CMTM6 Stabilizes PD-L1 Expression and Is a New Prognostic Impact Factor in Hepatocellular Carcinoma

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CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) was identified as a regulator of programmed death ligand 1 (PD-L1), which induces antitumor immunity in several cancers. This study aimed to clarify the relationship between CMTM6 and PD-L1 expression and clinical outcomes in patients with hepatocellular carcinoma (HCC). In total, 259 patients with HCC who had undergone hepatic resection were enrolled. Immunohistochemical staining for CMTM6 and PD-L1 was performed. The relationships between CMTM6 expression and the clinicopathological characteristics and outcomes were analyzed. Additionally, the stabilization of PD-L1 expression and regulation of malignant activities by CMTM6 were examined *in vitro*. Our patients were divided into high (n = 65, 25.1%) and low (n = 194, 74.9%) CMTM6 expression groups. High CMTM6 expression was significantly associated with malignant aggregates, including poor differentiation ($P < 0.0001$), microscopic intrahepatic metastasis ($P = 0.0369$), and multiple intrahepatic recurrences ($P = 0.0211$). CMTM6 expression was significantly correlated with PD-L1 expression in HCC tissues ($P < 0.0001$). The patients were classified into three groups: high CMTM6/PD-L1 positive (n = 21), high CMTM6/ PD-L1 negative (n = 44), and low CMTM6 (n = 194) expression pattern groups. Overall survival was significantly different among the three groups ($P < 0.0001$). Additionally, immunohistochemical double staining revealed that CMTM6 and PD-L1 were co-expressed on HCC cells. *In vitro*, PD-L1 expression was enhanced at late time points in the presence of CMTM6 expression. CMTM6 also regulated epithelial-to-mesenchymal transition and stemness phenotypes in HCC cells. *Conclusion:* Our large cohort study found that CMTM6 co-expressed with PD-L1 was strongly associated with the clinical outcome in patients with HCC. The evaluation of CMTM6 combined with PD-L1 in HCC might be useful for patient selection in immune checkpoint therapy. (*Hepatology Communications* 2021;5:334-348).

Hepatocellular carcinoma (HCC) is the most common primary liver neoplasm, the sixth most common neoplasm overall, and the third leading cause of cancer death.[1] Although hepatic resection has been established as a safe and effective treatment for patients with HCC, the prognosis of HCC remains poor.[2,3] To improve the outcomes of patients with HCC, a better understanding

Abbreviations: AFP, alpha-fetoprotein; CMTM6, CKLF-like MARVEL transmembrane domain containing 6; DAB, 3,3′-diaminobenzidine; DCP, des-gamma-carboxyprothrombin; EMT, epithelial-to-mesenchymal transition; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; IFN-γ, interferon γ; IHC, immunohistochemical; OS, overall survival; PD-1, programmed death protein 1; PD-L1, programmed death ligand 1; RFS, recurrence-free survival; TAM, tumor-associated macrophage.

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of the mechanisms of HCC progression is urgently needed. The malignant potential of tumor cells is regulated by the immune microenvironment.[4]

Recently, we and others have investigated that relationship between programmed death ligand 1 (PD-L1) and the prognosis of patients with HCC who had undergone hepatic resection.[5-7] Cancer immunotherapy has been used to suppress the immune checkpoint pathway in several types of cancers.[8] However, the response to programmed death protein 1 (PD-1) inhibitors in advanced HCC has been unclear.[9] A better understanding of the detailed mechanisms of PD-1/PD-L1 signaling is required to help effective anti-PD-1 immune checkpoint blockade treatment for HCC.

More recently, two studies showed that CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) is an important regulator of PD-L1 expression.[10,11] Burr et al. revealed that CMTM6 is a major regulator of PD-L1 expression in melanoma, breast, and lung cancer cell lines.[10] Mezzadra et al. demonstrated that the inhibition of CMTM6 expression resulted in impaired PD-L1 expression in all human tumor types.[11] PD-L1 is stabilized by CMTM6 on the tumor cell surface to efficiently inhibit T-cell activity, and the inhibition of CMTM6 might increase the effect of blocking the PD-L1 pathway. In addition, CMTM6 participates in the regulation of epithelial-to-mesenchymal transition (EMT) and stemness phenotypes in head and neck squamous cell carcinoma.[12] These findings suggest that CMTM6 potentially links EMT and stemness characteristics to tumor immunity. However, these linkages in HCC have not been reported.

In this study, we investigated the relationship between PD-L1 and CMTM6 expression by immunohistochemistry and examined the prognostic impact of PD-L1 and CMTM6 expression in patients with HCC. Additionally, we evaluated the stabilization of PD-L1 and the regulation of EMT and stemness phenotypes by CMTM6 *in vitro*.

Materials and Methods

PATIENTS AND PREPARATION OF SPECIMENS

In total, 259 patients with HCC who had undergone hepatic resection from January 2000 to December 2013 at the Department of Surgery and Science, Kyushu University Hospital, were enrolled in this study. The patients who had undergone resection for primary HCC without intravascular chemotherapy were selected retrospectively. The detailed surgical procedure and patient selection criteria for hepatic resection have been previously reported.[13] Pre-operation and postoperation de-identified clinical information was obtained from electronic and paper records. Complete charts for clinical data were available for all patients. Permission for the research use of the resected tissues was obtained.

Of these 259 patients, 202 were men, 57 were women, the median age was 68 years (range: 28–87 years), and the median overall survival (OS) and recurrence-free survival (RFS) rates were 6.1 years and 2.3 years, respectively. To evaluate the histological features, the specimens were fixed in 10% formalin solution, embedded in paraffin, and cut into 5-μm-thick slices. One section from each patient was counter-stained with hematoxylin and eosin for histological diagnosis. Additionally, this study was approved by the ethics committee at our hospital according to the Ethical Guidelines of the Japanese Government (Approval numbers 28-453 and 30-34).

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IMMUNOHISTOCHEMICAL EXAMINATION

PD-L1 immunostaining was performed as previously described.[7] The sliced sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Subsequently, the specimens were subjected to antigen retrieval at 121°C by autoclave for 10 minutes (Tris-EDTA buffer [pH 9.0] for PD-L1, and 10 mM citrate buffer [pH 6.0] for CMTM6 and CD68). The sections were incubated with 2% dry milk and 5% bovine serum albumin in phosphate-buffered saline to block nonspecific binding of the immune reagents. The primary antibodies antihuman PD-L1 rabbit monoclonal antibody (1:450 dilution; clone 28-8; Abcam, Cambridge, United Kingdom), antihuman CMTM6 rabbit polyclonal antibody (1:100 dilution; HPA026980; Atlas Antibodies, Stockholm, Sweden), and antihuman CD68 mouse monoclonal antibody (1:100 dilution; IR613; Agilent, Santa Clara, CA) were applied to the specimens and incubated overnight at 4°C. Next, the specimens were treated with 0.3% H_2O_2 for 5 minutes to inhibit the activity of endogenous peroxidase and then were labeled with streptavidin-biotin for 1 hour at room temperature. Color development was performed using 3,3′-diaminobenzidine, followed by counterstaining with Mayer's hematoxylin. The positive controls for CMTM6 and PD-L1 are shown in Supporting Fig. S1.

