ORIGINAL

Impact of CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) expression in gastric cancer

Masaaki Nishi, Mitsuo Shimada, Kozo Yoshikawa, Jun Higashijima, Takuya Tokunaga, Hideya Kashihara, Chie Takasu, Shohei Eto, and Toshiaki Yoshimoto

Department of Surgery, University of Tokushima Graduate School, Tokushima, Japan

Abstract : Background : CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) is the master regulator of programmed cell death-ligand 1 (PD-L1). We aimed to clarify the significance of CMTM6 expression in gastric cancer (GC). Methods : A total of 105 patients who had undergone curative surgical resection for stage II/III GC at Tokushima University Hospital were included in this study. The expression of CMTM6 was examined by immunohistochemistry. Additionally, the relationship of each expression level to several prognostic factors was examined using univariate and multivariate analyses. Results : CMTM6 was not positively correlated with any of the factors examined. The overall survival (OS) rates were significantly poorer in the CMTM6 high-expression group than in the CMTM low-expression group (5-year OS : 57.2% *vs.* 79.2%, respectively ; $p<0.05$). Disease-free survival (DFS) was significantly poorer in the CMTM high-expression group than in the CMTM6 low-expression group (5-year DFS : 52.8% *vs.* 72.4%, respectively ; $p<0.05$). Multivariate analysis confirmed CMTM6 expression as an independent prognostic factor in DFS ($p<0.05$). CMTM6 expression tended to be correlated with PD-L1 expression ($p=0.07$). Conclusions : CMTM6 is associated with a poor prognosis and immunotolerance through PD-L1 in GC. J. Med. Invest. 68 : 362-367, August, 2021

Keywords **:** *Gastric cancer, CMTM6, PD-L1*

BACKGROUND

Recent refinements in the investigation of tumor immunity and tumor microenvironments have led to an increasing number of advanced cancer treatments. Since the advent of the clinical use of immune-checkpoint inhibitors, the treatment strategies for advanced solid tumors have been changing. Blockade of the programmed cell death protein 1 (PD-1)/ PD-L1 : programmed cell death-ligand 1 (PD-L1) interaction is crucial to the inhibitory axis in the suppression of tumor-specific T-cell responses. We previously reported that PD-1 and PD-L1 expression are significant prognostic factors in gastric cancer (GC) patients after curative resection (1).

CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) belongs to the CMTM family (CMTM1–8) and is expressed at the cell membrane of various types of cells (2). Recently, CMTM6 was identified as a master regulator of the PD-L1 protein. CMTM6 binds PD-L1 and maintains its cell surface expression. In the absence of CMTM6, endocytosed PD-L1 is rerouted for lysosomal degradation (3, 4). Only a few reports have investigated the clinical significance of CMTM6 expression in malignancies, including lung cancer and hepatocellular carcinoma (5-8).

We aimed to clarify the significance of CMTM6 expression and analyze the relationship between CMTM6 and PD-L1 expression in patients with gastric cancer (GC).

PATIENTS AND METHODS

Patients

A total of 105 patients who had undergone surgical resection for stage II/III gastric cancer at Tokushima University Hospital between 2006 and 2012 were included in this study (stage II/III : 42/63). Patients' characteristics are shown in the Table 1. The study included 75 men and 30 women, with a mean age of 67.8 years (range, 38–95 years). The mean follow-up period

Table 1. Patients' characteristics

Variables	n = 105
Age	68 ± 13
Sex (Male/Female)	75/30
Differentiation (Dif/Undif)	52/53
Number of Lymph node mets, N (≤5/≥6)	60/45
Type (1/2/3/4)	10/24/62/9
Location (U/M/L)	23/39/43
Tumor size (<5/≥5 cm)	50/55
CEA (<5/≥ 5 ng/ml)	91/14
CA19-9 (<37/≥37 IU/ml)	81/24
DG/TG	54/51
Operative time (min)	298 ± 76

DG : distal gastrectomy, TG : total gastrectomy

Abbreviations :
CKLF-like MARVEL transmembrane domain containing 6 : CMTM6, PD-1 : programmed cell death protein 1, PD-L1 : programmed cell death-ligand 1, GC : gastric cancer, OS : overall survival, DFS : disease-free survival

Received for publication April 6, 2021 ; accepted August 22, 2021.

