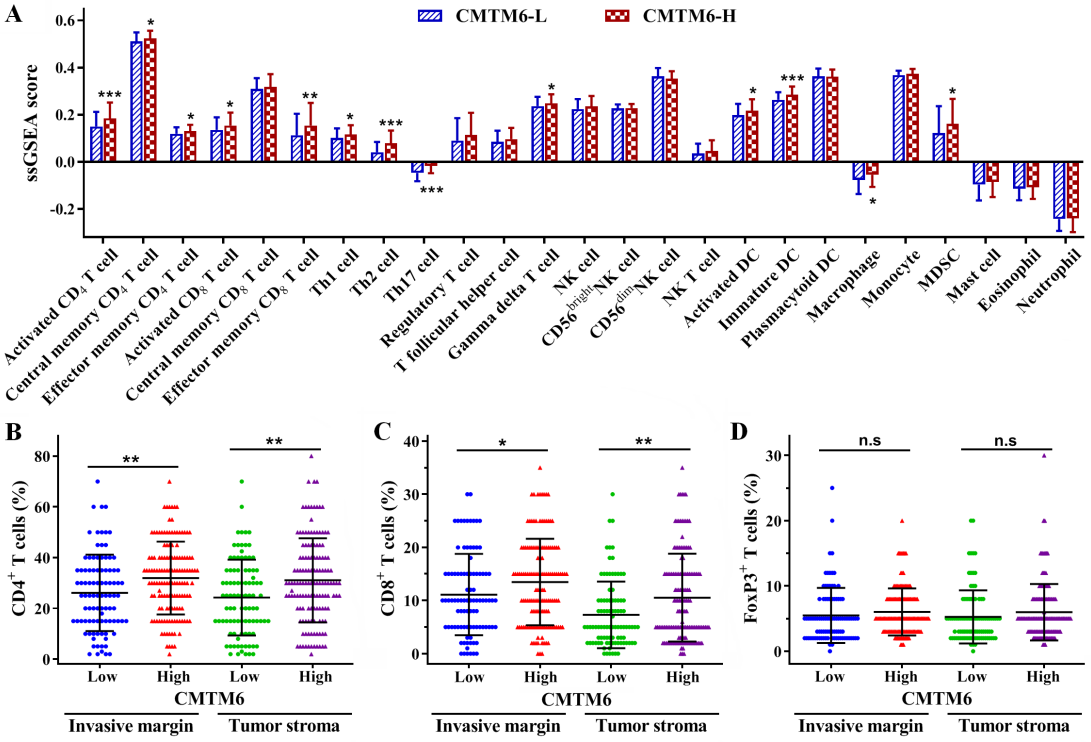

Figure 2 A comparison of immune cell infiltration in CRC samples with high or low levels of CMTM6 expression. (A) ssGSEA analysis of RNA-Seq data from the TCGA cohort. Immune cell types were defined by specific gene markers. (B–D) IHC analysis of (B) $CD4^+$, (C) $CD8^+$ and (D) $FoxP3^+$ T cell infiltration in CRC tissues with high or low CMTM6 expression from the SYSUCC cohort. *p<0.05; **p<0.01; ***p<0.001; n.s, no significant difference. CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; CRC, colorectal cancer; IHC, immunohistochemistry; ss-GSEA, gene set enrichment analysis; SYSUCC, Sun Yat-sen University Cancer Center; TCGA, The Cancer Genome Atlas.

CMTM6 expression status (high or low) was not associated with the status of PD-L1(whole) (positive or negative) (p=0.146; table 1). Furthermore, the positive rates of PD-L1 in CC (PD-L1(CC)) and in TS (PD-L1(TS)) were 23.6% (55/233) and 39.5% (92/233), respectively (online supplemental table S2). CMTM6 expression was significantly higher in $PD\text{-}L1(TS)^+$ than in $PD\text{-}L1(TS)^-$ CRC tissues (Student's t-test, p<0.05), but there was no difference between $PD\text{-}L1(CC)^+$ and $PD\text{-}L1(CC)^-$ tissues (online supplemental figure S3). Similar to PD-L1(whole) status, the PD-L1(TS) and PD-L1(CC) statuses were not associated with CMTM6 expression in CRC tissues (χ^2 test, p=0.103 and 0.532, respectively; table 1). These findings suggested a weak association between PD-L1 and CMTM6 protein levels in CRC. In addition, CMTM6 was identified to play a role in immune regulation beyond acting as a PD-L1 regulator, with PD-L1 expression being regulated by multiple factors other than CMTM6.