Double staining of CMTM6 and PD-L1 was performed using the EnVision G|2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red; Agilent) according to the manufacturer's instructions. The CMTM6 antigen was visualized using horseradish peroxidase (HRP)/DAB+, and the PD-L1 antigen was visualized using alkaline phosphatase/Permanent Red. Immunohistochemical (IHC) evaluations were performed independently by three experienced researchers (K.Y., S.I., and K.K.), who were blinded to the patients' clinical backgrounds. The final assessments were achieved by consensus. Both PD-L1 and CMTM6 were expressed on the cytoplasmic membrane of tumor cells. Regarding immunohistochemical evaluation, we set the cutoff point at 1% of total cancer cells for PD-L1.[7] The classification of CMTM6 expression was scored into four different categories (grades 0-3).

CELL LINES, CULTURE, CELL TRANSFECTION, AND TREATMENT WITH INTERFERON γ

The human HCC cell lines, Huh7, Hep3B, PLC/PRF/5 and HepG2 (Riken Cell Bank, Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA), supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. Short interfering CMTM6 (siCMTM6; Cat. No. 1299001) targeted messenger RNA (mRNA) of *CMTM6* (NM_0017801.2) and negative control (Cat. No. 12935300) were purchased from Stealth RNAi siRNA (Thermo Fisher Scientific); and siCMTM6 and negative control were transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. In addition, the CMTM6 plasmid (NM_017801; OriGene Technologies, Rockville, MD) and empty vector (pCMV6-Entry Vector; OriGene Technologies) were transfected into Hep3B cells using the jetPRIME kit (Polyplus Transfection, Illkirch-Graffenstaden, France) according to the manufacturer's instructions. Forty-eight hours after transfection, Hep3B cells were selected with 500 μg/mL G418 (Sigma-Aldrich, St. Louis, MO). The limiting dilution method was used to isolate single cell clones from the transfected Hep3B cells. Selected single cells were placed in each well of the culture plates, and the clonal populations growing from each single cell were isolated. To induce PD-L1 expression on HCC cells, Huh7 cells were treated with 10 ng/mL of recombinant human interferon γ (IFN-γ) (PeproTech, Rocky Hill, CT) for 24-72 hours, and followed by harvesting for protein extraction.

TRANSWELL INVASIVENESS, SCRATCH, AND COLONY FORMATION ASSAYS

The cell invasiveness assay used Falcon Permeable Support for 24-well plate with 15 μL of 5-mg/mL Matrigel Matrix (Corning Inc., Corning, NY) on an 8.0-μm Transparent PET Membrane (Corning Inc.). Stained cells using Diff-Quik (JACLaS,

Tokyo, Japan) on the transwell membrane surface were counted in five randomly selected fields at ×200 magnification and quantified using Image J software (https://imagej.net/). Huh7 and Hep3B cells were seeded in six-well plates and incubated until 90% confluent. Confluent cell monolayers were scratched by plastic tips. Images of scratched areas were captured over the following 24 hours, measured using Image J software and evaluated as percentage of migration (migration index: 1 − [length at 24 hours]/[length at 0 hours]). In addition, Huh7 and Hep3B cells were added to each well in six-well flat-bottomed plate in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS. After 10 days of culture, the small population of cells were fixed using 4% paraformaldehyde and stained using crystal violet. The number of colonies was counted. These experiments were repeated three times.

WESTERN BLOT ANALYSIS

The proteins of cells and isolated exosomes were separated by 10% SuperSep Ace gels (Fujifilm, Tokyo, Japan) and were transferred onto a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). Western blotting was processed using iBind Western Systems (Thermo Fisher Scientific) according to the manufacturer's instructions.[14] The protein bands were visualized using HRP-conjugated secondary antibodies and Chemi-Lumi One (Nakalai Tesque, Kyoto, Japan) on Amersham Imager 600 (GE Healthcare, Chicago, IL). The following antibodies were used: 1:1,000 dilution of antihuman PD-L1 rabbit monoclonal antibody (clone 28-8; Abcam) and 1:1,000 dilution of antihuman CMTM6 rabbit polyclonal antibody (HPA026980; Atlas Antibodies). Goat antirabbit and mouse secondary antibodies were used at 1:2,000 dilution (Cell Signaling Technology, Danvers, MA).

STATISTICAL ANALYSIS

Standard statistical analyses were used to evaluate descriptive statistics, including means, medians, frequencies, and percentages. Continuous variables were compared using the Mann-Whitney U-test and Kruskal-Wallis test. Categorical variables were compared using χ^2 test or Fisher's exact test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards model. Cumulative OS and RFS rates were calculated using the Kaplan-Meier method, and differences between curves were evaluated using the log-rank test. OS was calculated as years from the date of surgery to the date of the last follow-up or death. To identify postoperative prognostic factors, several variables that were found to be independent in univariate analysis were included in the overall multivariate Cox proportional hazards model to analyze both OS and RFS. All statistical tests were two-sided, and a value of $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using JMP14 software (SAS Institute, Cary, NC).

Results

CMTM6 AND PD-L1 EXPRESSION IN HCC

To evaluate the expression levels of CMTM6 and PD-L1 in HCC tissues, we performed IHC staining of 259 HCC tissues. As shown in Figure 1A,B, the cancer cells showed membranous staining for CMTM6 and PD-L1. First, we classified the patients with HCC into four different categories by intensity. Among the 259 patients, 111 (42.9%) patients were negative (grade 0), 83 (32.0%) patients were weak (grade 1), 48 (18.5%) patients were moderate (grade 2), and 17 (6.6%) patients were strong (grade 3, Fig. 1A). We divided the 259 patients with HCC into high (n = 65, 25.1%) and low (n = 194, 74.9%) CMTM6 expression groups. Next, we set the cutoff point for PD-L1 as a 1% positive proportion of total cancer cells, as previously described,[7] and divided the 259 patients into PD-L1-positive (n = 51, 19.7%) and PD-L1-negative (n = 208, 80.3%; Fig. 1B) patients. Tumor-associated macrophages (TAMs), a key component of the tumor immune microenvironment, play critical roles in tumor immune suppression.[15] In addition to the CMTM6 and PD-L1 expression, immunohistochemical CD68 staining was performed in our clinical population (Supporting Fig. S2A). The patients with HCC with PD-L1-positive samples had higher CD68+ cell counts than those with PD-L1-negative samples (median