Address correspondence and reprint requests to Masaaki Nishi, MD, PhD, F.A.C.S, Department of Surgery Tokushima University, 3-18-15 Kuramoto-cho, Tokushima, 770-8503 Japan and Fax : +81-88-631-9698.

was 34 months (range, 7–87 months). Seventy-three patients had received adjuvant chemotherapy following standard guidelines. The characteristic factors were defined according to the 15th edition of the Japanese Classification of Gastric Carcinoma published by the Japanese Gastric Cancer Association (9). This study was authorized in advance by the Institutional Review Board of Tokushima University, and all the patients provided written informed consent (No. 2901). These patients were also included in our previous study (1).

Immunohistochemistry

Immunohistochemistry was performed as previously described (8). The tissue samples were formalin-fixed and paraffin-embedded. Serial sections were cut at 5-μm intervals, dewaxed, deparaffinized in xylene, and rehydrated through a series of graded alcohols. The samples were boiled for 20 min in a microwave oven in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidases were blocked with 0.3% hydrogen peroxidase for 30 min, followed by incubation in 5% goat serum for 60 min to prevent nonspecific antigen binding. The slides were then incubated with primary antibodies overnight at 4°C. The following primary antibodies and dilutions were used : mouse monoclonal antibody for CMTM6 (1 : 100 ; Thermo Fisher Scientific ; PA5-34744). Secondary antibody binding was detected using the Envision Dual Link System-HRP (DAKO). A secondary peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins was applied for 60 min. The sections were developed in 3,3′-diaminobenzidine and counterstained with Mayer's hematoxylin. Each slide was dehydrated through graded alcohols and covered with a coverslip. The presence of positive cells on each slide was determined by a pathologist in a blinded manner. CMTM6 expression was predominantly located in the cytoplasm at the invasive front of the tumor. The staining intensity was graded as follows : 0 = no staining, 1+ = weak staining, 2+ = moderate staining and 3+ = strong staining. The proportion scores were as follows : 0, none ; 1, <10% ; 2, 10%–50% ; 3, 51%–80% ; 4, >80%. A total score greater than 4+ was defined as CMTM6-positive expression (Figure 1). Sixty-five patients (61.9%) were CMTM6 positive. We previously evaluated PD-L1 expression in gastric cancer tissue (1). Correlation between the CMTM6 expression and PD-L1 expression were evaluated.

Statistical analysis

All statistical analyses were performed using JMP 8.0.1 statistical software (SAS, Cary, NC, USA). Continuous variables were compared using the Mann–Whitney U-test, and categorical data were tested using χ^2 test. Survival curves were calculated using the Kaplan–Meier method and were compared using the log-rank test. The prognostic potentials of the parameters were analyzed by univariate analysis. Relative risk and 95% confidence intervals (CI) were estimated using the Cox proportional hazards model with stepwise forward selection. Statistical significance was defined as $p<0.05$.

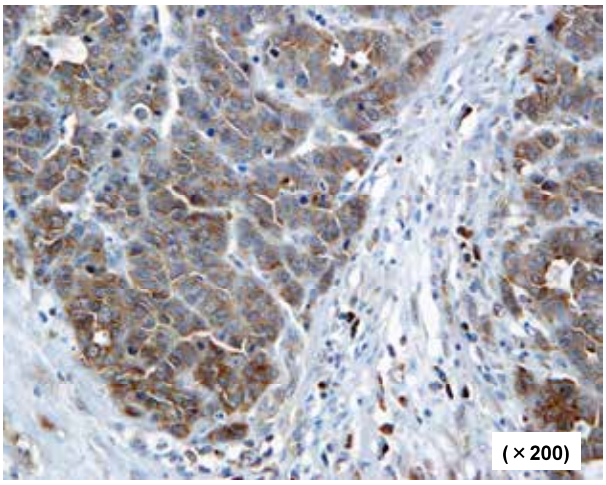

Figure 1. Immunohistochemistry of CMTM6 in gastric cancer tissue.