The association of PD-L1 expression with the infiltration of T cells and prognosis of patients with CRC

It has been reported that PD-L1 influences the tumor microenvironment by inhibiting the function of T lymphocytes in multiple cancers[18 34]; thus, we investigated the association of PD-L1 expression with the infiltration of T cells and the prognosis of patients with CRC. The results showed that the patients with CRC with $PD\text{-}L1(TS)^+$ had longer PFS and OS than those with $PD\text{-}L1(TS)^-$ (p=0.013 and 0.036, respectively), but there were no significant differences in survival (PFS and OS) between patients with $PD\text{-}L1(whole)^+$ and those with $PD\text{-}L1(whole)^-$ (p=0.140 and 0.243, respectively) or between patients with $PD\text{-}L1(CC)^+$ and those with $PD\text{-}L1(CC)^-$ (p=0.933 and 0.678, respectively; figure 3A–C). Further analysis of T cell infiltration revealed that there were no differences in the infiltration of $CD4^+$, $CD8^+$ or $FoxP3^+$ T cells in either TS or IM of CRC tissues between two groups classified based on PD-L1 expression status (figure 3D–F, online supplemental figure S4), except that there were more $CD4^+$ T cells in the TS of $PD\text{-}L1(TS)^+$ CRC tissues than in that of $PD\text{-}L1(TS)^-$ CRC tissues (figure 3D).

CMTM6 but not PD-L1 was an independent predictor of the survival of patients with CRC

Univariate Cox regression analyses of patients with CRC in the SYSUCC cohort revealed that in addition to higher T, N and M stages, clinical stage and CA19-9 level (≥35 kU/L), lower CMTM6 ($CMTM6^{Low}$) (PFS, HR=2.267, 95% CI 1.276 to 4.029, p=0.005; OS, HR=2.483, 95% CI 1.353 to 4.559, p=0.003) and PD-L1(TS) (PFS, HR=2.302, 95% CI 1.174 to 4.514, p=0.015; OS, HR=2.046, 95% CI 1.034 to 4.049, p=0.004) were associated with a higher risk of disease progression and death (table 2). Multivariate Cox regression analysis showed that CMTM6