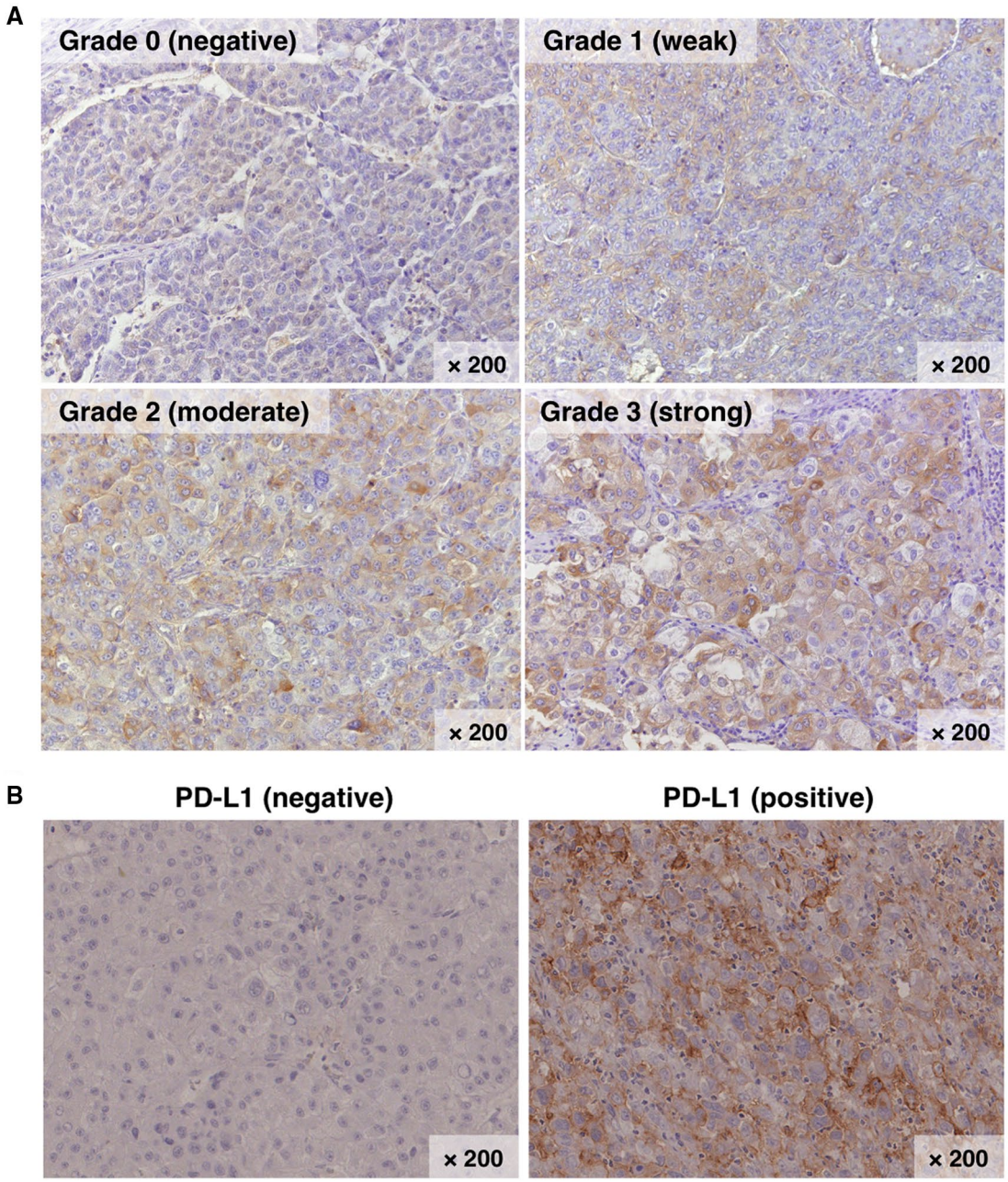

FIG. 1. IHC staining of CMTM6 and PD-L1 expression in HCC tissue. (A) A total of 111 (42.9%) patients showed negative expression (grade 0), 83 (32.0%) patients showed weak expression (grade 1), 48 (18.5%) patients showed moderate expression (grade 2), and 17 (6.6%) patients showed strong expression (grade 3, magnification, ×200). (B) Negative and positive membrane staining for PD-L1 (magnification, ×200).

96.0 vs. 143.3 cells/field; $P < 0.0001$; Supporting Fig. S2B). In addition, the patients with HCC with high CMTM6 expression had higher CD68+ cell counts than those with low CMTM6 expression (median 98.7 vs. 121.3 cells/field; $P = 0.0262$; Supporting Fig. S2C).

CORRELATION OF CMTM6 AND PD-L1 EXPRESSION IN HCC

We examined the relationship and localization between CMTM6 and PD-L1 in HCC tissues. Immunohistochemical double staining revealed that

