The association of CMTM6 expression with prognosis and PD-L1 expression in triple-negative breast cancer

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Background: Immune checkpoint inhibitors play a vital role in triple-negative breast cancer (TNBC) immunotherapy. A recent study showed that chemokine-like factor (CKLF)-like MARVEL transmembrane domain containing 6 (CMTM6) has a crucial role in programmed death-ligand 1 (PD-L1) stability. The aim of this study was to investigate the relationship between CMTM6 and PD-L1 in TNBC and the association with clinical characteristics.

Methods: A total of 143 patients, including 75 with human epidermal growth factor receptor 2 (HER2)-driven breast cancer and 68 with TNBC, were included in this study. In 83 paired primary breast cancers (PBCs) and metastatic breast cancers (MBC) comprising 45 HER2-driven breast cancers and 38 TNBC, CMTM6 and PD-L1 were detected based on immunohistochemistry (IHC) with FFPE tissues. Another 60 PBCs comprising 30 HER2-driven breast cancers and 30 TNBC in order to detect CMTM6 and PD-L1 mRNA expressions based on real-time polymerase chain reaction (RT-PCR) using frozen tissues. Furthermore, 153 patients comprising 30 TNBC and 123 HER2-driven breast cancer based on The Cancer Genome Atlas (TCGA) database were used to confirm the difference mRNA expression.

Results: The expression of CMTM6 in patients with TNBC was significantly higher than in those with HER2-driven PBC (IHC, P=0.036, mRNA, P=0.036, TCGA dataset, P=0.039). CMTM6 was correlated with PD-L1 based on IHC in triple-negative MBC (P=0.004); the same result was found based on mRNA data in triple- negative PBC (P=0.021). Moreover, a high expression of CMTM6 in TNBC was associated with poor progression-free survival (PFS) (P=0.030, 95% CI: 1.08–4.57, HR =2.22). After multiple Cox regression analysis, CMTM6 in TNBC emerged as an independent risk factor for PFS (P=0.027, 95% CI: 1.11–5.20, HR =2.40). The expression of PD-L1 was negatively correlated with lymph node metastasis (P=0.026) and was not associated with PFS.

Conclusions: The expression of CMTM6 was higher in TNBC than in HER2-driven breast cancer. In TNBC, CMTM6 was correlated with PD-L1 expression, and potentially could be used as an independent risk factor for predicting PFS.

Keywords: CMTM6; PD-L1; triple-negative breast cancer (TNBC); immunohistochemistry (IHC); polymerase chain reaction (PCR); prognosis

Submitted Oct 16, 2020. Accepted for publication Jan 14, 2021.

doi: 10.21037/atm-20-7616

View this article at: http://dx.doi.org/10.21037/atm-20-7616

Introduction

Breast cancer is the most common malignancy in women worldwide and its clinical and molecular heterogeneity is well-documented. Endocrine receptors for estrogen (ER) and progesterone (PR), and the aberrant expression of human epidermal growth factor receptor-2 (HER2) are the specific biomarkers for breast cancer most commonly used in clinical practice. Triple-negative breast cancer (TNBC), which lacks expression of ER, PR, and HER2, is found in 10–20% of all breast cancers. TNBC is an aggressive type of breast cancer that usually displays a higher grade and poorer outcome than other breast cancer subtypes (1,2). Therefore, effective therapeutic strategies for TNBC are urgently needed.

Programmed death-ligand 1 (PD-L1) is a ligand of the programmed cell death-1 (PD-1) receptor and can be expressed by the tumor cell surface as well as by tumor infiltration lymphocytes (TILs). Binding of PD-L1 to PD-1 can downregulate anti-tumor T cell responses which lead to tumor immune escape (3). To date, immune checkpoint inhibitors that block the interaction between PD-1 and PD-L1 have been used to treat many types of metastatic cancers (3-5). In primary breast cancer (PBC), the expression of PD-L1 is heterogeneous and associated with higher histological grades and more aggressive molecular subtypes [triple-negative (TN), basal, and HER2-driven] (6,7). However, the regulation mechanism of PD-L1 remains elusive.