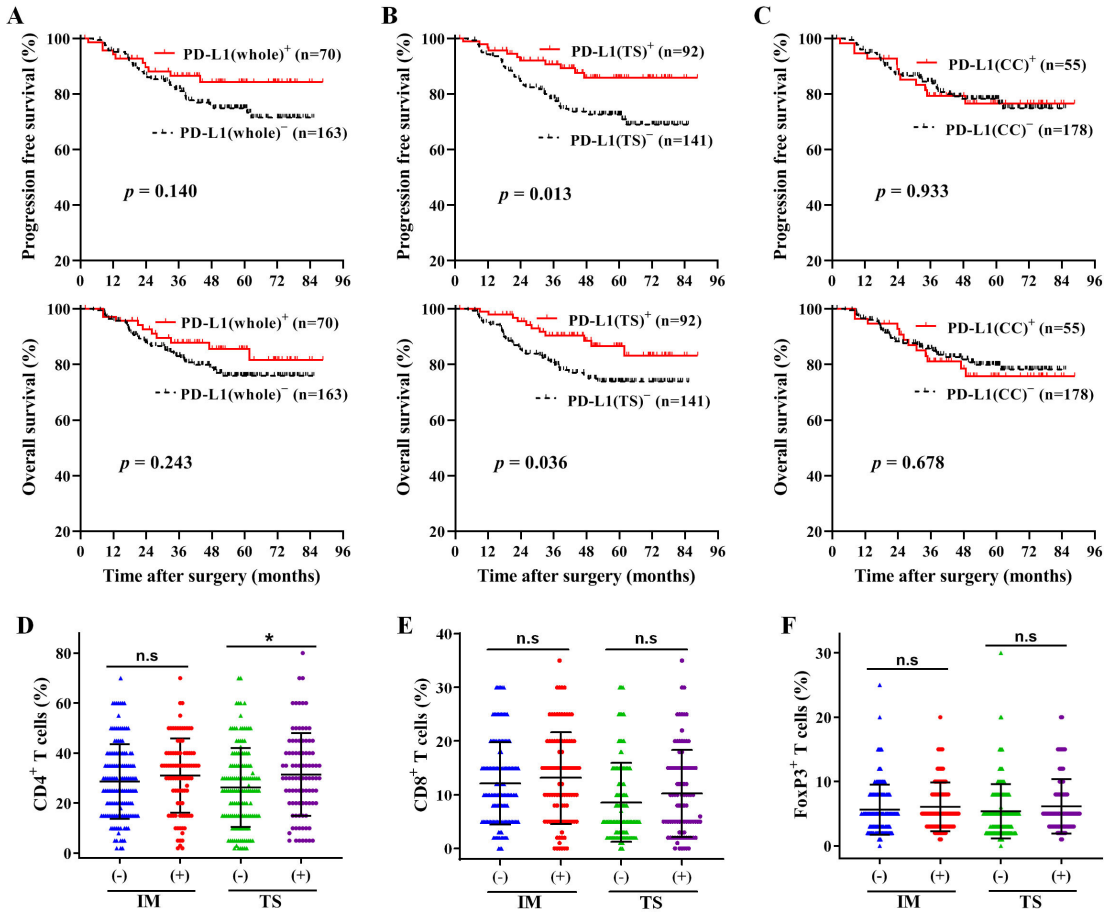

Figure 3 The association of PD-L1 expression with the infiltration of T cells and prognosis of patients with CRC. (A–C) Kaplan-Meier survival curves for PFS and OS of patients with CRC from the SYSUCC cohort based on the expression status of (A) PD-L1(whole), (B) PD-L1(TS) or (C) PD-L1(CC). (D–F) The infiltration of (D) $CD4^+$, (E) $CD8^+$ or (F) $FoxP3^+$ T cells in CRC tissues with $PD\text{-}L1(TS)^+$ or $PD\text{-}L1(TS)^-$ from the SYSUCC cohort. (+), $PD\text{-}L1(TS)^+$; (–), $PD\text{-}L1(TS)^-$. *p<0.05; n.s, no significant difference. CC, cancer cells; CRC, colorectal cancer; IM, invasive margin; OS, overall survival; PD-L1, programmed death-ligand 1; PFS, progression-free survival; SYSUCC, Sun Yat-sen University Cancer Center; TS, tumor stroma.

but not PD-L1(TS) was an independent predictor for PFS and OS in CRC (PFS: HR=1.833, 95% CI 1.005 to 3.343, p=0.048; OS: HR=1.953, 95% CI 1.040 to 3.669, p=0.037) (table 2).

Prognostic significance of coexpression of CMTM6 and PD-L1 in CRC

To better analyze the relationship between the expression of CMTM6 and/or PD-L1 and the prognosis and immune microenvironment of patients with CRC, we considered PD-L1 expression in combination with CMTM6 expression to generate a potential tool for predicting survival and tested it in survival analysis. We found that the expression status of PD-L1(TS), but not that of PD-L1(whole) or PD-L1(CC), could effectively differentiate the survival of patients with $CMTM6^{High}$ (PFS, p=0.033; OS, p=0.091), and the survival curves of patients with $CMTM6^{High}/PD\text{-}L1(TS)^-$ almost coincided with those of patients with $CMTM6^{Low}/PD\text{-}L1(TS)^+$ (PFS, p=0.757; OS, p=0.559; figure 4A, online supplemental figure S5). Thus, we classified these patients with CRC into three groups: group 1 ($CMTM6^{High}/PD\text{-}L1(TS)^+$; low risk), group 2 ($CMTM6^{High}/PD\text{-}L1(TS)^-$ or $CMTM6^{Low}/PD\text{-}L1(TS)^+$; moderate risk) and group 3 ($CMTM6^{Low}/PD\text{-}L1(TS)^-$; high risk) according to survival curves of PFS (p=0.001) and OS (p=0.002; figure 4B). The proportions of patients in groups 1, 2 and 3 were 26.2% (61/233), 46.8% (109/233) and 27.0% (63/233), respectively.

We next examined the associations of CMTM6/PD-L1(TS) coexpression with T lymphocyte infiltration. The data showed that the level of infiltrating $CD4^+$ T lymphocytes in both the IM and TS of CRC tissues was highest in group 1 compared with groups 2 and 3 (IM, p<0.05 and<0.01; TS, p<0.01 and<0.001; figure 4C (upper)). In terms of $CD8^+$ T lymphocytes, there were no significant differences between group 1 and group 2, but they were significantly higher in group 1 than in group 3 (IM: p<0.05; TS: p<0.001; figure 4C (middle)). However, there were no differences in the infiltration of $FoxP3^+$ Treg cells among the three groups (figure 4C (lower)).