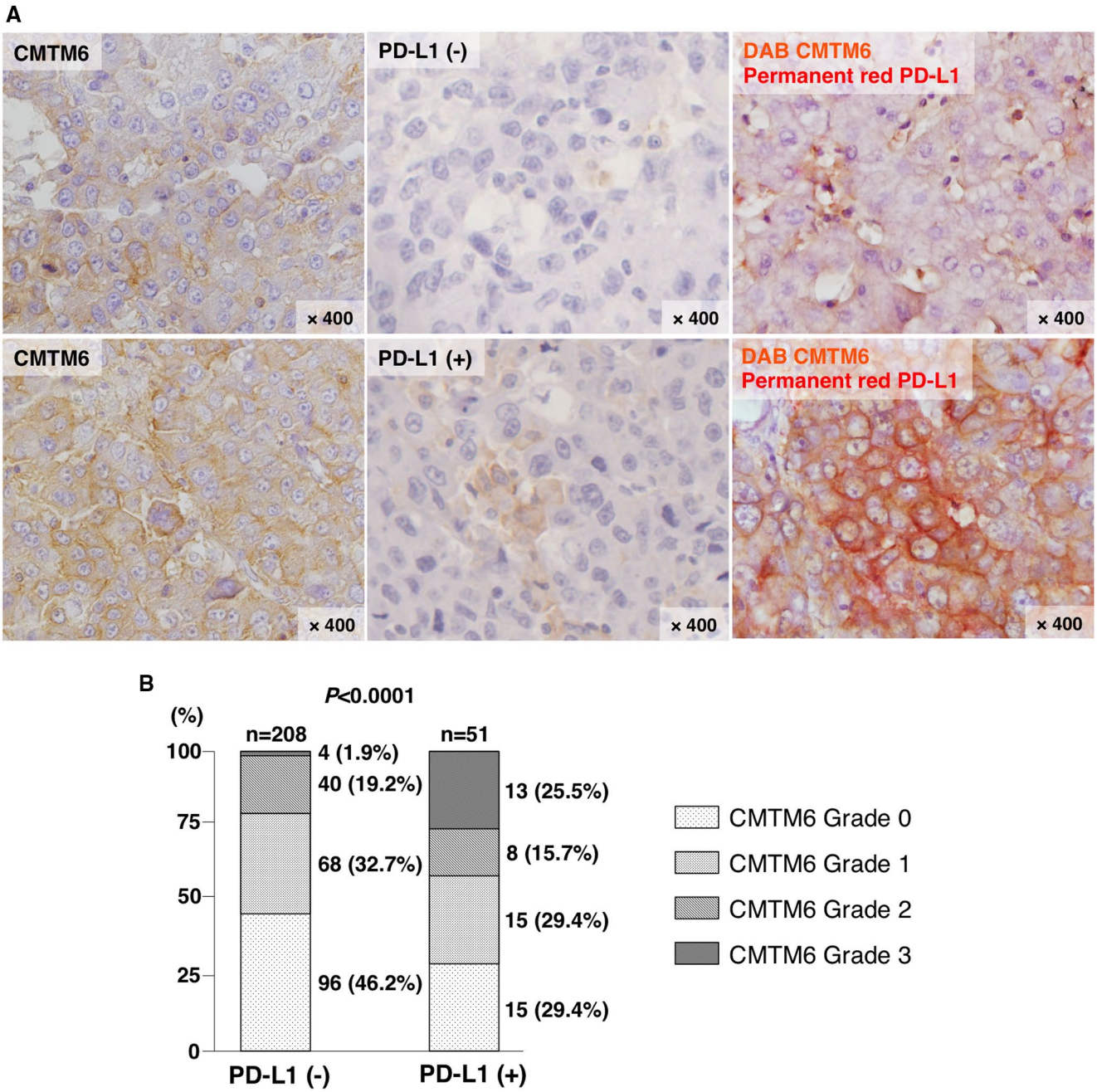

FIG. 2. CMTM6 and PD-L1 expression correlation. (A) Double staining of CMTM6 and PD-L1 expression. CMTM6 antigen was stained with DAB (brown), and the PD-L1 antigen was stained with permanent red (magnification, ×400). (B) Thirteen of 51 PD-L1-positive HCC tissues presented strong CMTM6 expression (grade 3, 25.5%), and 4 of 208 PD-L1-negative HCC tissues presented strong CMTM6 expression (grade 3, 1.9%, $P < 0.0001$).

CMTM6 and PD-L1 were co-expressed on HCC cells (Fig. 2A). In PD-L1-positive HCC tissue, the CMTM6 antigen was stained with 3,3′-diaminobenzidine (DAB; brown) and the PD-L1 antigen was stained with permanent red; however, in PD-L1-negative HCC tissue, PD-L1 was not visualized with CMTM6 using double staining. We next analyzed the proportion of CMTM6 among the PD-L1-positive

and PD-L1-negative groups (Fig. 2B). Thirteen of 51 PD-L1-positive patients with HCC had strong CMTM6 expression (grade 3, 25.5%); conversely, 4 of 208 PD-L1-negative patients with HCC had strong CMTM6 expression (grade 3, 1.9%). We observed that patients with HCC in the PD-L1-positive group had a higher grade of CMTM6 expression than those in the PD-L1-negative group. PD-L1 expression was significantly correlated with CMTM6 expression in HCC tissues ($P < 0.0001$).

CMTM6 EXPRESSION AND CLINICOPATHOLOGICAL FACTORS

The relationship between CMTM6 expression and the clinicopathological factors in patients with HCC was evaluated. Table 1 summarizes the clinicopathological characteristics of patients with high and low CMTM6 expression. Poorly differentiated HCC was more frequently observed in the high-CMTM6 group than in the low-CMTM6 group (51 of 194 [26.2%] vs. 34 of 65 [52.3%]; $P < 0.0001$). Additionally, the rate of microscopic intrahepatic metastasis was higher in patients with high CMTM6 expression than in those with low CMTM6 expression (31 of 194 [15.9%] vs. 18 of 65 [27.6%]; $P = 0.0369$). Notably, among 161 cases with intrahepatic recurrence, patients with high CMTM6 expression showed a higher incidence of multiple intrahepatic recurrent lesions (54 of 120 [45.0%] vs. 27 of 41 [65.9%]; $P = 0.0211$). Other host-related factors (e.g., age, sex, body mass index, chronic liver disease, serum albumin level) and tumor-related factors (e.g., tumor size, number of tumors, microscopic vascular invasion) were not significantly related to CMTM6 expression.

TABLE 1. CHARACTERISTICS OF THE PATIENTS WITH HCC WHO HAD UNDERGONE HEPATIC RESECTION

Variable	Low CMTM6 (n = 194)	High CMTM6 (n = 65)	*P* Value
Age (years)	67.5 (28-87)	69 (36-86)	0.5288
Sex, male/female	149/45	53/12	0.4252
BMI (kg/m^2)	22.62 (14.23-32.60)	22.67 (16.69-29.09)	0.6942
HBs-Ag positive	41 (21.1%)	15 (23.0%)	0.7419
HCV-Ab positive	103 (53.0%)	37 (56.9%)	0.6667
Albumin (g/dl)	4.0 (2.6-5.0)	3.9 (1.8-4.8)	0.2276
AFP (ng/ml)	15.0 (0.8-577660)	22.9 (0.8-410600)	0.1152
DCP (mAU/ml)	125 (8-75000)	247 (15-75000)	0.0906
Tumor size (cm)	3.5 (1.0-16.5)	3.4 (1.0-15.0)	0.7835
Solitary/Multiple	154/40	65/16	0.4981
Poorly differentiation	51 (26.2%)	34 (52.3%)	<0.0001†
Microscopic vascular invasion	84 (43.3%)	32 (49.2%)	0.4052
Microscopic intrahepatic metastasis	31 (15.9%)	18 (27.6%)	0.0369*
F3 or F4	85 (43.8%)	29 (44.6%)	0.9104
Any recurrence (n = 181)	133 (68.6%)	48 (73.8%)	0.4211
Intrahepatic recurrence (n = 161)	120 (90.2%)	41 (85.4%)	0.3623
Solitary/Multiple	66/54	14/27	0.0211*
Extrahepatic recurrence (n = 35)	23/133 (16.5%)	12/48 (25.0%)	0.2465

Note: The data are presented as n (%) or median (range).
*$P < 0.05$.
†$P < 0.001$.
Abbreviations: BMI, body mass index; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody.

CMTM6 AND PD-L1 AND PATIENT SURVIVAL

We next investigated whether CMTM6 expression influenced the outcomes for patients with HCC. The 10-year RFS and OS rates were 13.7% and 27.2%, respectively, for patients with high CMTM6 expression, and 23.6% and 56.5%, respectively, for patients with low CMTM6 expression (Fig. 3A,B). Patients with high CMTM6 expression showed a significantly worse prognosis than those with low CMTM6 expression for both RFS (log-rank $P = 0.0258$) and OS (log-rank $P < 0.0001$). As shown in Fig. 2, CMTM6 expression was significantly correlated with PD-L1 expression in HCC tissues. Moreover, consistent with previous findings, PD-L1-positive expression influenced the outcomes for patients with HCC for both RFS (log-rank $P = 0.0164$; Supporting Fig. S3A) and OS (log-rank $P < 0.0001$; Supporting Fig. S3B). Accordingly, we classified the patients into three groups: high CMTM6/PD-L1 positive (n = 21), high CMTM6/PD-L1 negative (n = 44), and low CMTM6 (n = 194) expression groups. High CMTM6 and PD-L1-positive expression was associated with malignant aggregates, including a high