RESULTS

The characteristics of the CMTM6-positive and -negative groups are shown in Table 2. Regarding the clinicopathological variables, CMTM6 expression was not positively correlated with any clinicopathological factor investigated.

Table 2. Characteristics of CMTM6-positive and CMTM6-negative patients

Variables	Positive (n = 65)	Negative (n = 40)	*p* Value
Age	68 ± 12	67 ± 14	0.64
Sex (Male/Female)	42/23	33/7	0.06
Differentiation (Dif/Undif)	34/31	18/22	0.53
Number of Lymph node mets, N (≤5/≥6)	35/30	25//15	0.38
Type (1/2/3/4)	3/16/40/6	7/8/22/3	0.14
Location (U/M/L)	14/26/25	9/13/18	0.56
Tumor size (<5/≥5 cm)	27/38	23/17	0.09
CEA (<5/≥5 ng/ml)	55/10	36/4	0.43
CA19-9 (<37/≥37 IU/ml)	50/15	31/9	0.95
DG/TG	35/30	19/21	0.53
Operative time (min)	296 ± 80	300 ± 68	0.75

DG : distal gastrectomy, TG : total gastrectomy

The overall survival (OS) rates were significantly poorer in the CMTM6-positive group than in the CMTM6-negative group (5-year OS : 57.2% *vs.* 79.2%, respectively ; $p<0.05$) (Figure 2a). Univariate analysis identified T factor, the number of positive lymph node metastases, the CA19-9 level, and CMTM6 expression as significant prognostic factors for OS ($p<0.05$). Multivariate analysis confirmed the number of positive lymph node metastases as an independent prognostic factor (relative risk : 2.44 ; $p<0.05$). CMTM tended to be an independent prognostic factor (relative risk : 2.47 ; $p<0.06$) (Table 3).

Disease-free survival (DFS) was significantly poorer in the CMTM6-positive group than in the CMTM6-negative group (5-year DFS : 52.8% *vs.* 72.4%, respectively ; $p<0.05$) (Figure 2b). Univariate analysis identified that the number of positive lymph node metastases, CA19-9 level, and CMTM6 expression are significant prognostic factors for DFS ($p<0.05$). Multivariate analysis confirmed that the number of positive lymph node metastases and CMTM6 expression are independent risk factors for recurrence ($p<0.05$) (Table 4). Additionally, both the CMTM6- and PD-L1-positive expression groups (n=20) had a poorer prognosis than the double-negative expression groups in OS ($p<0.05$) (Figure 3a) and DFS ($p<0.05$) (Figure 3b).

CMTM6 expression tended to be positively correlated with PD-L1 expression ($p=0.07$) (Figure 4).