Recently, chemokine-like factor (CKLF)-like Marvel Transmembrane Domain-containing 6 (CMTM6) has been identified as a key regulator of the PD-L1 protein, which is also thought to be involved in modulating tumor immunity (8,9). Dysfunction of CMTM6 impairs the expression of PD-L1 in many human tumor cell types, such as lung cancer, thyroid cancer, and melanoma (8). The depletion of CMTM6, via downregulated PD-L1, can reduce the suppression of tumor-specific T cell activity *in vitro* and *in vivo* assays (10). Previous studies have suggested the potential value of a therapeutic target that elicits an immune response and avoids the escape of immune surveillance. However, the role of CMTM6 in breast cancer remains unclear.

In the present study, we used immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR) to analyze the correlation between CMTM6 and PD-L1 in patients with TNBC. Furthermore, we assessed the association of CMTM6 or PD-L1 expression with patients' prognosis. This study gives a hint that the expression of CMTM6 may become a potential biomarker of immunotherapies through supplement to PD-L1 expression and could predict the patients' outcome. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7616).

Methods

Patients

A total of 143 patients with breast cancer who underwent surgical resection in the Cancer Hospital of the University of Chinese Academy of Sciences in China between January 2008 and December 2015 were enrolled in the study. Of these, 83 patients who had paired primary breast cancer tissues and matched metastatic cancer tissues were selected and comprised 45 HER2-driven breast cancer and 38 TNBC patients. All of these patients were without neoadjuvant chemotherapy at the first diagnosis and had sufficient archival tissue in formalin-fixed, paraffin-embedded (FFPE) blocks to perform IHC analyses. The remaining 60 individuals comprised 30 HER2-driven and 30 TNBC PBC patients with sufficient frozen tissue to perform mRNA expression detection (Figure S1).

The eighth edition of the tumor node metastasis (TNM) classification of the American Joint Commission on Cancer (AJCC) was used as a reference for pathological features and clinical stage. Progression-free survival (PFS) was defined as the time from the date of the initial treatment until the date of diagnosis of the initial recurrence or death from any cause. The PFS data were locked on August 30, 2020.

This study was approved by the Medical Ethics Committee of Zhejiang Cancer Hospital (IRB-2020-275). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Because of the retrospective nature of the research, the requirement for informed consent was waived.

IHC staining of CMTM6 and PD-L1

FFPE tissue specimens were collected from 83 patients with paired PBC and metastatic cancer tissues, and an IHC assay was performed to detect the expression of CMTM6 and PD-L1. FFPE tissue blocks were cut into 4-μm-thick sections and attached to a positively charged glass slide. Immunohistochemical staining of CMTM6 was carried out

with a Leica BOND-III automatic IHC staining device. The samples were incubated with an antibody against CMTM6 for 15 min at room temperature (dilution 1:200, recombinant monoclonal antibody, Abcam, EPR23015-45, US). The signal was subsequently detected with a Leica Bond Polymer Refine Detection Kit (Leica Biosystems, Buffalo Grove, US). Post-primary antibody incubation and polymer incubation were set at 8 min for CMTM6 and followed by immersion in diaminobenzidine (DAB) for signal visualization for 10 min at room temperature. CMTM6 expression was defined as any intensity in the cytomembrane on the tumor cells (Figure S2A,B).

For PD-L1 staining, the VENTANA PD-L1 (SP142) rabbit monoclonal primary antibody (Ventana Medical Systems Inc., Tucson, AZ, USA) was optimized for use as a fully automated IHC assay on the BenchMark ULTRA (Ventana Medical Systems Inc., Tucson, AZ, USA) staining platform using the OptiView DAB IHC Detection Kit and OptiView Amplification Kit (Ventana Medical Systems Inc., Tucson, AZ, USA) as previously described (11). PD-L1 expression was identified as any intensity in the cytomembrane on the tumor-infiltrating immune cells (Figure S2C,D).

RNA extraction and quantitative RT-PCR

Total RNA was isolated from 60 frozen cancer tissues using TRIzol (Invitrogen, USA), following the manufacturer's protocol. The concentration of RNAs was measured through a microvolume spectrophotometer. The 500 nanograms of RNA was reverse transcribed using a PrimeScript™ RT Reagent Kit (Takara, Dalian, China). Quantitative PCR (qPCR) was carried out as previously described (12). The primer sequences were: CMTM6-F: 5'-GCAACAATATCAGCAACTTCGT-3' and CMTM6-R: 5'-TTGGTCCTTAGGTGTGGTATCA-3'; PD-L1-F: 5'-CACCACCACCAATTCCAAGAG-3'; PD-L1-R: 5'- AGGATGTGCCAGAGGTAGTTC-3'; β-actin-F: 5'-TGGCACCCAGCACAATGAA-3', β-actin-R: 5'-CTAAGTCATAGTCCGCCTAGAAG CA-3'.

