Research Communication

CMTM6 expression as a potential biomarker for immunotherapy in metastatic renal cell carcinoma

The introduction of immune checkpoint inhibitors (ICIs) has revolutionized the treatment of metastatic RCC (mRCC) [1]. The expression of programmed death-ligand 1 (PD-L1), as assessed by immunohistochemistry (IHC), has been extensively studied as a predictive biomarker for patients receiving ICIs. However, results in clinical RCC trials concerning the predictive value of PD-L1 expression were highly controversial [2]. In the Checkmate 214 trial [3], patients with PD-L1 positivity were found to benefit most from ICI combination. By contrast, PD-L1 expression was not found to be predictive for nivolumab treatment in the second-line setting [4]. Thus, all ICIs (combination and monotherapy) were approved in mRCC, irrespective of PD-L1 status.

Expression of PD-L1 poses several disadvantages, including intratumoural heterogeneity between primary RCC and metastases or tumour cells (TC) and tumour-infiltrating immune cells (IC). Moreover, PD-L1 expression is highly dynamic and can be influenced by prior antiangiogenic treatments [1,2]. A possible explanation for this is the fact that the tumour microenvironment is highly heterogenous with the limitation having only a snapshot using single-core biopsies. Finally, several PD-L1 IHC assays using different thresholds are currently available, causing inter-assay variability and differences in sensitivity and specificity [1,2]. Thus, PD-L1 expression within the tumour microenvironment is not yet a reproducible and robust biomarker in clinical practice.

Post-translational modifications are an important mechanism for regulating PD-L1 expression [5]. Recently, the human chemokine-like factor (CKLF)-like MARVEL transmembrane domain containing family member 6 (CMTM6) has been identified as a key regulator of PD-L1 cell surface expression across various cancer entities. CMTM6 was shown to protect and stabilize PD-L1 via two independent mechanisms. CMTM6 promotes PD-L1 transportation to recycling endosomes, resulting in re-expression on the cell surface. Additionally, CMTM6 prevents proteasomal degradation via deubiquitination (Fig. 1A). In addition to causing T-cell dysfunction by stabilizing PD-L1 expression on the surface of tumour cells [6], secreted CMTM6 can induce M2-like macrophages, which promote tumour progression [7]. Moreover, CMTM6 may also directly affect tumour biology by enhancing the Wnt/β-catenin signalling pathway, which is essential for tumourigenesis, maintenance of cancer stem cells, and the epithelial-to-mesenchymal transition

characteristic of multiple cancer entities [8]. In RCC, the prognostic role of PD-L1 at the transcriptional level is dependent on the expression of CMTM6. In detail, high PD-L1 expression was only associated with poor survival when CMTM6 was overexpressed, but not in case of low CMTM6 expression [9]. CMTM6 may thus not only refine the prognostic value of PD-L1, but may play an independent role as a predictive factor for response to immune checkpoint inhibition [10].

After approval by our local institutional review board (Ethics Committee study number: 1202/2018), we evaluated for the first time expression of CMTM6 within the tumour microenvironment (TC and IC) of RCC and its predictive role when compared to PD-L1. The tissue was obtained from primary tumours of 16 patients receiving nivolumab after at least one prior antiangiogenic treatment. All specimens and individual tissue components were inspected and morphologically identified by one pathologist (A.B.) with a broad expertise in uropathology, who was blinded to patient outcome. Three primary antibodies were used for subtyping the tumour and the inflammatory infiltrate within the tumour microenvironment using an automated platform (VENTANA BenchMark ULTRA) to achieve optimal results. T cells were labelled using a CD3 antibody (Clone 2GV6, prediluted; Ventana Medical Systems, Tucson, AZ, USA) and immune checkpoint proteins were assessed using antibodies against PD-L1 (Clone CLA-10, prediluted; BioCare, Redditch, UK) and CMTM6 (polyclonal, dilution 1:100; Invitrogen, Thermo-Fisher, Waltham, MA, USA). CD3 staining was performed to determine the extent of infiltration, especially in the setting of a prominent fibrosing reaction around the tumour, where scattered lymphocytes may be difficult to assess. All IC, identified either by CD3 IHC or morphology, were taken into account when evaluating the stainings. Because the number of patients included in this preliminary analysis was small, differences in expression within different IC compartments (i.e. lymphocytes, macrophages) were only observed and not quantified. However, the extent of infiltration in and around the tumour can vary from only scattered lymphocytes to large aggregates, and the percentage of positively stained cells. As the overall extent of infiltration might play a role in response to treatment, percentage of positively stained cells alone could distort the results. Therefore, the extent of CMTM6 and PD-L1 expression on IC was assessed as a score built of the quantity of infiltration (1 = weak vs 2 = strong), evaluated with CD3 IHC multiplied by the extent of CMTM6