CMTM6/PD-L1 expression status had better prognostic value in patients with CRC receiving adjuvant chemotherapy than those not receiving chemotherapy

Considering that traditional chemotherapy (such as oxaliplatin) has been reported to induce immunogenic

Table 2 Univariate and multivariate analyses of prognostic factors correlated with progression-free survival and overall survival

	Progression-free survival						Overall survival					
	Univariate			Multivariate			Univariate			Multivariate		
Variables	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Gender (male vs female)	1.234	0.692–2.201	0.477				1.144	0.627–2.088	0.660			
Age (<60 vs ≥60)	0.766	0.434–1.350	0.356				0.692	0.382–1.254	0.225			
Pathology (AC vs MAC)	0.860	0.476–1.554	0.617				0.703	0.380–1.299	0.261			
Anatomy (colon vs rectum)	1.020	0.559–1.861	0.948				1.397	0.719–2.715	0.324			
Location (left vs right)	1.142	0.620–2.104	0.670				1.311	0.702–2.448	0.395			
pT classification (T_{1-3} vs T_4)	0.444	0.247–0.797	**0.006**	0.579	0.313–1.071	0.082	0.513	0.281–0.936	**0.030**	0.722	0.381–1.365	0.316
pN classification (N_0 vs N_{1-2})	0.341	0.187–0.621	**<0.001**	0.579	0.305–1.102	0.096	0.316	0.168–0.596	**<0.001**	0.557	0.282–1.103	0.093
pM classification (M_0 vs M_1)	0.057	0.031–0.107	**<0.001**	0.105	0.052–0.213	**<0.001**	0.044	0.023–0.085	**<0.001**	0.075	0.036–0.159	**<0.001**
Clinical stage (I–II vs III–IV)	0.263	0.139–0.497	**<0.001**				0.234	0.118–0.463	**<0.001**			
CEA (ng/mL) (<5 vs ≥5)	0.961	0.544–1.695	0.890				0.961	0.531–1.740	0.896			
CA19-9 (kU/L) (<35 vs ≥35)	0.447	0.251–0.793	**0.006**	0.647	0.356–1.173	0.151	0.427	0.235–0.776	**0.005**	0.603	0.324–1.120	0.109
CMTM6 (low vs high)	2.267	1.276–4.029	**0.005**	1.833	1.005–3.343	**0.048**	2.483	1.353–4.559	**0.003**	1.953	1.040–3.669	**0.037**
PD-L1 (whole) ((–) vs (+))	1.679	0.837–3.371	0.145				1.517	0.750–3.071	0.247			
PD-L1 (TS) ((–) vs (+))	2.302	1.174–4.514	**0.015**	1.528	0.762–3.065	0.233	2.046	1.034–4.049	**0.004**	1.259	0.618–2.564	0.525
PD-L1 (CC) ((–) vs (+))	0.972	0.506–1.869	0.933				0.869	0.448–1.687	0.678			

P value <0.05 in bold is statistically significant.
(–), negative; (+), positive; AC, adenocarcinoma; CA19-9, carbohydrate antigen 19-9; CC, cancer cell; CEA, carcinoembryonic antigen; CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; MAC, mucinous adenocarcinoma; PD-L1, programmed death-ligand 1; TS, tumor stroma.