Western blotting

Cells were collected and prepared as described earlier (13). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% non-fat milk in TBS-Tween 20 for 2 hours prior to overnight incubation with primary antibodies at 4 ℃, and were then incubated for 2 hours at room temperature with the secondary antibodies (Servicebio, Wuhan, China). Finally, the protein level was detected using an enhanced chemiluminescence (ECL) reagent (CwBio, Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. Primary antibodies were directed against CMTM6 (Abcam, USA, 1:1,000), PD-L1 (Proteintech, USA, 1:1,000), and GAPDH (Santa Cruz, CA, USA, 1: 5,000).

Web-based mRNA profiling

The RNA-sequencing data and clinical records of 30 HER2-driven PBC and 123 triple-negative PBC patients were downloaded from The Cancer Genome Atlas (TCGA) dataset (see URLs http://cancergenome.nih.gov/). The present study conforms to the publication guidelines.

Statistical analysis

Statistical analysis was performed using SPSS statistics version 22.0 (IBM Corp., Armonk, NY, USA). The continuous variables were described as means and standard deviations (SD), and the categorical variables were described as number (percentage). Any skewed distribution data were expressed with median and interquartile ranges. The Mann-Whitney test was adopted to evaluate the non-normal distribution dataset. Spearman's correlation analysis was used to describe the correlation between quantitative variables with skewed distribution data, and the Pearson Chi-square and McNemar's tests were used to compare categorical variables. Kaplan-Meier (K-M) analysis was used, and between-group differences were evaluated by the log-rank test. The multivariable Cox proportional hazard model was applied to calculate the adjusted hazard ratio (HR). A two-sided P value <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 143 patients with breast cancer, including 75 (52.4%) HER2-driven PBCs and 68 (47.6%) triple-negative PBCs were included in the study. The clinical features are shown in *Table 1*. All patients were female, with a mean

Table 1 General clinical characteristics

Variable	All	HER2-driven breast cancer	Triple-negative breast cancer
Primary total sample size, n (%)	143 (100%)	75 (52.4%)	68 (47.6%)
Age			
Mean ± SD, years	49.97±10.63	50.18±9.59	49.73±11.75
≤50	73 (51.0%)	39 (52.0%)	34 (50.0%)
>50	70 (49.0%)	36 (48.0%)	34 (50.0%)
Stage			
I	16 (11.2%)	6 (8.0%)	10 (14.7%)
II	60 (42.0%)	31 (41.3%)	29 (42.6%)
III	47 (32.9%)	27 (36.0%)	20 (29.4%)
IV	20 (14.0%)	11 (14.7%)	9 (13.2%)
Tumor size (cm)			
≤2	17 (11.9%)	6 (8.0%)	11 (16.2%)
2–5	96 (67.1%)	58 (77.3%)	38 (55.9%)
>5	30 (21.0%)	11 (14.7%)	19 (27.9%)
Lymph node status			
No metastasis	46 (32.2%)	18 (24.0%)	28 (41.2%)
Metastasis	97 (67.8%)	57 (76.0%)	40 (58.8%)

age of 49.97±10.63 years. Of these, 16 (11.2%) had stage I, 60 (42.0%) had stage II, 47 (32.9%) had stage III, and 20 (14.0%) had stage IV diagnoses of breast cancer. A total of 97 (67.8%) patients showed lymph node metastases. There were 17 (11.9%) patients with a tumor size smaller than 2 cm, 96 (67.1%) patients with a tumor size of 2–5 cm, and 30 (21.0%) patients with a tumor size larger than 5 cm. Of the total number of 143 patients, 83 had matched PBC and metastatic breast cancer (MBC). There was a 97.6% (81/83) concordance between ER, PR, and HER2 expression between the matched PBC and MBC patients. The median PFS of these 83 patients was 48 months (range, 29–76 months).

