High membrane expression of CMTM6 in hepatocellular carcinoma is associated with tumor recurrence

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Abstract
CKLF-like MARVEL transmembrane domain-containing protein 6 (CMTM6) maintains membrane PD-L1 expression by controlling its endosomal recycling. However, in patients with hepatocellular carcinoma (HCC), the correlation among CMTM6, B7 family ligands, and CD8-positive cytotoxic T lymphocytes (CTLs), and the molecular function of CMTM6 in HCC have not been established. We performed immunohistochemistry to evaluate the relationships among CMTM6 expression, clinicopathological factors, B7 family ligands expression, and CTL infiltration in HCC samples. Moreover, we established CMTM6-knockout human HCC cell lines to evaluate the function of human CMTM6 in immune regulation and tumor viability. CMTM6 expression was positively associated with membrane B7 family ligands expression and CTL infiltration in HCC samples. High CMTM6 expression in HCC tissues was associated with the expression of the proliferation marker Ki-67 and shorter recurrence-free survival. In vitro analysis showed the downregulation of membrane B7 family ligands and proliferation potency in the CMTM6-knockout human HCC cell line. High membrane CMTM6 expression was associated with tumor recurrence and proliferation via the regulation of membranous B7 family ligands expression. Thus, CMTM6 might be a biomarker to predict the risk of HCC recurrence and a therapeutic target to suppress tumor growth and increase CTL activity.

Keywords
biomarkers, cytotoxic T lymphocytes, hepatocellular carcinoma, immunohistochemistry, recurrence
Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related death in the world.\(^1\) HCC represents approximately 90% of all patients with primary liver tumors.\(^2\) Despite advances in HCC treatments, such as established surgical techniques, liver transplantation, ablation treatment, and molecular targeting drugs, post-treatment recurrence is difficult to completely control.\(^3,4\) The 5-year recurrence rate of patients with HCC undergoing radical resection is approximately 80%.\(^4\) Therefore, the establishment of useful biomarkers and therapeutic targets is urgently required to improve the prognosis of refractory HCC patients.

Therapeutic strategies targeting immune checkpoints, such as the blockade of programmed cell death-1 (PD-1) on T lymphocytes and its ligand (PD-L1) on tumor cells using anti-PD-1 and anti-PD-L1 antibodies, have been proposed for several cancers.\(^5\)–\(^9\) Anti-PD-1/PD-L1 antibody blockade exerts its therapeutic effects via the activation of CD8-positive cytotoxic T lymphocytes (CTLs), which target tumor cells.\(^10\)–\(^12\) Therefore, the direct immunohistochemical assessment of PD-L1 in tumor tissues is used as a companion diagnostic technique to predict the effectiveness of PD-1/PD-L1 antibodies as immune checkpoint inhibitors (ICIs).\(^13\) To date, several PD-L1 regulation strategies, such as STAT3 activation by growth factors and cytokines including interferon-gamma (IFN-\(\gamma\)), hypoxic conditions, and microRNAs, have been proposed.\(^14\)–\(^17\) In this study, we focused on CKLF-like MARVEL transmembrane domain-containing 6 (CMTM6) as a candidate PD-L1 regulator. CMTM6 has been reported to colocalize with membrane PD-L1 in tumor cells, and it was shown to inhibit the lysosome-mediated degradation of recycling endosomal PD-L1. This causes the accumulation of membrane PD-L1.\(^18\) The positive correlation between CMTM6 and membrane PD-L1 expression has already been validated in clinical cancer samples\(^19\)–\(^21\) and hepatocytes.\(^22\)

Additionally, other B7 family ligands, such as PD-L2, B7-H3, and B7-H4, have been reported to play essential roles in tumor immune evasion.\(^23\) The structures and expression patterns of the B7 family ligands are similar to those of PD-L1. We therefore speculated that CMTM6 might regulate membrane expression of the B7 family ligands PD-L2, B7-H3, and B7-H4 by inhibiting lysosome-mediated degradation in the same manner as it regulates PD-L1. A previous study reported that CMTM6 expression was positively correlated with B7-H3 and B7-H4 expression in head and neck squamous cell carcinoma.\(^19\) However, little is known about the molecular function of CMTM6 in HCC tumors or the clinical correlation among CMTM6, B7 family ligands, and CTL in patients with HCC.