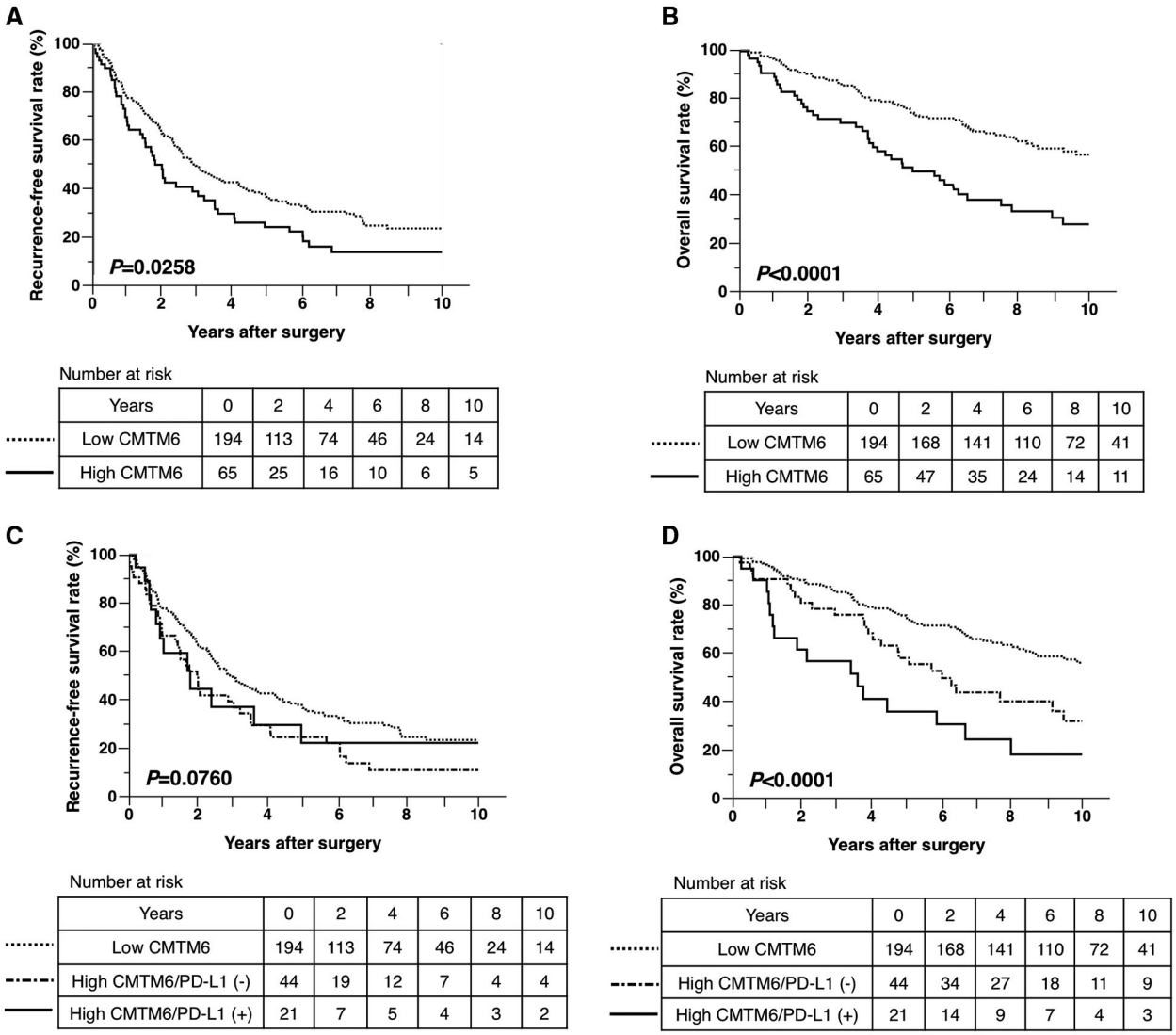

FIG. 3. Kaplan-Meier survival curves after hepatic resection of HCC according to CMTM6 and PD-L1 expression: RFS (A) and OS (B) according to CMTM6 expression; RFS (C) and OS (D) according to three different expression patterns (high CMTM6/PD-L1 positive, high CMTM6/PD-L1 negative, and low CMTM6).

serum alpha-fetoprotein (AFP) (median: 14.6 vs. 164.4 ng/mL; P = 0.0085) and poorly differentiated HCC (29.4% [70 of 238] vs. 71.4% [15 of 21]; P < 0.0001). Importantly, among 161 intrahepatic recurrence-positive cases, our analysis showed more frequent multiple intrahepatic recurrences in patients with high CMTM6 and PD-L1-positive expression than those with other expression patterns (47.0% [71 of 151] vs. 100% [10 of 10]; P = 0.0012; Supporting Table S1). We found that OS was significantly different among the three groups (log-rank P < 0.0001; Fig. 3D), whereas RFS was not significantly so (log-rank P = 0.0760; Fig. 3C).

UNIVARIATE AND MULTIVARIATE ANALYSES OF PROGNOSTIC FACTORS FOR RFS AND OS

Tables 2 and 3 provide the results of univariate and multivariate analyses that were used to identify the factors that were significantly associated with RFS and OS after hepatic resection in patients with HCC.

TABLE 2. UNIVARIATE AND MULTIVARIATE ANALYSES OF FACTORS RELATED TO RFS IN PATIENTS WITH HCC WHO UNDERWENT HEPATIC RESECTION (COX PROPORTIONAL HAZARDS ANALYSIS)

	Univariate			Multivariate		
Variable	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI	*P* Value
Age (years)	1.005	0.991-1.019	0.4716			
Sex: male	1.747	1.173-2.602	0.0060*	1.729	1.145-2.610	0.0092*
HBs-Ag	1.034	0.727-1.471	0.8490			
HCV-Ab	1.335	0.993-1.795	0.0552			
Albumin	0.548	0.385-0.787	0.0010*	0.544	0.376-0.790	0.0014*
AFP	1.000	1.000-1.000	<0.0001†	1.000	1.000-1.000	0.0286*
DCP	1.000	1.000-1.000	<0.0001†	1.000	1.000-1.000	0.0155*
Tumor size	1.094	1.039-1.147	0.0004*	1.005	0.938-1.074	0.8830
Tumor number (multiple)	2.173	1.559-3.030	<0.0001†	1.401	0.914-2.148	0.1216
Poorly differentiation	1.612	1.189-2.185	0.0021*	0.920	0.631-1.343	0.6671
Microscopic vascular invasion	1.379	1.029-1.849	0.0311*	0.872	0.612-1.242	0.4484
Microscopic intrahepatic metastasis	3.539	2.469-5.073	<0.0001†	3.000	1.866-4.824	<0.0001†
F3 or F4	1.254	0.936-1.680	0.1285			
High CMTM6/PD-L1 (+)	1.230	0.684-2.212	0.4888			

*$P < 0.05$.
†$P < 0.001$.
Abbreviations: CI, confidence interval; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody.

TABLE 3. UNIVARIATE AND MULTIVARIATE ANALYSES OF FACTORS RELATED TO OS IN PATIENTS WITH HCC WHO UNDERWENT HEPATIC RESECTION (COX PROPORTIONAL HAZARDS ANALYSIS)

	Univariate			Multivariate		
Variable	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI	*P* Value
Age (years)	1.019	1.001-1.038	0.0375*	1.033	1.013-1.053	0.0012*
Sex: male	1.658	1.003-2.742	0.0485*	1.687	1.003-2.836	0.0487*
HBs-Ag	0.833	0.532-1.303	0.4251			
HCV-Ab	1.298	0.899-1.874	0.1637			
Albumin	0.415	0.272-0.641	<0.0001†	0.402	0.258-0.631	<0.0001†
AFP	1.000	1.000-1.000	0.0344*	1.000	0.999-1.000	0.4904
DCP	1.000	0.999-1.000	0.1133			
Tumor size	1.103	1.042-1.162	0.0004†	1.033	0.981-1.129	0.1420
Tumor number (multiple)	1.883	1.258-2.818	0.0021*	0.935	0.555-1.577	0.8022
Poorly differentiation	1.831	1.271-2.637	0.0012*	0.970	0.620-1.517	0.8929
Microscopic vascular invasion	1.872	1.299-2.699	0.0008†	1.110	0.726-1.695	0.6314
Microscopic intrahepatic metastasis	3.754	2.519-5.594	<0.0001†	4.046	2.341-6.993	<0.0001†
F3 or F4	1.051	0.731-1.512	0.7854			
High CMTM6/PD-L1 (+)	2.960	1.740-5.035	<0.0001†	2.231	1.218-4.085	0.0093*

*$P < 0.05$.
†$P < 0.001$.
Abbreviations: CI, confidence interval; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody.