expression on IC (0 = no expression, 1 = <1% positive cells, 2 = 1–5% positive cells and 3 = >5% positive cells). The intensity of staining and the percentage of positively stained tumour cells within a representative slide were evaluated to quantify the extent of CMTM6 and PD-L1 expression on TC. A score was built by multiplying the extent of expression (0 = no expression, 1 = <1% positive cells, 2 = 1–5% positive cells and 3 = >5% positive cells) by intensity of expression (0 = negative, 1 = weak and 2 = strong). Both scores, ranging from 0 to 6, take into account the percentage of positively stained cells related to the total amount of IC or TC and thus should be comparable.

For statistical analysis, baseline characteristics and expressions levels of IHC markers were compared using Student's t-test. Correlations between variables were assessed with Spearman's P correlation coefficient (r_s). The median CMTM6 and PD-L1 score on IC and TC was used as a cut-off (score 3) to dichotomize patients into two groups (low vs high) for Kaplan–Meier survival analysis and comparison by the log-rank test. A significance level of $\alpha = 0.05$ (two-tailed) was applied for all P values. SPSS, version 22.0 (SPPS Inc., Chicago, IL, USA) was used for statistical analysis. Graphic diagrams were produced with GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All values are presented as means ± SD.

Sixteen patients (eight women and eight men) with metastatic RCC who have progressed after prior treatment with at least one vascular endothelial growth factor receptor-targeting tyrosine kinase inhibitor were enrolled in this study. Histology of primary RCC confirmed clear-cell RCC in 15 patients (93.8%) and chromophobe RCC in one patient. International Society of Urological Pathology tumour grading showed grade 1/2 in seven patients (43.7%), and grade 3/4 in nine patients (56.3%). The mean (range) age was 66.5 (52–78) years. All eligible patients had a Karnofsky performance status score of ≥70%, had adequate organ and marrow function, and received antiangiogenic tyrosine kinase inhibitor monotherapy in the first-line setting, including nine patients (56.4%) with pazopanib and seven (43.6%) with sunitinib. Most patients ($n = 11$, 68.8%) were treated with nivolumab as a second-line regimen, whereas five patients (32.2%) had two ($n = 2$) or three ($n = 3$) prior antiangiogenic therapy regimens. Approximately two-thirds of patients (62.5%) were assigned to an intermediate/poor International Metastatic RCC Database Consortium (IMDC) risk group and had more than four metastases at the beginning of nivolumab treatment. The mean (range) durations of progression-free survival (PFS) and overall survival (OS) during nivolumab treatment were 12.8 (1–35) and 27 (6–43) months, respectively.