The purpose of this study was to evaluate the clinical significance of CMTM6, B7 family ligands, and immune cells in clinical HCC patients. Furthermore, we sought to clarify whether CMTM6 targeting can regulate HCC growth and membrane B7 family ligand expression in vitro. We therefore carried out immunohistochemistry (IHC) to analyze the relationships among CMTM6 expression, clinicopathological factors, and B7 family ligands, including PD-L1, B7-H3, and B7-H4, as well as CTL infiltration, in clinical HCC samples. Moreover, we established a CMTM6-knockout HCC cell line to evaluate the function of human CMTM6 in HCC growth and regulation of membrane B7 family ligands.

## MATERIALS AND METHODS

### Patients and samples

Eighty-four HCC patients who had undergone operations at Gunma University Hospital between 1996 and 2014 were included in the study. The ages of the patients ranged from 48 to 89 years. The tumor stage was classified according to the 6th Japanese Tumor-Node-Metastasis Classification of the Liver Cancer Study Group of Japan. All patients were eligible for our study in accordance with institutional guidelines and the Declaration of Helsinki at Gunma University Hospital (approval number: HS2019-143). Patient consent was obtained using the opt-out method.

### Tissue microarrays

Formalin-fixed, paraffin-embedded (FFPE) samples from HCC patients were stored in the archives of the Clinical Department of Pathology, Gunma University Hospital. After checking the hematoxylin and eosin-stained slides, two representative tumor areas were marked on FFPE tissue blocks. Tumor cores with a diameter of 2.0 mm were punched out using a cylinder. A manual arraying instrument (Beecher Instruments) was used to assemble the paraffin blocks into TMAs, as previously described.\(^24\)

### Immunohistochemistry

Four-micrometer sections were cut from the TMA blocks. Immunostaining was performed using the following primary antibodies: anti-CMTM6 monoclonal antibody (HPA026980, 1:50 dilution; Sigma Aldrich), anti-PD-L1 monoclonal antibody (E1L3N, 1:200 dilution; Cell Signaling), anti-B7-H3 monoclonal antibody (ab105922, 1:400 dilution; Abcam plc), anti-B7-H4 monoclonal antibody (ab108336, 1:600 dilution; Abcam), anti-CD8 monoclonal antibody (clone C8/144B, 1:50 dilution; Dako), anti-Ki67 (30-9, 1:50 dilution; Ventana Medical Systems), and anti-granzyme B polyclonal antibody (Roche Diagnostics). Each mounted section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. Antigen retrieval for CMTM6, B7-H3, B7-H4, CD8, and Ki-67 was performed by boiling slides in 10 mmol/L citrate buffer (pH 6.0) at 98–100°C for 45 minutes. Nonspecific binding sites were blocked.
by incubating with Protein Block Serum-Free (Dako) for 30 minutes at room temperature. The sections were washed in PBS, and the primary antibody was visualized using the Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei). The chromogen 3,3-diaminobenzidine tetrahydrochloride (Dojindo Laboratories) was applied as a 0.02% solution containing 0.005% H₂O₂ in 50 mmol/L Tris-HCl buffer (pH 7.6). The sections were lightly counterstained with Mayer's hematoxylin and mounted. Negative controls were established by omitting the primary antibody.