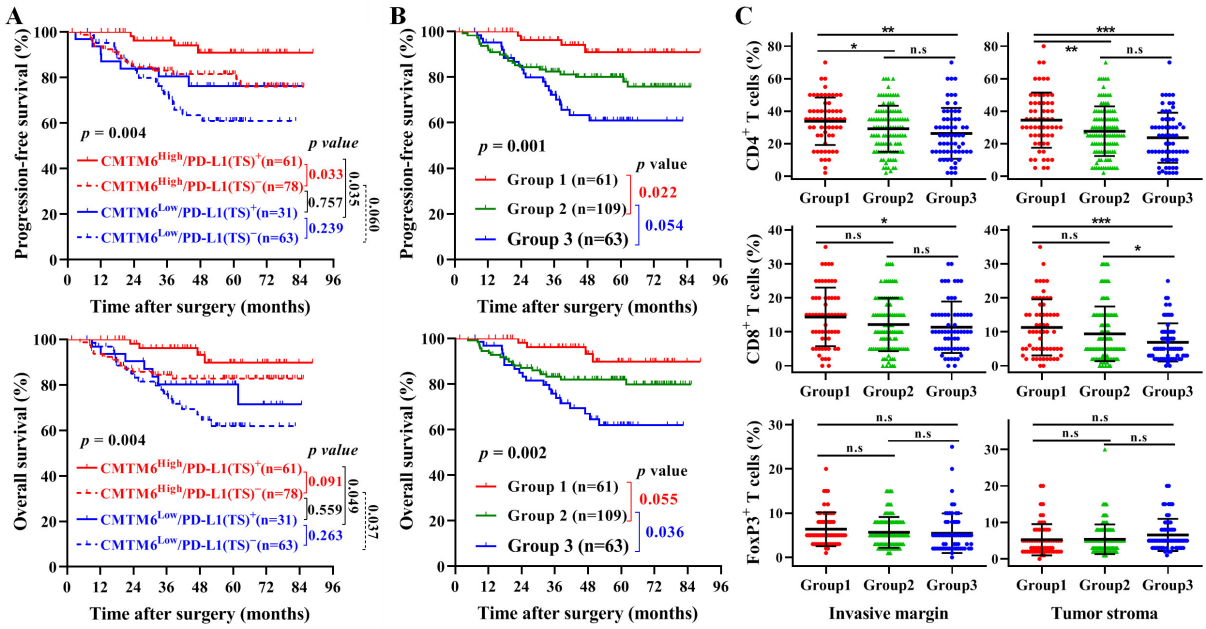

Figure 4 The association of coexpression of CMTM6/PD-L1 with the infiltration of T cells and the prognosis of patients with CRC. (A) Kaplan-Meier survival curves for progression-free survival (PFS) and overall survival (OS) of patients with CRC from the SYSUCC cohort based on the coexpression status of CMTM6/PD-L1(TS). (B) Kaplan-Meier survival curves for PFS and OS of patients with CRC in three groups: group 1, $CMTM6^{High}$/PD-L1(TS)$^+$; group 2, $CMTM6^{High}$/PD-L1(TS)$^-$ or $CMTM6^{Low}$/PD-L1(TS)$^+$ and group 3, $CMTM6^{Low}$/PD-L1(TS)$^-$. (C) The infiltration of $CD4^+$ (upper), $CD8^+$ (middle) or $FoxP3^+$ (lower) in CRC tissues from groups 1, 2 and 3. *p<0.05; **p<0.01; ***p<0.001; n.s, no significant difference. CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; CRC, colorectal cancer; PD-L1, programmed death-ligand 1; SYSUCC, Sun Yatsen University Cancer Center; TS, tumor stroma.

cell death, thereby improving the tumor immune microenvironment,[35] we performed subgroup survival analysis stratified by whether patients received adjuvant chemotherapy or not. Kaplan-Meier analyses revealed that patients with CRC with $CMTM6^{High}$ had a significantly longer PFS (p<0.001) and OS (p<0.001) than those with $CMTM6^{Low}$ in the subgroup received adjuvant chemotherapy (figure 5A), but there were no significant differences in PFS (p=0.246) or OS (p=0.270) between patients with $CMTM6^{High}$ and $CMTM6^{Low}$ in the subgroup not received adjuvant chemotherapy (figure 5B). Similar phenomena were observed for PD-L1(TS) expression status in differentiating the survival of patients with CRC: p=0.019 for PFS and p=0.054 for OS in the adjuvant chemotherapy subgroup; p=0.442 for PFS and p=0.455 for OS in the non-adjuvant chemotherapy subgroup (figure 5C,D). Notably, PD-L1(whole), a factor without obvious prognostic value in the whole CRC cohort, displayed a significant predictive value for the survival of patients with CRC who received adjuvant chemotherapy (PFS, p=0.032; OS, p=0.070; online supplemental figure S6A), although PD-L1(CC) still showed no prognostic value for patients in the adjuvant chemotherapy subgroup (online supplemental figure S6B).