CMTM6 and PD-L1 expression in HER2-driven breast cancer and TNBC

IHC was performed to test the expression of CMTM6 and PD-L1 in the 83 paired breast cancers. The typical CMTM6 and PD-L1 staining images from breast cancer samples are shown in Figure S2. Western blot was used to confirm the expression detected by IHC (Figure S3). In PBC, 56 (67.5%) cases were negative for CMTM6, while 27 (32.5%) showed CMTM6-positive staining. The positive expression rate of CMTM6 in patients with triple-negative PBC was significantly higher than in those with HER2-driven PBC (63.0% *vs.* 37.0%, P=0.036, *Table 2*). A similar result was found in the MBC patients (61.1% *vs.* 38.9%, P=0.048, *Table 2*). Moreover, the mRNA expression level of CMTM6 showed the same result (the median CMTM6 mRNA expression was 0.037 in triple-negative PBC and 0.026 in HER2-driven PBC, P=0.036, Table S1, Figure S4A).

For the PD-L1 expression, 50 (60.2%) showed negative staining, while 33 (39.8%) showed positive staining. However, no significant difference in PD-L1 expression was found between HER2-driven breast cancer and TNBC, either in PBC or in MBC samples (*Table 2*). Moreover, a significant difference was found in the PD-L1 mRNA expression level between triple-negative PBC and HER2-driven PBC (the median expression level was 0.00026 and 0.00012, respectively, P=0.040, Table S1, Figure S4B).

To further verify our findings, we performed CMTM6 and PD-L1 mRNA expression analyses in HER2-driven

Table 2 CMTM6 and PD-L1 protein expression in HER2-driven breast cancer and triple-negative breast cancer

Protein expression status	Primary			Metastasis		
	Her2-driven (%)	Triple-negative (%)	P	Her2-driven (%)	Triple-negative (%)	P
CMTM6 negative	35 (62.5)	21 (37.5)	0.036	29 (61.7)	18 (38.3)	0.048
CMTM6 positive	10 (37.0)	17 (63.0)		14 (38.9)	22 (61.1)	
PD-L1 negative	28 (56.0)	22 (44.0)	0.822	31 (50.0)	31 (50.0)	0.621
PD-L1 positive	17 (51.5)	16 (48.5)		12 (57.1)	9 (42.9)	

Pearson Chi-square test.

Table 3 CMTM6 and PD-L1 protein expression in matched PBC and MBC

Matched MBC protein expression status	CMTM6 expression in primary cancer			PD-L1 expression in primary cancer		
	Negative (%)	Positive (%)	P	Negative (%)	Positive (%)	P
All metastasis samples						
Negative	39 (83.0)	8 (17.0)		43 (69.4)	19 (30.6)	
Positive	17 (47.2)	19 (52.8)	0.108	7 (33.3)	14 (66.7)	0.031
Metastasis HER2-driven samples						
Negative	26 (86.7)	4 (13.3)		22 (66.7)	11 (33.3)	
Positive	9 (60.0)	6 (40.0)	0.267	6 (50.0)	6 (50.0)	0.332
Metastasis triple-negative samples						
Negative	13 (76.5)	4 (23.5)		21 (72.4)	8 (27.6)	
Positive	8 (38.1)	13 (61.9)	0.388	1 (11.1)	8 (88.9)	0.039

McNemar's test. PBC, primary breast cancer; MBC, metastatic breast cancer.

PBC and triple-negative PBC using the TCGA dataset. Although the expression level of PD-L1 between HER-2 driven PBC and triple-negative PBC did not differ (P=0.283, Table S2), a higher expression level of CMTM6 was observed in triple-negative PBC (P=0.039, Table S2).

CMTM6 and PD-L1 expression in the matched PBC and MBC samples

As shown in *Table 3*, we evaluated whether there was a difference in the expression levels of CMTM6 and PD-L1 in the matched PBC and MBC samples. Across the 83 matched samples, patients with a PD-L1 positive expression in the MBC group were more likely to have a PD-L1 positive expression in the matched PBC group (P=0.031). A similar result was found in the triple-negative samples (P=0.039), while no significant difference was found for the HER2-driven samples. There was no correlation between the expression of CMTM6 in the matched breast cancer samples in this study.

Correlation of CMTM6 expression and PD-L1 expression in breast cancers

The correlation between the expression of CMTM6 and PD-L1 was further analyzed. In MBC, we found that CMTM6 protein expression was positively correlated with PD-L1 protein expression. McNemar's test showed that the expression of CMTM6 and PD-L1 were significantly correlated across the entire MBC sample (P=0.012, *Table 4*), but especially in the triple-negative MBC sample (P=0.004, *Table 4*). However, no significant correlation was observed in the individual PBC or HER2-driven MBC groups (*Table 4*).