Multivariate analysis identified five prognostic factors that influenced RFS: male, a low serum albumin level, high levels of AFP, and des-gamma-carboxyprothrombin (DCP), and positivity of microscopic intrahepatic metastasis. Multivariate analysis identified five prognostic factors that influenced OS: old age, male, a low serum albumin level, positivity of microscopic intrahepatic metastasis, and a high CMTM6/PD-L1-positive expression pattern (hazard ratio, 2.231; 95% confidence interval, 1.218-4.085; $P = 0.0093$).

CMTM6 STABILIZES PD-L1 EXPRESSION AND REGULATES EMT AND STEMNESS PHENOTYPES *IN VITRO*

We next confirmed the function by which CMTM6 regulates PD-L1 expression in HCC cells. First, we examined the constant expression of CMTM6 and expression of PD-L1 induced by human recombinant IFN-γ (10 ng/mL) in HCC cell lines (Huh7, Hep3B, PLC/PRF/5, and HepG2). Of the cell lines, Huh7 showed the strongest CMTM6 expression and Hep3B showed the weakest CMTM6 expression (Supporting Fig. S4A). In Huh7 and Hep3B, PD-L1 protein was detected after IFN-γ stimulation (Supporting Fig. S4B); therefore, we selected Huh7 and Hep3B for further examination. To determine the function of CMTM6 in stabilizing PD-L1, we treated CMTM6-knockdown and negative-control Huh7 cells, and CMTM6-overexpressed and negative-control Hep3B cells with IFN-γ at different time points. Importantly, although Huh7 cells were stimulated by IFN-γ, the expression level of PD-L1 was reduced in CMTM6-knockdown Huh7 cells but enhanced in negative control cells after 72 hours (Fig. 4A). Conversely, the expression level of PD-L1 was increased gradually in CMTM6-overexpressed Hep3B cells compared with negative control cells (Fig. 4B). Next, to explore effects of CMTM6 on HCC cell motility and stemness, transwell invasion, scratch, and colony formation assays were used. These results showed that the down-regulation of CMTM6 significantly inhibited cell motility of both Huh7 and Hep3B cells (Fig. 5A,B). In addition, overexpressed CMTM6 significantly promoted cell motility and stemness of both Huh7 and Hep3B cells (Fig. 5C,E).

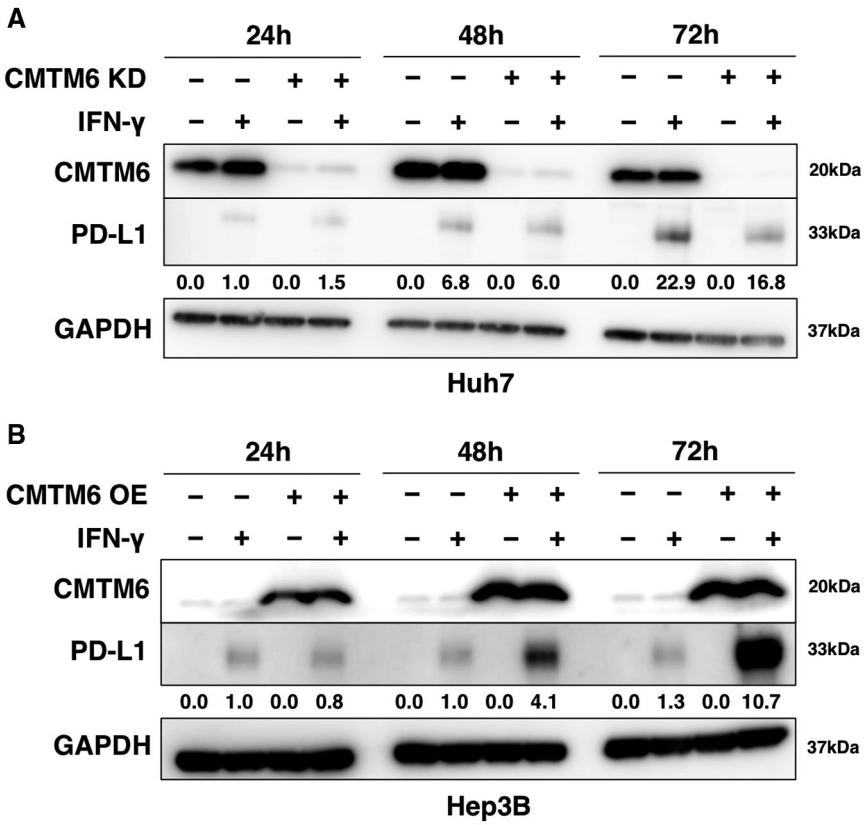

FIG. 4. CMTM6 regulates PD-L1 stability. (A) Time course of PD-L1 protein levels in CMTM6 knockdown and negative-control Huh7 cells following IFN-γ stimulation. (B) Time course of PD-L1 protein levels in CMTM6 overexpression and negative-control Hep3B cells following IFN-γ stimulation. Abbreviations: Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, knockdown; OE, overexpression.