### 2.4 Immunohistochemical evaluation

Each tissue section was evaluated independently by two observers, who were blinded to the patient outcomes. The immunohistochemical expression of CMTM6 and B7-H3 was evaluated based on the intensity of cell membrane staining in positive cases, and B7-H4 was evaluated according to the intensity of cell membrane and cytoplasm staining. The intensity was scored as a grade of 0, 1+, and 2+ for CMTM6 and B7-H4; grade 0 and 1+ staining was defined as low expression, whereas grade 2+ denoted high expression. The intensity was scored as 0 and 1+ for B7-H3; grade 0 staining was defined as low expression, whereas grade 1+ denoted high expression. PD-L1 expression was evaluated as the percentage of positive tumor cell membrane staining. A staining percentage >1% indicated high PD-L1 expression and <1% indicated low expression. Intratumoral CD8-positive CTLs (CD8+) and granzyme B–positive lymphocytes were counted in three selected hotspots by light microscopy at a 200× magnification. We defined patients with a CTL count >10 as part of the high–CTL-infiltration group and patients with a granzyme B–positive lymphocytes count >50 as part of the high-infiltration group of granzyme B–positive lymphocytes. The Ki-67 labeling index was evaluated as the percentage of positive tumor cell nuclei based on >500 tumor cells, regardless of the staining intensity. Activated lymphocytes were determined by counting the number of Ki-67–positive lymphocytes in three selected hotspots of stromal area by light microscopy at a 200× magnification, and we defined patients with a Ki-67–positive lymphocytes count >10 as part of the high-infiltration group of activated lymphocytes.

### 2.5 Cell lines

The human HCC line PLC/PRF/5 (RRID: CVCL_0485) was purchased from the JCRB Cell Bank (Osaka, Japan) and Hep3B (RRID: CVCL_XE51) was obtained from the American Type Culture Collection (ATCC). The cell lines were cultured at an appropriate density in Dulbecco's Modified Eagle Medium (high glucose) with L-glutamine, phenol red, and sodium pyruvate (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained at 37°C in an atmosphere of humidified air with 5% CO₂ and cultured in 100 cm² dishes, and the medium was changed every 2-3 days. IFN-γ treatment was performed over a period of 48 hours at a concentration of 25 ng/mL, unless indicated otherwise.

### 2.6 Establishment of a CMTM6-knockout human HCC cell line

To establish CMTM6-knockout cells, plasmid pRP [CRISPR] expressing hCas9 and dual guide RNAs (gRNAs) were designed and synthesized by Vector Builder Inc. The target sequences for human CMTM6 were 5′-CGGACGGCCCTGCTGCCCTA-3′ and 5′-TTGCTCCGGCCGCGGTCTCCTCA-3′. The plasmid pRP [CRISPR] with dual gRNA sequences targeting CMTM6 was introduced into PLC cells by the electroporation method using an electric pulse generator (CUY-21; Bex) according to the manufacturer's instructions. Single colonies were then collected for further analysis.

### 2.7 Protein extraction and Western blotting

Whole-cell proteins were extracted using RIPA buffer (FUJIFILM Wako Pure Chemical Corporation) from cultured cells according to the manufacturer's protocol. Cell membrane and cytosolic proteins were extracted using the Mem-PER Plus membrane protein extraction kit (Thermo Fisher Scientific). Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% TGX gels (Bio-Rad) and transferred to nitrocellulose membranes by the wet transfer method. The membranes were blocked with 5% skim milk and incubated at 4°C overnight using the following primary antibodies: anti-CMTM6 monoclonal antibody (HPA026980, 1:500 dilution; Sigma Aldrich), anti-PD-L1 monoclonal antibody (E1L3N, 1:1000 dilution; Cell Signaling), anti-PD-L2 monoclonal antibody (ab20037, 1:1000 dilution; Abcam), anti-B7-H3 monoclonal antibody (ab105922, 1:500 dilution; Abcam), anti-B7-H4 monoclonal antibody (ab108336, 1:1000 dilution; Abcam), β-actin mouse monoclonal antibody (A5316, 1:1000 dilution; Sigma Aldrich), and anti-E-cadherin monoclonal antibody (610 182, clone 36, 1:1000 dilution; BD Transduction Laboratories). β-actin expression was used as a loading control for whole-cell or cytosolic protein, and E-cadherin expression was used as a loading control for cell membrane protein. The membranes were then treated with horseradish peroxidase–conjugated secondary antibodies. The blots were detected using the ECL Western blot analysis detection system and an Image Quant LAS 4000 machine (Cytiva).