Next, we performed correlation analyses between CMTM6 and PD-L1 expression in the mRNA data using

Table 4 The associated protein expression between CMTM6 and PD-L1 in breast cancers

CMTM6 expression status	PD-L1 expression in primary cancer			PD-L1 expression in metastasis		
	Negative (%)	Positive (%)	P	Negative (%)	Positive (%)	P
CMTM6 in all samples						
Negative	38 (67.9)	18 (32.1)		39 (83.0)	8 (17.0)	
Positive	12 (44.4)	15 (55.6)	0.361	23 (63.9)	13 (36.1)	0.012
CMTM6 in Her2-driven samples						
Negative	23 (65.7)	12 (34.3)		24 (80.0)	6 (20.0)	
Positive	5 (50.0)	5 (50.0)	0.143	9 (60.0)	6 (40.0)	0.607
CMTM6 in triple-negative samples						
Negative	15 (71.4)	6 (28.6)		15 (88.2)	2 (11.8)	
Positive	7 (41.2)	10 (58.8)	>0.999	14 (66.7)	7 (33.3)	0.004

McNemar's test.

Spearman's correlation coefficient analysis. The results showed that the mRNA expression level of CMTM6 was positively correlated with the mRNA expression level of PD-L1 in total breast cancers ($r_{Spearman}$=0.419 and P=0.001, *Figure 1A*), in HER2-driven PBC ($r_{Spearman}$=0.373 and P=0.042, *Figure 1B*), and in TNBC ($r_{Spearman}$=0.421 and P=0.021, *Figure 1C*).

Clinicopathological characteristics by CMTM6 and PD-L1 expression

CMTM6 expression was not significantly associated with clinicopathological characteristics (*Table 5*) but was significantly associated with PFS. In the total sample, those with CMTM6 expression were at higher risk for disease progression compared with those without CMTM6 expression, especially in TNBC (HR =1.83, 95% CI: 1.13–2.96, P=0.014 for the total sample; and HR =2.22; 95% CI: 1.08–4.57, P=0.030 for TNBC, *Table 6*). Median PFS times for those with CMTM6 expression in the total sample and triple-negative cases were 46.8 (IQR, 38.3–50.0) and 47.0 (IQR, 39.5–52.7) months, respectively, while the median for those without CMTM6 expression was over 51 months. The Kaplan-Meier survival analysis demonstrated similar results (*Figure 2A,B,C*). After adjusting for age and TNM stage, significant associations were found in CMTM6 (HR =2.21, 95% CI: 1.32–3.71, P=0.003), especially in the TNBC patients (HR =2.40, 95% CI: 1.11–5.20, P=0.027, *Table 6*).

In the case of PD-L1, the absence of PD-L1 expression was correlated with lymph node metastasis (P=0.026, *Table 5*). No other clinicopathologic characteristics were associated with PD-L1 expression. Moreover, our data showed that the expression of PD-L1 was not significantly associated with disease progression (*Figure 2D,E,F*, *Table 6*).

Discussion

Our data suggested that the expression of CMTM6 was higher in TNBC than in HER2-driven breast cancer and that CMTM6 was also an independent risk factor of PFS, especially in TNBC. The expression level of CMTM6 was positively correlated with the expression of PD-L1. Moreover, the expression of PD-L1 in triple-negative metastatic breast cancer was positively correlated with that in primary breast cancer. And the expression of PD-L1 was negative with metastases in lymph nodes in PBC.

TNBC has a more aggressive biological behavior than other types of breast cancer. Patients with TNBC do not benefit from hormonal or trastuzumab-based therapy because of the loss of the target receptors HER2, ER, and PGR (2,14); chemotherapy and surgery appear to be the only available treatment modalities (15). Recent studies have shown that TNBC has a higher mutational burden than other subtypes and presents with tumor-infiltrating lymphocytes (TILs). Moreover, patients with TNBCs combined low-TILs and high PD-L1 expression showed unfavorable outcome, which may be benefit from immune therapy (1,7,16-18). According to these results, many clinical trials are currently evaluating the role of