Because PD-L1(TS) showed more significant prognostic value than PD-L1(whole), we considered the expression of PD-L1(TS) and CMTM6 jointly and analyzed its association with the survival of patients with CRC who received adjuvant chemotherapy. The results were similar to those from the analysis of the whole cohort (figure 4A): in the adjuvant chemotherapy subgroup, patients with $CMTM6^{High}$/PD-L1(TS)$^+$ had the best prognosis, those with $CMTM6^{Low}$/PD-L1(TS)$^-$ had the worst prognosis, and those with $CMTM6^{High}$/PD-L1(TS)$^-$ and those with $CMTM6^{Low}$/PD-L1(TS)$^+$ showed similar survival curves, especially for PFS (online supplemental figure S7). Thus, patients in adjuvant chemotherapy subgroup were divided into three groups, as was done for the whole cohort, for subgroup survival analysis. The results showed that this new classification (the coexpression status of CMTM6 and PD-L1(TS)) could divide patients with CRC who received adjuvant chemotherapy into three groups with low, moderate or high risk (PFS, p<0.001; OS, p<0.001; figure 5E) but was unable to predict prognosis for patients who did not receive adjuvant chemotherapy (PFS, p=0.419; OS, p<0.442; figure 5F). These findings suggest that CMTM6/PD-L1 expression may have better prognostic value for patients with CRC receiving adjuvant chemotherapy than for those not receiving adjuvant chemotherapy. Similar to the analysis of T lymphocyte infiltration in the whole cohort (shown in figure 4C), there were higher infiltration of $CD4^+$ or $CD8^+$ T cells in the tumor tissues of group 1 than in those of group 2 and/or group 3 in adjuvant chemotherapy subgroup (online supplemental figure S8).

DISCUSSION

Over the past decades, the implications of the tumor immune microenvironment on the therapeutic efficacy