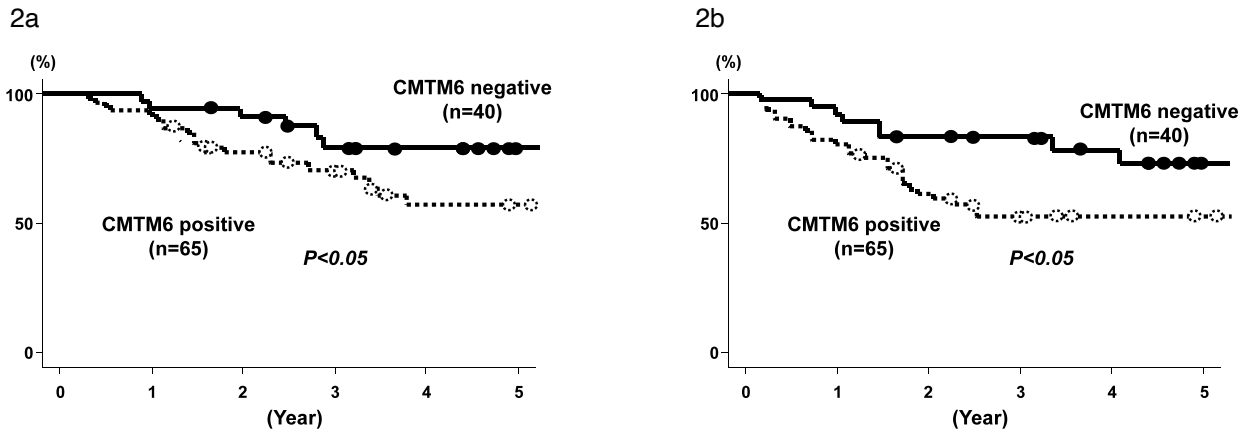

Figure 2. Kaplan–Meier analysis of OS and DFS for CMTM6 (a, b).

Table 3. Univariate and multivariate analysis for OS

Variables	Univariate		Multivariate	
	5-year OS rate (%)	*p*-Value	HR (95% CI)	*p*-Value
Age in years (<70/≥70)	71.5/61.2	0.17		
Sex (Male/Female)	59.2/82.3	0.08	2.03 (0.67–6.13)	0.21
Dif/Undif	64.9/67.5	0.76		
T 1–3/4	76.0/45.0	<0.05	2.24 (0.98–5.13)	0.06
Number of Lymph node mets, N (≤5/≥6)	75.6/51.6	<0.05	2.44 (1.05–5.65)	<0.05
fStage II / III	75.4/60.4	0.18		
Tumor size (<5/≥5 cm)	70.2/61.7	0.59		
CEA (<5/≥5 ng/ml)	68.9/58.4	0.19		
CA19-9 (<37/≥37 IU/ml)	74.2/45.0	<0.05	2.47 (0.95–4.49)	0.11
DG/TG	68.8/63.0	0.68		
CMTM6 (-/+)	79.2/57.2	<0.05	2.47 (0.97–6.21)	0.06

DG : distal gastrectomy, TG : total gastrectomy

Table 4. Univariate and multivariate analysis for DFS

Variables	Univariate		Multivariate	
	5-year OS rate (%)	*p*-Value	HR (95% CI)	*p*-Value
Age in years (<70/≥70)	69.4/48.3	0.08	1.65 (0.78–3.47)	0.19
Sex (Male/Female)	52.6/75.6	0.08	0.62 (0.29–1.60)	0.32
Dif/Undif	60.3/59.6	0.73		
T 1–3/4	63.9/51.1	0.13		
Number of Lymph node mets, (≤5/≥6)	71.6/41.3	<0.05	2.43 (1.16–5.10)	<0.05
fStage II/III	68.1/53.7	0.14		
Tumor size (<5/≥5 cm)	65.5/49.9	0.18		
CEA (<5/≥5 ng/ml)	61.1/53.9	0.24		
CA19-9 (<37/≥37 IU/ml)	64.7/42.3	<0.05	1.79 (0.86–3.73)	0.12
DG/TG	58.4/61.4	0.82		
CMTM6 (-/+)	72.4/52.8	<0.05	2.22 (0.99–5.00)	<0.05