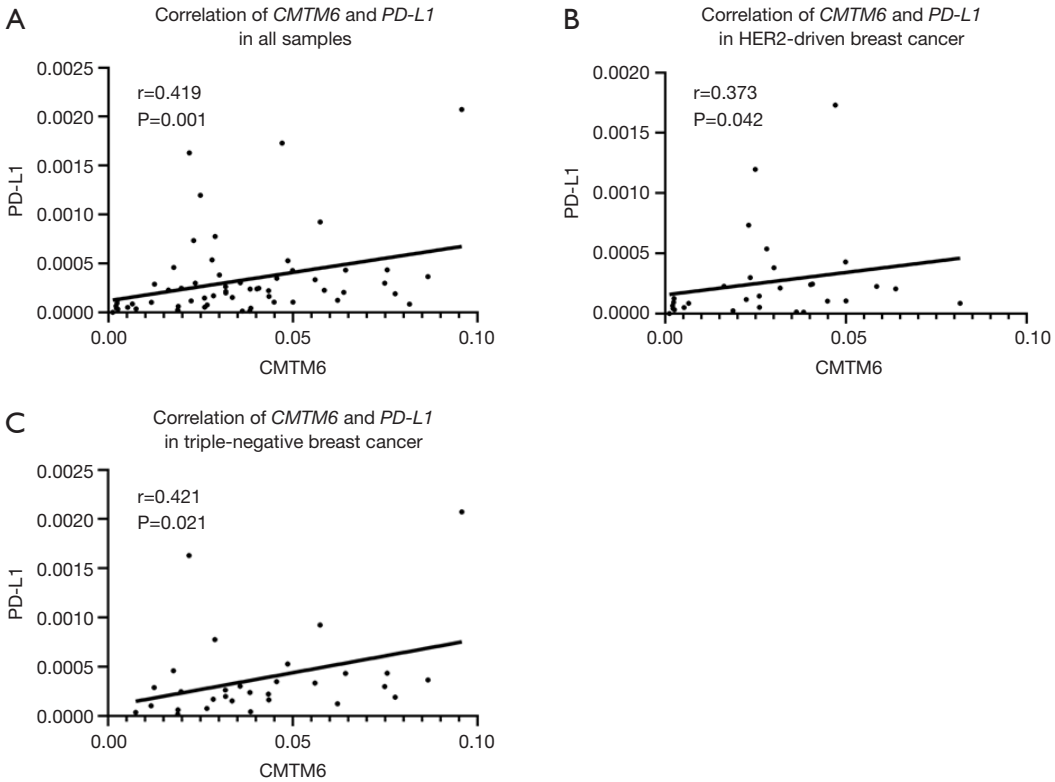

Figure 1 The correlation of *CMTM6* and *PD-L1* mRNA expression in all 60 samples (A), in HER2-driven breast cancer (B), and in triple-negative breast cancer (C). P values for correlation analysis are determined with the Spearman correlation coefficient.

Table 5 Clinical features by CMTM6 and PD-L1 protein expression

Clinical features	CMTM6 expression			PD-L1 expression		
	Negative	Positive	P	Negative	Positive	P
Age, n (%)						
≤50	31 (64.6)	17 (35.4)		29 (60.4)	19 (39.6)	
>50	25 (71.4)	10 (28.6)	0.636	21 (60.0)	14 (40.0)	>0.999
Size, n (%)						
≤2	5 (50.0)	5 (50.0)		4 (40.0)	6 (60.0)	
2–5	35 (70.0)	15 (30.0)		29 (58.0)	21 (42.0)	
≥5	16 (69.6)	7 (30.4)	0.453	17 (73.9)	6 (26.1)	0.164
Stage, n (%)						
I, II	19 (61.3)	12 (38.7)		16 (51.6)	15 (48.4)	
III, IV	37 (71.2)	15 (28.8)	0.468	34 (65.4)	18 (34.6)	0.251
LN stage, n (%)						
Negative	8 (47.1)	9 (52.9)		6 (35.3)	11 (64.7)	
Positive	48 (72.7)	18 (27.3)	0.079	44 (66.7)	22 (33.3)	0.026

Pearson Chi-square test.