We found high intratumoural heterogeneity for both CMTM6 and PD-L1 expression (extent), with a significantly higher expression of both markers on IC compared to TC (CMTM6: $P < 0.001$; PD-L1: $P = 0.008$ [Fig. 1B,C]). Focusing on IC, CMTM6 expression was significantly higher compared to PD-L1 expression ($P = 0.0016$ for extent; $P = 0.03$ for score [Fig. 1D]). One possible explanation for these observations could be the presence of CMTM6 in both lymphocytes and macrophages, whereas PD-L1 was primarily expressed on macrophages (Fig. 1E). In line with previous studies [3,4], CMTM6 expression was positively correlated with PD-L1 on TC ($r_s = 0.441$; $P = 0.047$) and IC ($r_s = 0.487$; $P = 0.057$ [Fig. 1F]). Patients with CMTM6-IC high scores (CMTM6-IC^{high}) had a significantly shorter PFS on nivolumab when compared to patients with CMTM6-IC low scores (CMTM6-IC^{low}; median, low vs high: 24 vs 7 months; $P = 0.032$). No significant differences between CMTM6-IC^{high} and CMTM6-IC^{low} patients were found for OS; however, the median OS has not yet been reached (median, low vs high: not reached vs 23.5 months; $P = 0.133$ [Fig. 1G]).