DG : distal gastrectomy, TG : total gastrectomy

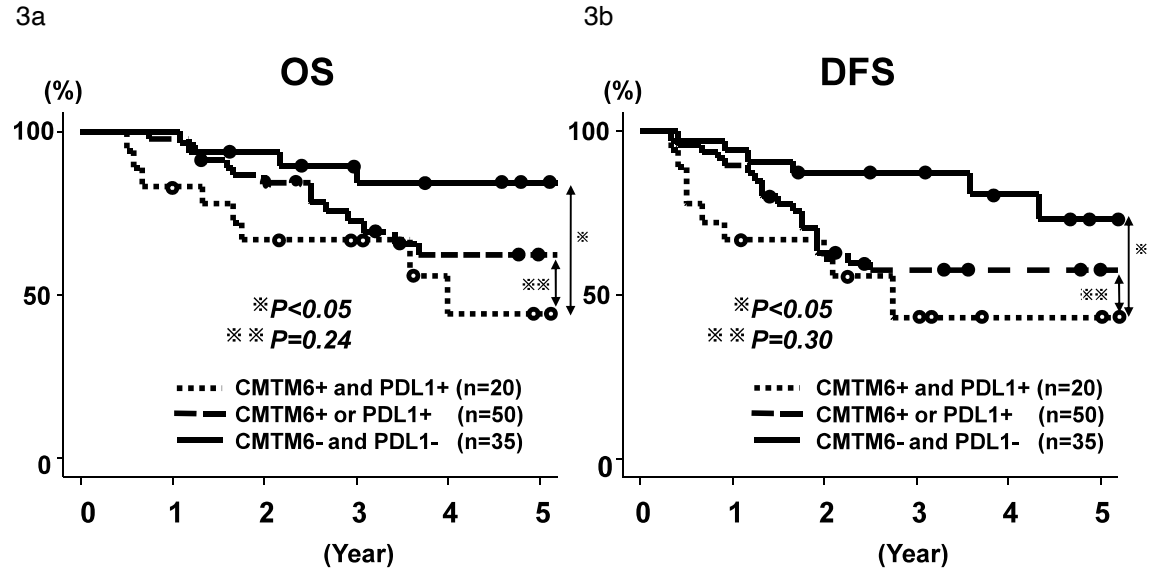

Figure 3. Kaplan–Meier analysis of OS and DFS for Survival for double CMTM6 and PD-L1 expression (a, b).

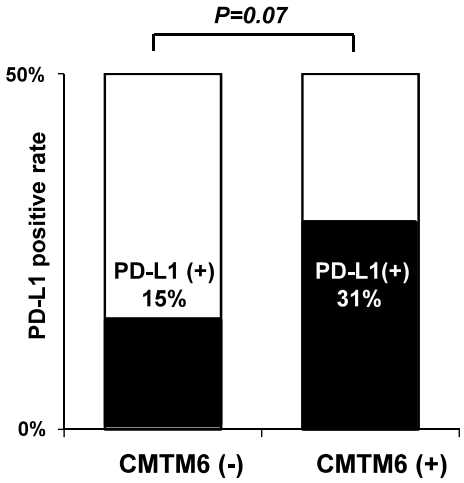

Figure 4. Correlation between CMTM6 and PD-L1.

DISCUSSION

The results of this study demonstrated that CMTM6 expression is a marker for poor prognosis in terms of both OS and DFS, and CMTM6 expression tended to correlate with PD-L1 expression in patients with stage II/III gastric cancer after curative resection.

CMTM comprises eight subtypes (CMTM1–8). CMTM shows broad-spectrum chemotactic activity and plays important roles in the hematopoietic, immune, cardiovascular, and male reproductive systems (10-14). CMTM6 was identified as a master regulator of PD-L1 in 2017. CMTM6 directly or indirectly modifies one of the lysines in the PD-L1 cytoplasmic domain(3, 4). The T-cell inhibitory capacity of PD-L1-expressing tumor cells is enhanced by CMTM6. CMTM6 shows significant specificity for PD-L1. CMTM6 is a therapeutic target that enhances the effectiveness of current immunotherapy : PD-L1/PD-1 blocking therapies (3, 4).

To our best knowledge, this is the first report concerning CMTM6 in GC. Only a few reports have investigated CMTM6 in other solid malignancies (5-8). In lung adenocarcinoma, CMTM6 expression was positively correlated with PD-L1 in both the mRNA and protein levels. Furthermore, CMTM6 expression was positively correlated with immune-infiltrating cells, resting dendritic cells, eosinophils, M1 or M2 macrophages, neutrophils, and CD4 T cells (5). Kan demonstrated that CMTM6 could predict the clinical response to PD-1 inhibitors (6). In hepatocellular carcinoma, the survival time of the patients was different between the CMTM6-positive and -negative groups. Additionally, the downregulation of CMTM6 is related to distant metastasis (7).