Table 6 Survival analysis of gene expressions in PBC

Variable	HR_a (95% CI)	P_a	HR_b (95% CI)	P_b
All samples				
CMTM6	1.83 (1.13–2.96)	0.014	2.21 (1.32–3.71)	0.003
PD-L1	1.06 (0.68–1.65)	0.795		
HER2-driven				
CMTM6	1.83 (0.89–3.76)	0.102		
PD-L1	0.81 (0.43–1.49)	0.490		
Triple-negative				
CMTM6	2.22 (1.08–4.57)	0.030	2.40 (1.11–5.20)	0.027
PD-L1	1.36 (0.70–2.61)	0.364		

P_a value for PFS was determined with Cox proportional hazards regression. P_b value was measured by multivariate analyses of PFS (Cox proportional hazards regression model) after adjustment for age and TNM stage. HR_a, hazard ratio (HR) was measured by Cox proportional hazard model; HR_b, the adjusted hazard ratio (HR) was measured by multivariate Cox proportional hazard model after adjustment for age and TNM stage. PBC, primary breast cancer; PFS, progression-free survival.

checkpoint inhibitors, PD-1, and/or PD-L1 in TNBC with encouraging results (19,20).

Although anticancer therapies based on immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway have emerged as a critical treatment option, it seems that only a small subset of patients can benefit from these (20,21). To solve this challenge, some studies have focused on investigating the regulation of PD-L1 expression identifying a type-3 transmembrane protein, CMTM6, as a regulator of PD-L1 expression (8,10). In our study, the expression of CMTM6 was higher in TNBC than in HER2-driven breast cancer, according to the TCGA dataset. The expression of CMTM6 was positively correlated with PD-L1 in PBC and MBC in mRNA expression and IHC data, respectively. Koh *et al.* and Gao *et al.* reported similar results in non-small cell lung cancer (9,22). Moreover, our data revealed no significantly different expression of CMTM6 between paired triple-negative PBC and MBC. Considering the transient PD-L1 expression in tumor cells which tends to disappear rapidly, the intratumoral heterogeneity of PD-L1 expression is frequently observed (23). Thus, the expression of CMTM6 may be a new potential biomarker of immunotherapies in TNBC.

In our study, TNBC patients with a high expression of CMTM6 had a poorer PFS. CMTM6 could therefore serve as an independent risk factor to predict patient outcome. At the mRNA expression level, Mamessier *et al.* found that a CMTM6-high group had a shorter overall survival than a CMTM6-low group in pancreatic adenocarcinomas. They also found that CMTM6-high and PD-L1-high groups were associated with better metastasis-free survival in triple-negative PBC and that the CMTM6 expression enhanced the prognostic value of PD-L1 expression (24). Furthermore, some studies reported that individuals diagnosed with non-small cell lung cancer and hepatocellular carcinoma who showed a high expression of CMTM6 had better overall survival (22,25). On the other hand, some studies have provided evidence that a high expression of CMTM6 is associated with poor prognosis in gliomas and gastric cancer (26,27). Tumor heterogeneity and ethnic differences may be responsible for these conflicting results, and a larger cohort of a specific cancer type based on multicenter studies is recommended.

This study has several limitations. Firstly, this is a retrospective study with a relatively small sample size. A larger cohort of TNBC patients and multicenter studies are recommended for future studies. Secondly, some cases were from biopsy samples that showed few tumor-infiltrating immune cells, which may explain the lack of significant correlation between the CMTM6 and PD-L1 protein expression in primary TNBC. Moreover, it was not possible to evaluate the PD-L1 expression in cancer cells. Thirdly, the exact mechanism of CMTM6 regulation in TNBC remains unclear and requires further investigation.

In conclusion, and to the best of our knowledge, this is the first study that has reported a higher expression of CMTM6 in triple-negative compared to HER2-