### 2.8 Cell viability assay

The cells were plated in 96-well plates in a volume of 100 μL and at an approximate density of 3000 cells/well. A Cell Counting Kit-8
MURANUSHI et al. (Dojindo Laboratories) was used to quantify cell viability. Ten microliters of cell-counting solution was added to each well, after which the plates were incubated at 37°C for 2 hours. The cell proliferation rate was then determined by measuring the absorbance of each well using an absorbance spectrophotometer (Bio-Rad) at 450 nm with the reference wavelength set at 650 nm.

2.9 | Statistical analysis

The $\chi^2$ test and Student’s t-test were used to evaluate associations among CMTM6 expression, clinicopathological factors, B7 family ligand expression, and CTL infiltration in the immunohistochemical analysis of HCC samples. To calculate and analyze recurrence-free survival (RFS) and overall survival (OS), we used the Kaplan-Meier method and the log-rank test. Independent predictors for postoperative recurrence were tested by univariate and multivariate analyses, which were based on the Cox proportional hazards regression model. A statistically significant $P$-value was considered to be less than 0.05. All statistical analyses were performed using the JMP software (SAS Institute).

3 | RESULTS

3.1 | Immunohistochemical staining of CMTM6 in HCC tissues

CMTM6 expression was evaluated in 84 HCC samples by IHC. The expression of CMTM6 in HCC cells was primarily observed in both the cellular membrane and cytoplasm (Figure 1A). The membrane expression of CMTM6 in cancerous tissues (25%) was higher than that in noncancerous tissues (7%). We therefore divided the HCC samples into two groups according to the amount of membrane CMTM6 in the cancerous tissues. In total, 21 of 84 HCC samples (25%) were considered to have high membrane CMTM6 expression, whereas 63 HCC samples (75%) had low CMTM6 expression (Figures 1B and S1).

3.2 | CMTM6 expression in HCC tissues and its correlation with clinicopathological factors, B7 family ligand expression, and intratumoral CTL infiltration

Correlations between CMTM6 expression in HCC samples and patient age, gender, liver cirrhosis, differentiation, T classification, tumor growth pattern, cancerous capsule infiltration, intrahepatic metastasis, hepatic vein invasion, portal vein invasion, hepatic artery invasion, tumor size, serum AFP level, serum PIVKII level, and the Ki-67 labeling index are shown in Table 1. High CMTM6 expression was correlated with the progression of the Ki-67 labeling index (Table 1; $P = .0024$). We also examined the relationship between CMTM6, PD-L1, B7-H3, and B7-H4 expression. High CMTM6 expression was strongly associated with the accumulation of membrane PD-L1, B7-H3, and B7-H4 expression (Table 1; $P = .0001$, $P = .0088$, $P = .0002$, respectively). High CD8-positive CTL infiltration was associated with CMTM6, PD-L1, and B7-H3 expression (Table 2; $P = .043$, $P = .014$, and $P = .0002$, respectively). Low infiltration of Ki-67-positive lymphocytes and granzyme B-positive lymphocytes was correlated with high CMTM6 expression (Table S1; $P = .028$ and $P = .026$, respectively).