CMTM6 controls PD-L1 expression (3, 4). We previously reported PD-1 expression in gastric cancer patients and that PD-L1 expression or Indoleamine 2, 3-dioxygenase (IDO) correlated with a poor prognosis in gastric cancer after curative resection (1, 15). Furthermore, PD-1 expression was correlated

with PD-L1 expression (1). The correlations between CMTM6 and PD-1, IDO, FoxP3, and TGFβ were investigated in the present study. However, no significant correlations were observed (data not shown).

Immunogenic agents targeting T-cell immune checkpoints, such as PD-1, PD-L1, and cytotoxic T lymphocyte-associated antigen-4, are currently being applied in the treatment of several types of cancers (16-18). Nivolumab was adapted for unresectable or recurrent GC. However, the Attraction 2 trial revealed a median overall survival of 5.26 months and a 12-month overall survival rate of 26.2% in the nivolumab group (19). Koh showed that CMTM6 is an independent predictor of the response to PD-1 inhibitors. CMTM6 expression can be a promising predictor useful for therapeutic decision-making regarding PD-1 inhibitors. (20)

In conclusion, CMTM6 expression is associated with a poor prognosis in patients with GC. Thus, CMTM6 expression may represent a useful new therapeutic target for GC treatment.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

MN analyzed and interpreted the patient data regarding the gastric cancer after curative resection. CT and HK performed the histological examination. MN and MS were major contributors in the writing of the manuscript. TT, KY, JH, SE and TY contributed to data interpretation. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGEMENTS

We thank Nicole Okoh, PhD, from Edanz Group (https://en-authorservices.edanzgroup.com/) for editing a draft of this manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed in studies involving human participants were completed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent : Informed consent was obtained from all individual participants included in the study.

REFERENCES

1. Eto S, Yoshikawa K, Nishi M, Higashijima J, Tokunaga T, Nakao T, Kashihara H, Takasu C, Iwata T, Shimada M : Programmed cell death protein 1 expression is an independent prognostic factor in gastric cancer after curative resection. Gastric Cancer 19(2) : 466-471, 2016
2. Han W, Ding P, Xu M, Wang L, Rui M, Shi S, Liu Y, Zheng Y, Chen Y, Yang T, Ma D : Identification of eight genes encoding chemokine-like factor superfamily members 1-8 (CKLFSF1-8) by in silico cloning and experimental validation. Genomics 81(6) : 609-17, 2003
3. Mezzadra R, Sun C, Jae LT, Gomez-Eerland R, de Vries E, Wu W, Logtenberg MEW, Slagter M, Rozeman EA, Hofland I, Broeks A, Horlings HM, Wessels LFA, Blank CU, Xiao Y, Heck AJR, Borst J, Brummelkamp TR, Schumacher TNM : Identification of CMTM6 and CMTM4 as PD-L1 protein regulators. Nature 549(7670) : 106-110, 2017
4. Burr ML, Sparbier CE, Chan YC, Williamson JC, Woods K, Beavis PA, Lam EYN, Henderson MA, Bell CC, Stolzenburg S, Gilan O, Bloor S, Noori T, Morgens DW, Bassik MC, Neeson PJ, Behren A, Darcy PK, Dawson SJ, Voskoboinik I, Trapani JA, Cebon J, Lehner PJ, Dawson MA : CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity. Nature 549(7670) : 101-105, 2017
5. Wang H, Gao J, Zhang R, Li M, Peng Z, Wang H : Molecular and immune characteristics for lung adenocarcinoma patients with CMTM6 overexpression. Int Immunopharmacol 83 : 106478, 2020
6. Gao F, Chen J, Wang J, Li P, Wu S, Wang J, Ji Y : CMTM6, the newly identified PD-L1 regulator, correlates with PD-L1 expression in lung cancers. Biochem Biophys Rep 20 : 100690, 2019
7. Zhu X, Qi G, Li C, Bei C, Tan C, Zhang Y, Shi W, Zeng W, Kong J, Fu Y, Tan S : Expression and Clinical Significance of CMTM6 in Hepatocellular Carcinoma. DNA Cell Biol 38(2) : 193-197, 2019
8. Guan X, Zhang C, Zhao J, Sun G, Song Q, Jia W : CMTM6 overexpression is associated with molecular and clinical characteristics of malignancy and predicts poor prognosis in gliomas. EBioMedicine 35 : 233-243, 2018
9. Japanese classification of gastric carcinoma : 5th English edition. Kanahara 2017.
10. Zhang M, Xu Y, Liu Y, Cheng Y, Zhao P, Liu H, Wang Y, Ma X : Chemokine-Like Factor 1 (CKLF-1) is Overexpressed in Keloid Patients : A Potential Indicating Factor for Keloid-Predisposed Individuals. Medicine (Baltimore) 95(11) : e3082, 2016
11. Ihsan C, Laura LM, Maarten B, Christian GM van D, Petra B, Changbin Z, Johan M K, Dirk J D, Caroline C : CMTM3 (CKLF-Like Marvel Transmembrane Domain 3) Mediates Angiogenesis by Regulating Cell Surface Availability of VE-Cadherin in Endothelial Adherens Junctions. Arteriosclerosis, thrombosis, and vascular biology 37 : 1098-1114, 2017
12. Li G, Li GY, Ji HJ, Zhao WJ, Chu SF, Chen NH : [Effect of testosterone on the expression of CMTM family of the male spermatogenesis suppression rats]. Yao Xue Xue Bao 45(8) : 995-1000, 2010
13. Li T, Han W, Yang T, Ding P, Rui M, Liu D, Wang Y, Ma D : Molecular cloning and identification of mouse Cklfsf2a and Cklfsf2b, two homologues of human CKLFSF2. Int J Biochem Cell Biol 38(3) : 420-9, 2006
14. Zhang JW, Liu TF, Chen XH, Liang WY, Feng XR, Wang L, Fu SW, McCaffrey TA, Liu ML : Validation of aspirin response-related transcripts in patients with coronary artery