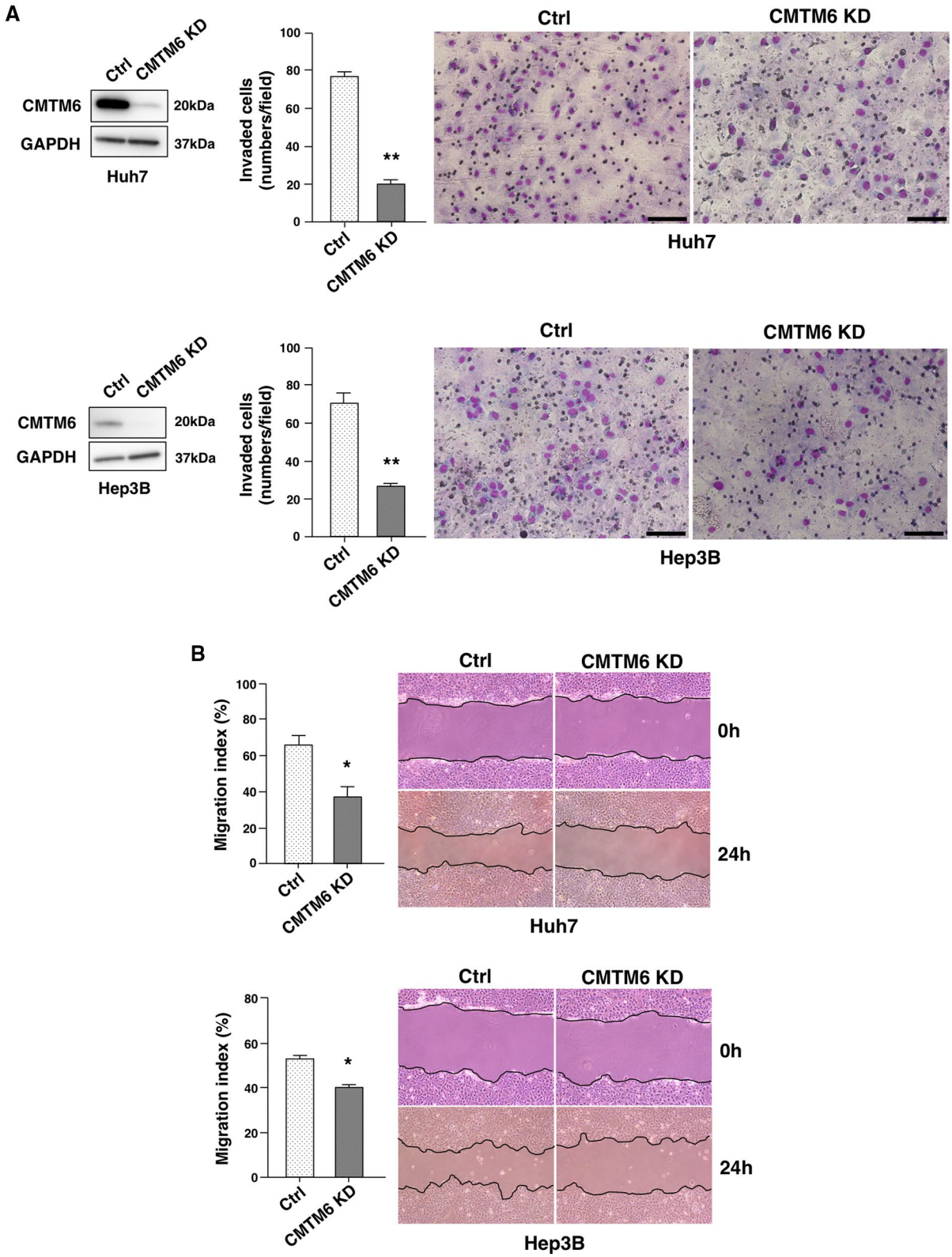

FIG. 5. CMTM6 promotes HCC cell motility. CMTM6 knockdown significantly inhibited invasion (A) and scratch assay of Huh7 and Hep3B cells (B). (C) CMTM6 overexpression in Huh7 and Hep3B cells promoted invasion (D) and scratch colony formation assay (E). $^{*}P < 0.05$ and $^{**}P < 0.005$. Abbreviations: Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, knockdown; OE, overexpression.

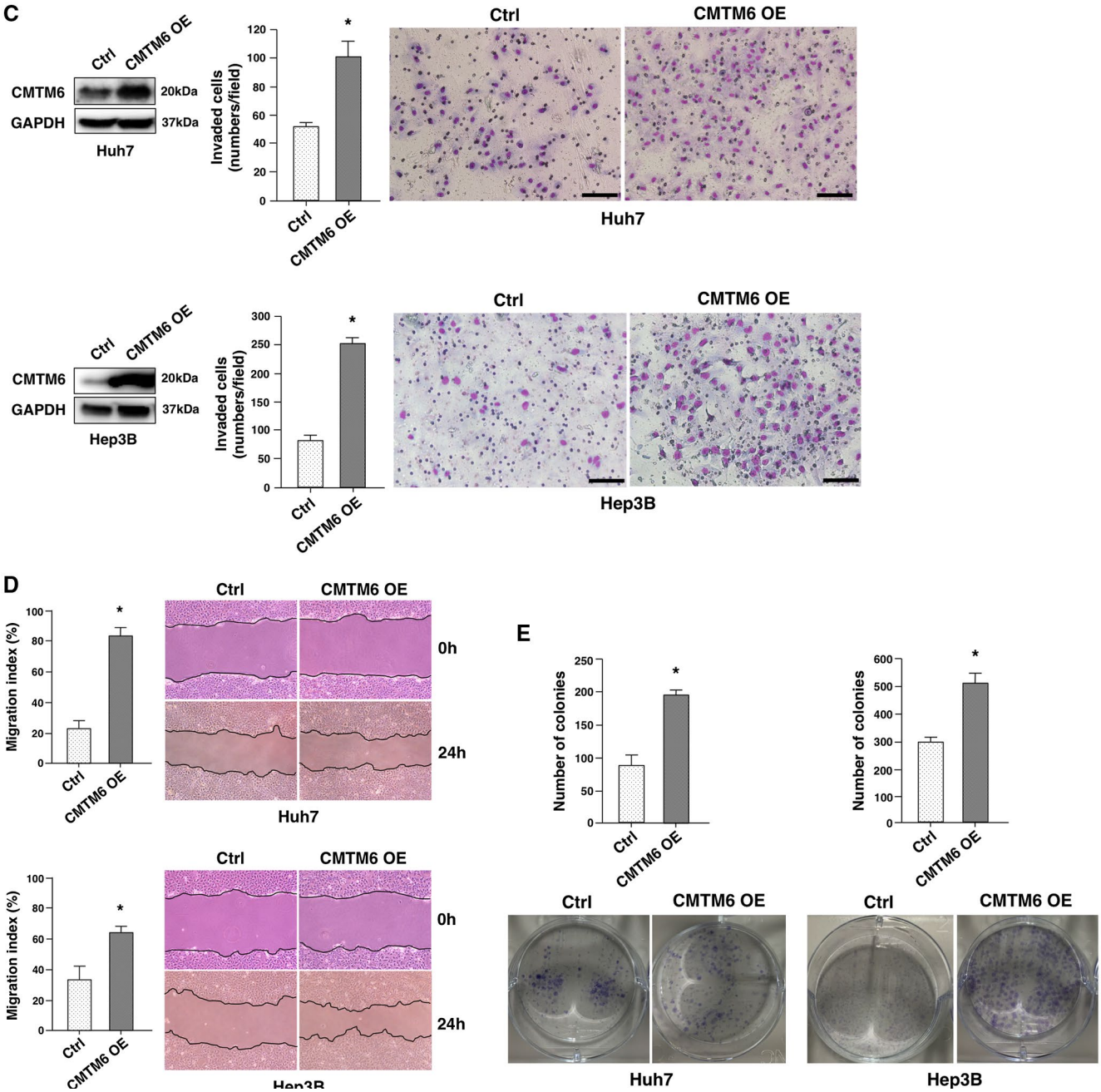

FIG. 5. Continued.

Discussion

In the present study, we investigated the relationship between CMTM6 and PD-L1 expression in patients with HCC who had undergone hepatic resection. The CMTM6 expression was co-expressed with PD-L1 on the HCC cell membrane and was significantly correlated with PD-L1 expression. Additionally, a high CMTM6 and PD-L1-positive expression pattern was a significant prognostic factor that influenced OS for patients with HCC. We found that PD-L1 expression was enhanced at late time points in the presence of CMTM6 expression, indicating that CMTM6 stabilizes PD-L1 expression in HCC cells. We also showed that CMTM6 was significantly associated with EMT and stemness of HCC cells *in vitro*. These

results suggest that CMTM6 contributes to HCC progression by maintaining immune suppression and promoting malignant activities.