3.3 | Prognostic significance of CMTM6, B7 family ligands, and intratumoral CTL in clinical HCC patients

The RFS and OS of patients with HCC are shown in Figure 2. Patients with high CMTM6 expression had significantly shorter RFS compared with patients with low expression ($P = .0013$), but the OS was not significantly shorter (Figure 2A; $P = .34$). High PD-L1, B7-H3, and B7-H4 expression or high CTL infiltration was not associated

![FIGURE 1 Immunohistochemical staining of CMTM6 and B7 family ligands in representative clinical hepatocellular carcinoma (HCC) samples. A, High membrane CMTM6 expression was detected in cancer tissue, but not in the noncancerous tissue from identical patients. B, Low CMTM6 and low B7 family ligand expression was detected in HCC tissue from an identical patient. High CMTM6 and high B7 family ligand expression was detected in HCC tissue from another identical patient. Magnification 400x, Scale bar: 100 μm](image-url)
with shorter RFS and OS (Figure 2B, S2A, B and S3). High CMTM6 expression in HCC was a predictive factor for shorter RFS according to the univariate analysis (Table 3; hazard ratio [HR] 2.93, 95% confidence interval [CI] 1.44-5.68, \( P = .004 \)). According to the multivariate analysis, CMTM6 expression was an independent predictor for postoperative recurrence in HCC patients (Table 3; HR 2.7, 95% CI 1.31-5.58, \( P = .002 \)).

Next, we stratified all patients according to the CTL infiltration group and evaluated the association between RFS and the expression of CMTM6 or B7 family ligands in HCC patients. High CMTM6 expression was associated with shorter RFS rather than cases with low CMTM6 and B7 expression and high CD8-positive CTL infiltration (Figure 3; \( P = .044 \) and \( P = .0043 \)). On the other hand, high PD-L1 expression was associated with shorter RFS rather than low PD-L1 expression in cases with high CD8-positive CTL infiltration (\( P = .044 \)), but not in cases with low CTL infiltration (Figure 3; \( P = .64 \)).

3.4 | CMTM6 depletion suppresses membrane PD-L1 expression and viability in human HCC cell lines

CMTM6 was knocked out in two different human HCC cell lines with the Crispr/Cas9 system. We further verified that CMTM6 expression in knockout clones was depleted by Western blot analysis (Figures 4A and S4A). Next, we evaluated the membrane expression of CMTM6 and B7 family ligands such as PD-L1, PD-L2, B7-H3, and B7-H4. The membrane expression of PD-L2, B7-H3, and B7-H4 in CMTM6-knockout cells was significantly suppressed.

### TABLE 1 (Continued)

| Parameters                        | Low expression n = 63 | High expression n = 21 | \( P \) value |
|----------------------------------|-----------------------|------------------------|--------------|
| Serum PIVKA II level (mAU/mL)    |                       |                        |              |
| Normal (≤40)                     | 23                    | 7                      | .83          |
| High (>40)                       | 29                    | 10                     |              |
| Ki-67 labeling index Median (range) | 2.8 (1-40.7)         | 7.7 (1-44.2)           | .0024        |
| PD-L1                            |                       |                        |              |
| Low expression                   | 57 (83.8%)            | 11 (16.2%)             | .0001        |
| High expression                  | 6 (37.5%)             | 10 (62.5%)             |              |
| B7-H3                            |                       |                        |              |
| Low expression                   | 49 (83.1%)            | 10 (16.9%)             | .0088        |
| High expression                  | 14 (56%)              | 11 (44%)               |              |
| B7-H4                            |                       |                        |              |
| Low expression                   | 53 (85.5%)            | 9 (14.5%)              | .0002        |
| High expression                  | 10 (45.5%)            | 12 (54.5%)             |              |

(Continues)
(Figures 4B and S4B). However, we could not detect the baseline expression of PD-L1 in both control and CMTM6-knockout cells (Figures 4B and S4B). We therefore tried to induce PD-L1 expression in HCC cells via IFN-γ treatment. As a result, CMTM6 expression was largely detected in the cell membrane in the IFN-γ-treated controls (Figures 4C and S4C). As expected, membrane PD-L1 expression in the IFN-γ-treated CMTM6-knockout cells was significantly suppressed compared with that in the controls (Figures 4C and S4C).