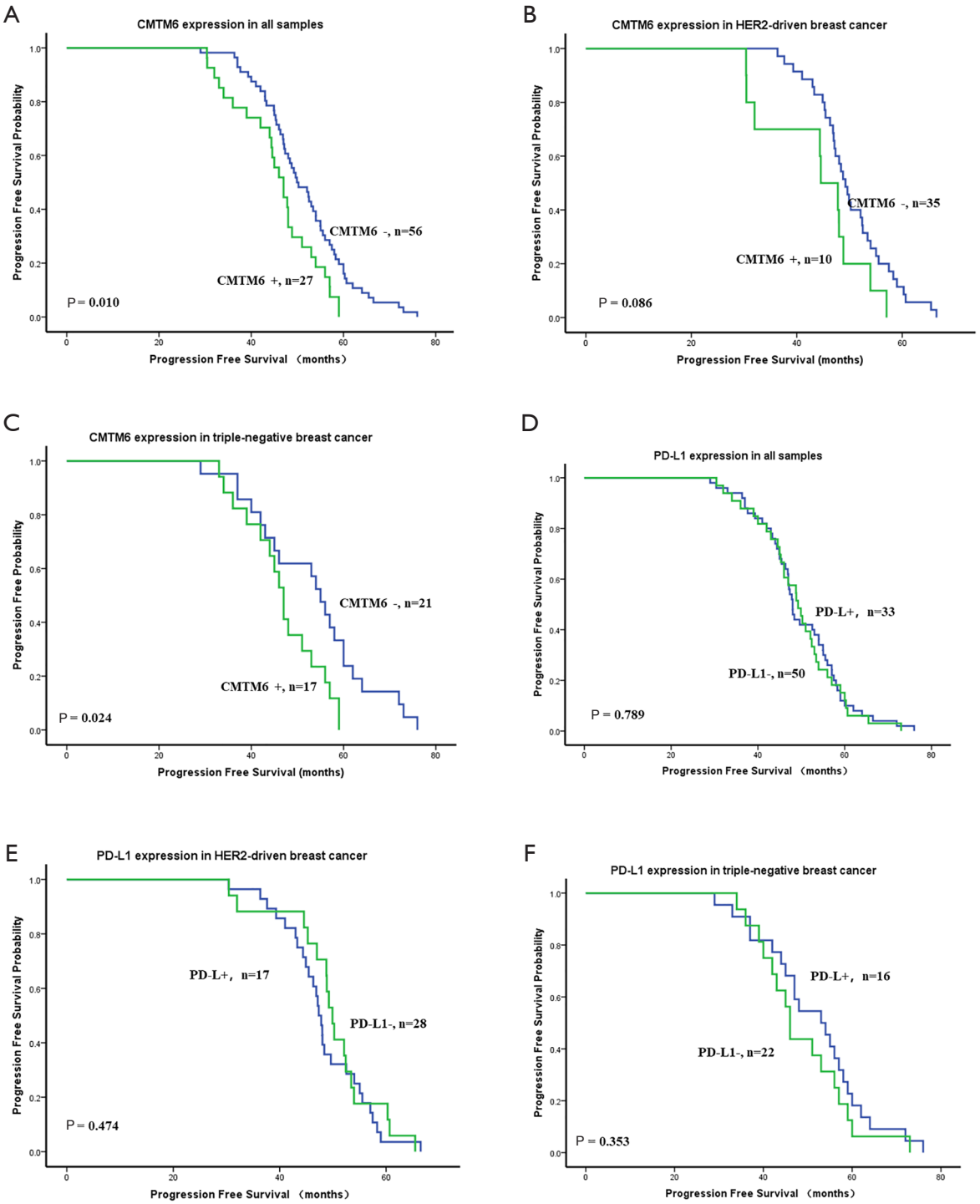

Figure 2 The Kaplan-Meier survival analysis of CMTM6 and PD-L1 expression from 83 primary breast cancers (A,D), 45 primary HER-2 driven breast cancers (B,E), and 38 primary triple-negative breast cancers (C,F). P values for PFS are determined with the log-rank test. N, number of patients.

driven breast cancer. The expression of CMTM6 was correlated with PD-L1 in TNBC and could be used as an independent risk factor to predict PFS. The results from this study suggest that CMTM6 may be able to guide prognosis and would become a new potential biomarker of immunotherapies as a supplement to PD-L1 expression in TNBC.

Acknowledgments

Funding: This study was supported by the Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (2018KY030) and the Natural Science Foundation of Zhejiang Province (LQ21H260001).

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-7616

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-7616

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-7616). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the ethics committee of Zhejiang Cancer Hospital (No. IRB-2020-275). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Because of the retrospective nature of the research, the requirement for informed consent was waived.

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(English Language Editor: D. Fitzgerald)

Cite this article as: Tian Y, Sun X, Cheng G, Ji E, Yang S, Feng J, Zheng L. The association of CMTM6 expression with prognosis and PD-L1 expression in triple-negative breast cancer. Ann Transl Med 2021;9(2):131. doi: 10.21037/atm-20-7616