Fig. 1 (A) Schematic overview of post-translational modifications of programmed death-ligand 1 (PD-L1) adapted from Motzer et al. [4]. CMTM6 is a transmembrane protein that co-localizes with PD-L1, both on the plasma membrane and in recycling endosomes. CMTM6 has been found to promote PD-L1 transportation to recycling endosome, resulting in a decreased distribution to late endosome and lysosome, thus decreasing its lysosomal degradation. Moreover, CMTM6 stabilizes PD-L1 by suppressing its ubiquitination and thus protecting PD-L1 from proteasomal degradation. **(B)** Expression levels of CMTM6 and PD-L1 (extent) stratified by cell type within the tumour microenvironment (tumour cells [TC] vs immune cells [IC]). Data represent mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ according to independent t-test). **(C)** Clear-cell RCC without CMTM6 expression in tumour cells; only occasional perivascularly located IC show CMTM6 expression (arrow; left); the same case was negative for PD-L1 in TC (right). Immunohistochemistry (IHC) for CMTM6 and PD-L1; all micrographs = magnification 20×. **(D)** IC expression of CMTM6 (extent and score) in comparison to PD-L1. Data represent mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ according to independent t-test). **(E)** Representative IHC images for CD3, CMTM6 and PD-L1 staining. Clear-cell RCC with a strong CD3$^+$ IC infiltration (upper left) with >5% of IC (lymphocytes and macrophages) expressing CMTM6 (upper middle) as well as PD-L1, which is mainly restricted to intermingled macrophages as identified morphologically (upper right). Clear-cell RCC with weak CD3$^+$ IC infiltration (lower left), expressing CMTM6 in the majority of IC (lower middle), while occasional macrophages (arrow) are positive for PD-L1 (lower right), IHC for CD3, CMTM6 and PD-L1; lower right: magnification = 40×; all other micrographs: magnification = 20×. **(F)** Spearman correlation analyses between CMTM6 and PD-L1 immunohistochemical expression on TC und IC based on TC/IC score (0–6). r_s = Spearman's rank correlation coefficient; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **(G)** Kaplan–Meier survival analysis was applied to examine the CMTM6 expression and its associations with survival. CMTM6 score (IC and TC) was dichotomized into a high and low group using the median (score 3) as cut-off level, providing a significant association between CMTM6-IC score and progression-free survival (PFS). OS, overall survival.
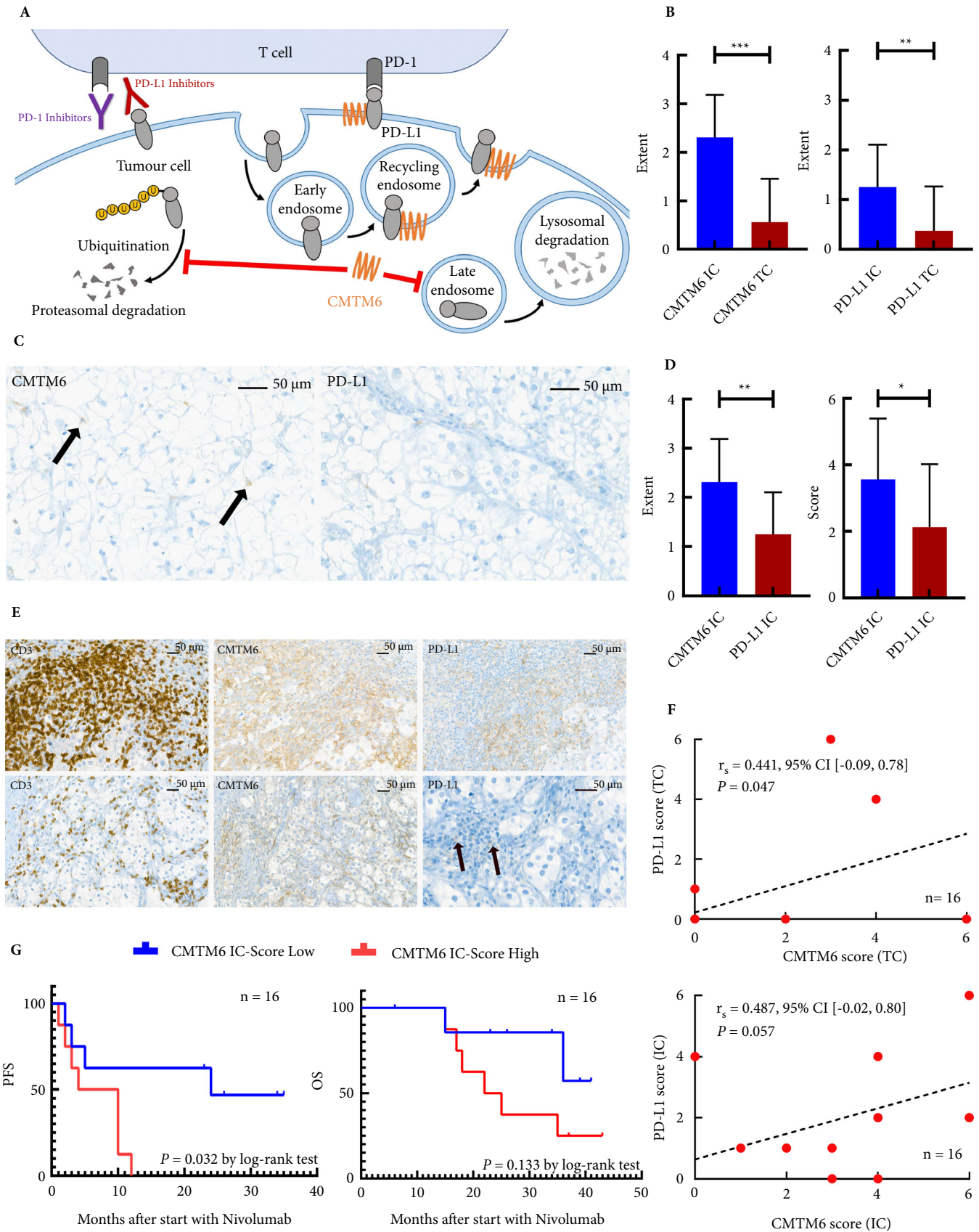
Our data suggest that CMTM6 plays an important role in the RCC tumour microenvironment. Although both markers CMTM6 and PD-L1 were more frequently expressed on IC than on TC, CMTM-IC expression was significantly higher compared to PD-L1. Moreover, in contrast to PD-L1, CMTM6-IC expression appears to predict PFS in patients receiving nivolumab. Thus, PD-L1 IHC may be supplemented by IHC measurement of CMTM6 to increase its prognostic role as a predictive biomarker.

One of the major limitations of this pilot study is the small sample size, with the study cohort having heterogeneous clinicopathological characteristics (including one patient with non-clear-cell RCC) and oncological results that were retrospectively evaluated. Thus, further research in a larger patient population is needed to confirm our preliminary findings and to define whether CMTM6 may serve as a potential target for cancer immunotherapy.

Conflicts of Interest

The authors declare no conflicts of interest.

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Abbreviations: IC, immune cells; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; mRCC, metastatic RCC; OS, overall survival; PD-L1, programmed death-ligand 1; PFS, progression-free survival; TC, tumour cells.