In Figure 4C, the cytosol PD-L1 expression level was significantly low compared with the membrane PD-L1 expression level. To evaluate the CMTM6-knockout effect on cytosol PD-L1 expression at different exposure times, we performed Western blot analysis and found that cytosol PD-L1 expression in IFN-γ-treated CMTM6-knockout cells was significantly suppressed compared with that in the controls, suggesting an association between membrane PD-L1 and CMTM6 depletion (Figure S5).

Additionally, the proliferation rate of CMTM6-knockout HCC cells was significantly decreased compared with that of the controls (Figures 4D and S4D).

### 4 DISCUSSION

In the present study, we demonstrated that CMTM6 expression is positively associated with membrane B7 family ligands expression and that CTL infiltration is correlated with CMTM6, PD-L1, and B7-H3 expression in clinical HCC samples. High CMTM6 expression in HCC tissues was associated with the expression of the proliferation marker Ki-67 and shorter RFS. In vitro analysis clearly showed downregulation of the membrane B7 family ligands and proliferation potency in the CMTM6-knockout human HCC cell lines.

In this study, we showed the distribution of CMTM6 in clinical HCC samples. CMTM6 expression was detected in both the cytoplasm and cellular membrane, and the membrane expression of CMTM6 in HCC cells was significantly higher than that in noncancerous cells. High expression of membrane CMTM6 in cancerous tissue is consistent with the results of previous studies. Yamamoto et al have reported inducible factors of CMTM6 to be the CDR1-AS RNA and the anti-HBV drug entecavir and suppressive factors to

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**TABLE 2** Correlation among CD8-positive cytotoxic T lymphocytes infiltration, CMTM6 expression, and B7 family ligand expression

| Parameters | Low infiltration | High infiltration | P value |
|------------|------------------|-------------------|---------|
|            | n=27             | n=57              |         |
| CMTM6      |                  |                   |         |
| Low expression | 24 (38.1%)       | 39 (61.9%)        | .043    |
| High expression | 3 (14.3%)        | 18 (85.7%)        |         |
| PD-L1      |                  |                   |         |
| Low expression | 26 (38.2%)       | 42 (61.8%)        | .014    |
| High expression | 1 (6.3%)         | 15 (93.7%)        |         |
| B7-H3      |                  |                   |         |
| Low expression | 25 (42.4%)       | 34 (57.6%)        | .002    |
| High expression | 2 (8%)           | 23 (92%)          |         |
| B7-H4      |                  |                   |         |
| Low expression | 23 (37.1%)       | 39 (62.9%)        | .10     |
| High expression | 4 (18.2%)        | 18 (81.8%)        |         |

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**FIGURE 2** Postoperative recurrence-free survival and overall survival rates of patients stratified by CMTM6 and PD-L1 expression in hepatocellular carcinoma. All survival curves were plotted using the Kaplan-Meier method. A, High versus low CMTM6 expression (P = .0013, P = .34). B, High versus low PD-L1 expression (P = .12, P = .60)
be WEE1 inhibitors and ATM inhibitors. However, our data might be insufficient to explain the relationship between such factors and membrane CMTM6 expression in HCC cells. Conversely, database analysis yielded interesting findings regarding CMTM6 regulation in many cancer cell lines. The data clarified that the expression levels of CMTM6 in several cancer cell lines, including HCC, were significantly associated with CMTM6 DNA copy number alteration (Figure S6). This suggests the importance of DNA copy number alterations in