disease and preliminary investigation on CMTM5 function. Gene 15 ; 624 : 56-65, 2017
15. Nishi M, Yoshikawa K, Higashijima J, Tokunaga T, Kashihara H, Takasu C, Ishikawa D, Wada Y, Shimada M : The Impact of Indoleamine 2,3-dioxygenase (IDO) Expression on Stage III Gastric Cancer. Anticancer Res 38(6) : 3387-3392, 2018
16. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M : Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366(26) : 2443-54, 2012
17. Schlößer HA, Drebber U, Kloth M, Thelen M, Rothschild SI, Haase S, Garcia-Marquez M, Wennhold K, Berlth F, Urbanski A, Alakus H, Schauss A, Shimabukuro-Vornhagen A, Theurich S, Warnecke-Ebertz U, Stippel DL, Zippelius A, Büttner R, Hallek M, Hölscher AH, Zander T, Mönig SP, von Bergwelt-Baildon M : Immune checkpoints programmed death 1 ligand 1 and cytotoxic T lymphocyte associated molecule 4 in gastric adenocarcinoma. Oncoimmunology 9 ; 5(5) : e1100789, 2015
18. Gibney GT, Weiner LM, Atkins MB : Predictive biomarkers for checkpoint inhibitor-based immunotherapy. Lancet Oncol 17(12) : e542-e551, 2016
19. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yeh KH, Yoshikawa T, Oh SC, Bai LY, Tamura T, Lee KW, Hamamoto Y, Kim JG, Chin K, Oh DY, Minashi K, Cho JY, Tsuda M, Chen LT : Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2) : a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 390(10111) : 2461-2471, 2017
20. Koh YW, Han JH, Haam S, Jung J, Lee HW : Increased CMTM6 can predict the clinical response to PD-1 inhibitors in non-small cell lung cancer patients. Oncoimmunology Jun 14 ; 8(10) : e1629261, 2019