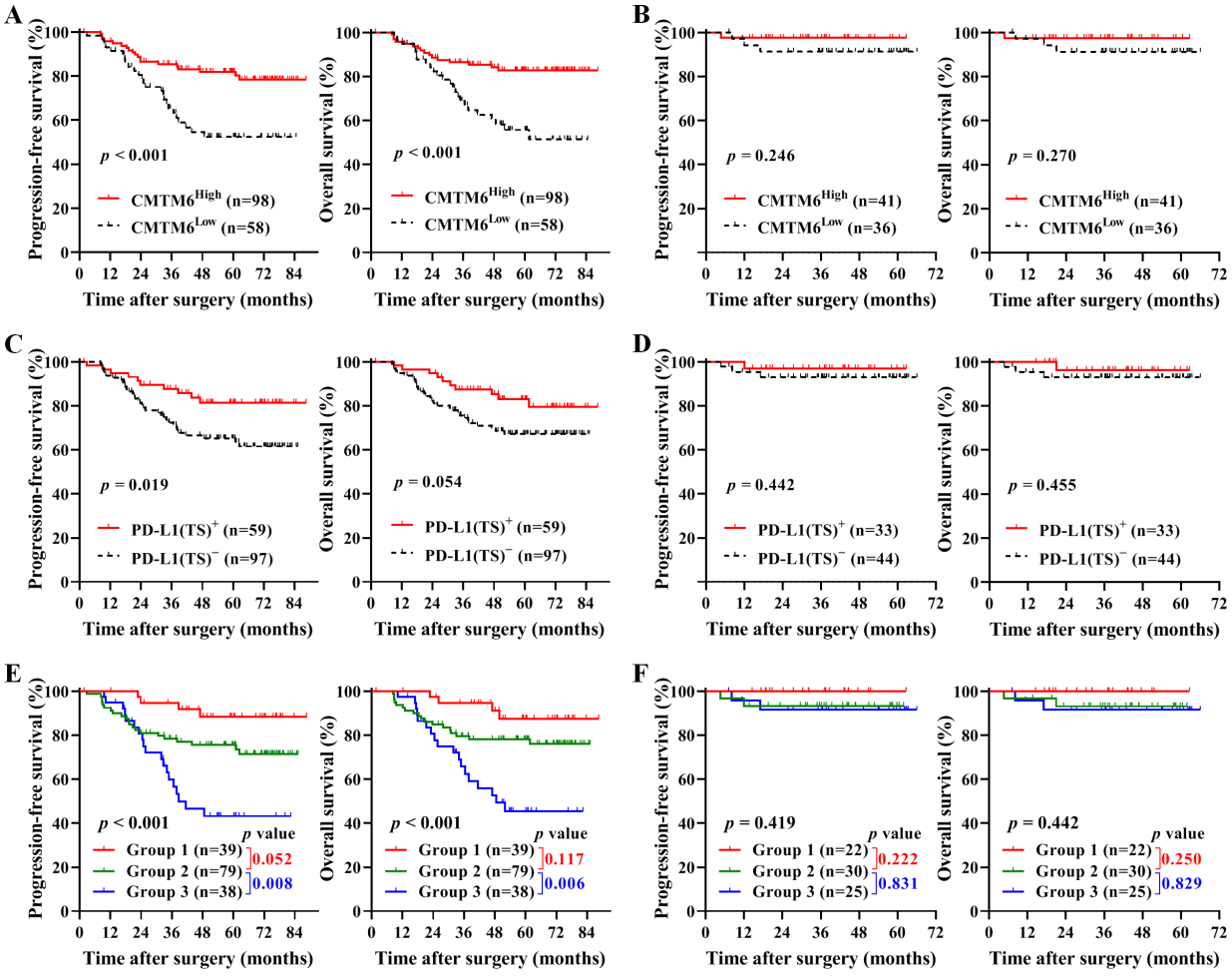

Figure 5 Subgroup survival analysis for patients with CRC receiving or not receiving adjuvant chemotherapy. Kaplan-Meier survival curves for progression-free survival and overall survival of patients treated with (A, C, E) or not treated with (B, D, F) adjuvant chemotherapy based on (A, B) CMTM6 expression, (C, D) PD-L1(TS) expression, or (E, F) the coexpression status of CMTM6/PD-L1(TS) (group 1, $CMTM6^{High}$/PD-L1(TS)$^+$; group 2, $CMTM6^{High}$/PD-L1(TS)$^-$ or $CMTM6^{Low}$/PD-L1(TS)$^+$; and group 3, $CMTM6^{Low}$/PD-L1(TS)$^-$). p<0.05 indicating significant difference. CMTM6, CKLF-like MARVEL transmembrane domain-containing 6; CRC, colorectal cancer; PD-L1, programmed death-ligand 1; PD-L1(TS), PD-L1 in the tumor stroma; TS, tumor stroma.

and prognosis of cancer patients have received increasing attention.[36 37] Immunotherapy targeting immune checkpoints (such as PD1/PD-L1) has become an approved treatment option for patients with CRC with mismatch repair deficiency or high microsatellite instability.[38] CMTM6 has been identified to maintain the stability of PD-L1 by inhibiting the ubiquitination-induced degradation of PD-L1 and is involved in the regulation of the tumor microenvirnment.[1 2] However, CMTM6 has displayed different prognostic significance in various types of malignancies; high levels of CMTM6 were related to a favorable prognosis in hepatocellular carcinoma,[4] lung adenocarcinoma[5] and triple-negative breast cancer,[39] but predicted a poor prognosis in HNSCC,[3] pancreatic adenocarcinoma[39] and glioma.[40] In this study, we first examined the profile of CMTM6 expression in CRC and found that CMTM6 was significantly upregulated in CRC tissues compared with normal colorectal tissues, but CMTM6 levels were lower in advanced CRC than in early-stage CRC tissues. Considering that CMTM6 expression has also been reported to be correlated with increased activity of Wnt/β-catenin signaling, which is essential for tumorigenesis, maintenance of cancer stem cells and epithelial-to-mesenchymal transition in multiple cancers,[3] we hypothesized that CMTM6 may mainly play a tumor promoting role during the carcinogenic transformation of the colorectum and instead serve as an immunoregulator in the development stage of CRC. Responding to the supposition above, we found that high CMTM6 levels were associated with a tumor microenvironment with an activated adaptive immune phenotype, specifically increased infiltration of activated $CD4^+$ and $CD8^+$ T cells, and longer OS and PFS. These findings were consistent with those reported in hepatocellular carcinoma and lung adenocarcinoma.[4 5]

Although CMTM6 binds PD-L1 to maintain the stability of PD-L1 on the cell surface, it does not affect PD-L1 mRNA levels or compromise antigen presentation by MHC class I.[2 40] We found that CMTM6 expression was much higher than PD-L1 expression in CRC, and CMTM6 levels showed

only a weak association with PD-L1 expression on the cell surface in CRC samples. This may have been the results of the low expression of PD-L1 in CRC tissues, especially in cancer cells. The key factor promoting tumor PD-L1 expression is IFN-γ, which is mainly produced by infiltrating lymphocytes in the TS, but CMTM6 expression is independent of the IFN-γ pathway,[1 41] and CMTM6 does not participate in the transcription and translation of PD-L1.[1 2] These findings suggest that PD-L1 expression is regulated by multiple factors other than CMTM6 and that some factors suppress PD-L1 expression in CRC tissues.