### Clinicopathologic Variable Results

| Clinicopathologic variable | Univariate analysis | Multivariate analysis |
|----------------------------|--------------------|---------------------|
|                            | HR     | 95%CI  | P value | HR     | 95%CI  | P value |
| CMTM6 expression (low/high) | 2.93   | 1.44-5.68 | .004 | 2.7    | 1.31-5.58 | .002 |
| PD-L1 expression (low/high) | 1.83   | 0.78-3.81 | .15 |                  |        |        |
| B7-H3 expression (low/high) | 1.05   | 0.53-1.95 | .89 |                  |        |        |
| B7-H4 expression (low/high) | 1.03   | 0.46-2.08 | .94 |                  |        |        |
| CD8 infiltration (low/high) | 0.57   | 0.31-1.10 | .094 |                  |        |        |
| Age (yr) (≤65/>65)          | 0.79   | 0.41-1.54 | .49 |                  |        |        |
| Sex (male/female)           | 1.89   | 0.91-4.42 | .089 |                  |        |        |
| Liver Cirrhosis (negative/positive) | 1.69 | 0.92-3.09 | .092 |                  |        |        |
| Differentiation (well or moderate/poor) | 2.52 | 0.89-7.15 | .082 |                  |        |        |
| T classification (T1-T3/T4) | 1.15   | 0.28-3.19 | .82 |                  |        |        |
| Pattern of tumor growth (expansive/infiltrative) | 1.87 | 0.84-3.76 | .12 |                  |        |        |
| Tumor size (≤20 mm/>20 mm)  | 4.48   | 1.37-27.52 | .009 | 3.8    | 0.88-16.52 | .0044 |
| AFP level (normal/high)     | 1.19   | 0.61-2.38 | .62 |                  |        |        |
| PIVKA II level (normal/high) | 1.38  | 0.69-2.81 | .36 |                  |        |        |

**Abbreviations:** HR, hazard ratio; CI, confidence interval.

### Table 3

| Clinicopathologic variable | Univariate analysis | Multivariate analysis |
|----------------------------|--------------------|---------------------|
|                            | HR     | 95%CI  | P value | HR     | 95%CI  | P value |
| CMTM6 expression (low/high) | 2.93   | 1.44-5.68 | .004 | 2.7    | 1.31-5.58 | .002 |
| PD-L1 expression (low/high) | 1.83   | 0.78-3.81 | .15 |                  |        |        |
| B7-H3 expression (low/high) | 1.05   | 0.53-1.95 | .89 |                  |        |        |
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**Abbreviations:** HR, hazard ratio; CI, confidence interval.

### Figure 3

**Figure 3** Postoperative recurrence-free survival rates of patients stratified according to the CD8-positive cytotoxic T lymphocytes group. CMTM6 expression and B7 family ligand expression were evaluated in patients with hepatocellular carcinoma. All survival curves were plotted using the Kaplan-Meier method.
the regulation of CMTM6 expression. Further studies are needed to clarify the main regulators of CMTM6 in HCC.

Previous studies have reported that high CMTM6 expression in cancerous tissue is a significant prognostic marker for several cancers.\textsuperscript{19,26} In this study, we clarified that high membrane CMTM6 expression in HCC cells is correlated with a high Ki-67 index and shorter RFS. Additionally, high CMTM6 expression was associated with shorter RFS not only in the high–CTL-infiltration group but also in the low-infiltration group. Meanwhile, high PD-L1 expression was associated with only the high–CTL-infiltration group. These findings indicated that CMTM6 might be a predictive marker for postoperative recurrence independent of intratumoral immune cell infiltration. Our data demonstrated the expression of CMTM6 in HCC was positively associated with PD-L1 expression and CD8-positive T cell infiltration. Positive correlation between PD-L1 expression and CD8-positive T cell infiltration in HCC is consistent with previous report.\textsuperscript{27} It was suggested that high PD-L1 expression induced by CMTM6 might impair antitumor effect via interaction to PD-1 on CD8-positive T cell. These are supported by our data, namely that the CMTM6 expression was negatively correlated with the granzyme B and Ki-67 expression of tumor-infiltrating immune cells in the tumor stromal area.