We and other researchers, using large sample sizes, have shown the prognostic effect of PD-L1 expression in HCC.[7,16,17] In this study, we also confirmed that patients with PD-L1-positive expression had worse outcomes for RFS and OS. Although PD-L1 expression is a significantly predictive biomarker for a promising response to immune checkpoint inhibitor (ICI) therapy in cancer patients, only a few of these patients benefit from this therapy. Currently, combination regimens with ICI therapy targeting anti-PD-L1 or anti-PD-1, including anti-angiogenic agents, have been discussed to improve the objective response and survival of patients with HCC.[18] Thus, to improve ICI therapeutic effects, a biological factor that regulates PD-L1 expression has been investigated worldwide. Burr et al. and Mezzadra et al. identified CMTM6 as a PD-L1 regulator in several tumor types.[10,11] However, the status of CMTM6 in the clinical samples of HCC and the relationship between CMTM6 and PD-L1 has been unclear. The present clinical study, with a large sample size, shows a significant correlation between CMTM6 and PD-L1 expression in HCC.

The number of reports on the relationship between CMTM6 and PD-L1 in several tumor types has been increasing. In glioma, high CMTM6 expression is closely related to a malignant phenotype and is correlated with the clinical outcomes of glioma patients through transcriptomic and genomic profiling data.[19] In pancreatic cancer, high CMTM6 expression and high PD-L1 expression are associated with shorter OS in patients with primary pancreatic ductal adenocarcinoma based on mRNA-expression data.[20] In lung cancer, CMTM6 expression is associated with PD-L1 expression in IHC staining of lung cancer tissues.[21] Consistent with these reports, our results show that CMTM6 expression is significantly correlated with PD-L1, and high-CMTM6/PD-L1-positive expression is a prognostic impact factor for patients with HCC. Regarding this finding, CMTM6 was suggested to be involved in immune functions through modulating T-cell-mediated antitumor immunity. In contrast to our results, in triple-negative breast cancer[20] and lung adenocarcinoma, CMTM6 expression increased the favorable prognostic value of PD-L1 expression.[22] Because of the different nature of the enrolled patients (e.g., cancer origin, malignant grade, race), controversy exists regarding the effect of CMTM6 on prognosis. To clarify the CMTM6 function, the accumulation of analyses is urgently required.

In this study, clinicopathological characteristics and host-related factors, including age, sex, body mass index, chronic hepatitis disease, and serum albumin level, were not significantly different between the CMTM6 expression groups. However, tumor-related factors (poor differentiation and microscopic intrahepatic metastasis) were more frequently found in patients with high CMTM6 expression. More recently, Chen et al. identified that CMTM6 is associated with EMT-related and cancer stem cell–related genes in head and neck squamous cell carcinoma.[12] It is well known that EMT is a critical part of the process of metastasis. Furthermore, stemness is closely related to the formation of a small subpopulation of cancer cells that lead to cancer recurrence and metastasis. In our experiments *in vitro*, CMTM6 promoted both cell invasiveness and stemness, which reflects pathological results (poor differentiation and microscopic intrahepatic metastasis). Thus, CMTM6 regulates not only T-cell dysfunction but also EMT and stemness. The tumor malignant activities that were induced by CMTM6 might contribute to the poor outcome of patients with HCC; conversely, the malignant status might up-regulate CMTM6 expression and lead to an immune response against tumor cells. However, the detailed molecular mechanisms of CMTM6 expression in HCC cells remain unknown and need to be addressed in further studies.

Kaplan-Meier curves showed that high CMTM6 and PD-L1-positive expression significantly influenced OS but not RFS. It is well known that the prognoses of patients with HCC are strongly influenced by frequent recurrence after curative resection. Therefore, treatment strategies for local recurrence and intrahepatic metastatic recurrence are major issues to improve the postoperative outcome of HCC.[23] Multiple intrahepatic recurrences lead to a poor prognosis because of the limited resectability that is affected by the size, number, and distribution of recurrent lesions, as well as individual hepatic reserve.[24] Our results showed that high CMTM6 and PD-L1-positive expression are significantly associated with multiple hepatic recurrences, which are considered to be correlated with poor OS.

In the tumor immune microenvironment, TAMs are strongly related to expression of PD-L1 induced by IFN-γ in HCC tissues.[15,25] An increasing number of reports have shown that there is an association between PD-L1 and TAMs in patients with HCC.[26,27] Consistent with these reports, we identified a significant correlation between PD-L1 and CD68+ cells. Interestingly, CMTM6 was also related to CD68+ cells in HCC. These results indicate that TAMs, which release IFN-γ, may affect PD-L1 expression and lead to the maintenance of tumor immunity through CMTM6 expression in the immune microenvironment of HCC.

We next examined the mechanism of the interaction between CMTM6 and PD-L1 *in vitro*, revealing that CMTM6 affects PD-L1 protein stability at late time points after IFN-γ stimulation. CMTM6 binds PD-L1 at the cell surface and inhibits lysosomal degradation of PD-L1. CMTM6 is not required for the trafficking of PD-L1 from the endoplasmic reticulum to the cell surface, but it may be required for stable expression of PD-L1 at the plasma membrane. Therefore, PD-L1 protein levels are decreased in the absence of CMTM6 at later time points.[10] Consistent with this, PD-L1 was detected at early time points after IFN-γ was added in the absence of CMTM6; however, beyond the 24-hour time point, PD-L1 protein levels were decreased in the absence of CMTM6. Moreover, we confirmed the localization of CMTM6 by double staining. The results of double-staining experiments revealed that the CMTM6 expression pattern was different from that of PD-L1. The expression of CMTM6 was diffuse and the expression of PD-L1 was localized or heterogeneous in HCC tissues. These results suggest that CMTM6 expression may be related to other functions affecting HCC progression, such as EMT and stemness characteristics. Thus, CMTM6 may have other roles in addition to the suppression of tumor immunity through the PD-L1 pathway.

CMTM6 is largely located at the cancer cell surface co-expressed with PD-L1, indicating that CMTM6 protects PD-L1 from degradation in HCC cells. Recently, Chen et al. showed that PD-1-inhibitor responder patients with non-small cell lung cancer had higher CMTM6 expression, which was also found to be an independent predictor of the response to PD-1 inhibitors.[28] Therefore, the evaluation of CMTM6 should be routinely scored to predict the response for ICI therapy, a strategy that might help patient selection for PD-1 inhibitor therapy in patients with HCC.

In summary, our large cohort study revealed that CMTM6 co-expressed with PD-L1 was strongly associated with the clinical outcome in patients with HCC. Additionally, we found co-localization of CMTM6 and PD-L1 and the stability of PD-L1 in the presence of CMTM6 at late time points. We also found that CMTM6 promoted the malignant activities of HCC cells. The evaluation of CMTM6 combined with PD-L1 in HCC might be useful for predicting patients' outcomes and patient selection for ICI therapy.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1643/suppinfo.