Although PD-L1 expression in tumor tissues has been reported as a prognostic factor in patients receiving conventional treatments as well as in patients receiving anti-PD-1/PD-L1 immunotherapy,[42] previous studies have reported conflicting results about the prognostic value of PD-L1 expression in CRC.[18 22–26] Li *et al* and Droeser *et al* reported that higher expression of PD-L1 correlates with better prognosis in patients with CRC.[22 24] Masugi *et al* and Eriksen *et al* posited that PD-L1 expression in tumor cells does not provide any prognostic impact for CRC.[18 23] However, two other studies revealed that the prognostic value of PD-L1 depends on the cell type of the tumor tissues expressing PD-L1,[25 26] and PD-L1 expression in different cells can predict different prognoses.[26] To accurately elucidate the prognostic value of PD-L1, we evaluated PD-L1 expression in CC (PD-L1(CC)) and TS (including immune cells) in the cancer nest (PD-L1(TS)) and found that PD-L1(TS) but not PD-L1(CC) was significantly associated with the prognosis of CRC, positivity of PD-L1 expression in the TS (especially in infiltrating lymphocytes) predicted lower risks of disease progression and death than PD-L1 negativity. We assume that these conflicting results regarding the prognostic value of PD-L1 resulted from the different methods used to evaluate PD-L1 expression (eg, assessment of mRNA level or protein level, and assessment in overall cells or in specific cell types) and the various study cohorts analyzed (eg, cohorts with various clinical/pathological stages and different treatment strategies). In support of our assumption, we also found that the expression of PD-L1(TS) showed better prognostic value in CRC treated with adjuvant chemotherapy than in CRC not treated with chemotherapy.

Furthermore, we found that the expression of PD-L1(TS) modulated the prognostic significance of CMTM6 in patients with CRC, and the coexpression status of CMTM6 and PD-L1(TS) divided patients into three groups with low, moderate and high risk of progression and death. Additionally, there was the highest infiltration of $CD4^+/CD8^+$ T cells, suggesting an activated immune microenvironment, in low risk group, which is consistent with the best prognosis in this group.

Interestingly, the expression status of CMTM6 and PD-L1(TS) showed better prognostic value in patients with CRC receiving adjuvant chemotherapy (fluorouracil only or fluorouracil combined with oxaliplatin, excluding immunotherapy) than in those not receiving adjuvant chemotherapy. Some published studies may help to explain our findings. It has been reported that fluorouracil may change the expression of PD-L1 in CRC cells,[43] and a low dose of 5-FU can specifically induce apoptosis of myeloid-derived suppressor cells,[44] thus reversing immunosuppression. Oxaliplatin can cause immunogenic cell death[35] and encourage CRC cells to express chemokines to induce T cell activation,[45] thereby activating T cell immunity. In other words, adjuvant chemotherapy in CRC may have the potential to mediate the antitumor immune response. High expression of CMTM6 or/and PD-L1(TS) positivity are related to high infiltration of $CD4^+/CD8^+$ T cells and an activated immune microenvironment. Adjuvant chemotherapy is expected to boost antitumor immunity in patients with CRC with $CMTM6^{High}$ or/and $PD\text{-}L1(TS)^+$, although the expression status of CMTM6 and PD-L1(TS) during and after chemotherapy remains to be studied.

In summary, CMTM6 expression was upregulated in CRC tissues, especially in early-stage CRCs, and high expression of CMTM6 correlated with an active immune microenvironment and a favorable prognosis. PD-L1 was expressed at a low level in CRC tissues, and PD-L1 positivity in the TS, but not in cancer cells, was associated with the infiltration of $CD4^+$ T cells and increased survival. The coexpression status of CMTM6 and PD-L1(TS) could divide patients with CRC into three groups with low, moderate and high risk of progression and death. Moreover, the prognostic value of CMTM6/PD-L1 expression status was more significant in patients with CRC receiving adjuvant chemotherapy than in those not receiving chemotherapy. However, the results of the survival analyses were from only one cohort from a single center, and the sample size was limited, especially for the subgroup not receiving chemotherapy, so a sample selection bias was inevitable. Therefore, we cannot conclude that the expression of CMTM6/PD-L1 is unable to predict the prognosis of patients with CRC not receiving chemotherapy. To address the shortcomings of this study, a large-scale and multicenter retrospective clinical study is needed.

Contributors R-YL contributed to conception and design. Q-HP, C-HW, G-YW and XY performed the experiments. Q-HP, H-MC, R-XZ, C-HW, Z-ZP, Z-HL and WH contributed to acquisition and interpretation of data. Q-HP and R-YL contributed to drafting the article. R-YL, XY and WH contributed to manuscript reviewing and editing. All authors approved the manuscript.

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Competing interests No, there are no competing interests.

Patient consent for publication Not required.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Data may be obtained from a third party and are not publicly available. The data used in the current study are available from the corresponding author on reasonable request. The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (https://www.researchdata.org.cn/), with the approval number RDDB2021000958.

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