Zhu et al reported that positive CMTM6 expression in HCC tissues is significantly lower than that in adjacent nontumor tissues and that high CMTM6 expression is negatively associated with survival time.\textsuperscript{28} This is contrary to the findings of our study. In this study, we focused on membrane CMTM6 expression, as the immunohistochemical evaluation was consistent with our in vitro study, which demonstrated that the HCC cell line largely expressed CMTM6 in the membrane. This suggests that membrane CMTM6 expression could serve as a better indicator for evaluating its function in HCC than the cytoplasmic protein.

This study demonstrates that CMTM6 might be a common regulator of B7 family ligands in HCC cells. Tumor PD-L1 signaling could regulate features related to cancer aggressiveness, such as cancer stemness, epithelial-mesenchymal transition, and chemoresistance,
via activation of cancer-related factors, including the PI3K/AKT and RAS/ERK pathways, and the upregulation of MDR1.19 High PD-L2, B7-H3, and B7-H4 expression was found to be associated with poor prognosis and CTL infiltration in HCC, showing a similar relationship to that between PD-L1 and CTL. Additionally, Chen et al reported that CMTM6 activates Wnt/β-catenin signaling, which controls proliferation potency, metastasis initiation, and therapeutic resistance in several cancers.15 In this study, we demonstrated that membrane CMTM6 expression was significantly higher in cancerous tissues than in noncancerous tissues. Furthermore, CMTM6 depletion clearly suppressed HCC growth and the membrane expression of B7 family ligands, including PD-L1, PD-L2, B7-H3, and B7-H4. CMTM6 targeting is therefore suggested to be a promising therapeutic strategy to inhibit both HCC growth via the aforementioned cancer-related factors and by inhibiting CTL activity mediated by membrane B7 family ligands. To the best of our knowledge, this is the first report showing that human CMTM6 can regulate PD-L1, PD-L2, B7-H3, and B7-H4 expression in HCC tumor cells by in vitro analysis.

PD-L1 regulation via CMTM6 is reportedly controlled by the inhibition of recycling endosomes and subsequent lysosomal degradation.18 To validate the CMTM6-altered PD-L1 regulation via lysosomal degradation, we evaluated the expression levels of B7 family ligands, including PD-L1, by treating with chloroquine (CQ), a lysosome inhibitor. Contrary to the expectations, the IFN-γ–induced PD-L1 and PD-L2 expression levels in CMTM6-knockout cells were not altered by CQ treatment; however, PD-L1 expression level in CQ-treated control cells was higher than that in cells not treated with CQ (Figure S7A). Interestingly, the expression levels of B7-H3 and B7-H4 in CMTM6-knockout cells were restored to the same levels as control cells (Figure S7B). Therefore, these data suggested that the expression of B7 family ligands might be controlled by CMTM6 via the lysosomal degradation system, and that IFN-γ–induced PD-L1 expression levels might be regulated by CMTM6 via not only lysosomal degradation but also other unknown mechanisms.

Our study has several limitations. First, this was a retrospective single-institution study with a small sample size, which might bias the results. Further large-scale clinical trials will be needed to clarify the potential of CMTM6 as a new predictive biomarker for HCC recurrence. Second, we discussed the potential of CMTM6 evaluation in the first report showing that human CMTM6 can regulate PD-L1, PD-L2, B7-H3, and B7-H4 expression in HCC tumor cells by in vitro analysis. The present study was supported in part by a grant from the Japanese Society for Gastroenterological Carcinogenesis. The authors thank Bilguun Erkhem-Ochir, Navchaa Gombodorj, and Tadashi Handa for useful discussions and excellent technical assistance for this study. We would like to thank Editage (www.editage.com) for English language editing.

ConFLICT OF INTEREST
The authors have no conflict of interest.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
This study was performed in compliance with the Declaration of Helsinki. All patients were eligible for our study in accordance with institutional guidelines at Gunma University Hospital (approval number: HS2019-143). Patients’ consent was obtained by the opt-out method.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.