

Original Article

Immunomodulatory molecules in renal cell cancer: CD80 and CD86 are expressed on tumor cells

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Abstract: Despite modern therapies with tyrosine kinase inhibitors (TKI), the management of patients with metastatic renal cell carcinoma (mRCC) remains a challenge. Significant immunosuppression has been described in patients with mRCC. Therefore, immunotherapeutic strategies such as checkpoint inhibitors have been developed. To further elucidate the underlying mechanisms of immunosuppression and response to therapy, different features of the immune microenvironment (expression of HIF-1- α , VEGFR-1, FOXP3, TGF- β 1, CD80, CD86, PD-1, and PD-L1) were analyzed in tumor tissues within different subgroups of mRCC patients (responders vs. non-responders to therapy). Results: The most interesting finding was low-level CD80 and CD86-expression on tumor tissue samples (n = 18) of nearly all mRCC patients. This finding was in line with CD86 expression, which could also be found in renal carcinoma cell lines. To the best of our knowledge, this is the first report on CD80/CD86 expression in human renal cell carcinoma-possibly reflecting an immunomodulatory mechanism of the tumor.

Keywords: Renal cell carcinoma, expression, CD80, CD86

Introduction

Over the last decade, potent targeted therapies have evolved for patients with mRCC [1-6]. Sequential use of these agents has led to an improvement in progression-free survival and (partially) overall survival as compared to cytokine therapy [7-9]. However, despite the successful development of novel targeted therapies, the management of patients with mRCC remains challenging and relevant subgroups of patients are refractory to TKI treatment [10, 11]. Recently, immune checkpoint pathways have been increasingly recognized and their inhibition has been incorporated into the treatment strategies of several tumors, including mRCC [12-15]. Immune checkpoints pose an important immune evasion mechanism utilized by cancer cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD-1) protein are the key receptors on T cells

mediating inhibitory interactions [16]. CTLA-4 is solely expressed on T cells and its inhibition leads to enhanced T cell activation via increased co-stimulation by CD28 [14, 16-19]. PD-1 is an immune-checkpoint receptor mediating immunosuppression and is expressed by activated and follicular T cells, B cells, and NK-cells [20]. In tumors, these activated T cells may bind to their immunosuppressive ligands PD-L1 and PD-L2 [14], that are expressed by tumor cells and in the tumor microenvironment. PD-L1 may also be expressed on macrophages, myeloid-derived suppressor cells (MDSC), dendritic cells (DC), B and T cells [21, 22]. It has recently been shown, that blockade of PD-1 or PD-L1 has significant antitumor effects in RCC [12, 15, 23]. Multiple efforts have been made to correlate the expression of PD-L1 in tumor tissue with the clinical response to anti-PD-1 directed therapy [12, 13, 24, 25]. So far, no clear relationship could be established. However, the rele-

CD80 and CD86 expression in RCC

Table 1. Patients' characteristics

Patient	Age in years	Histologic subtype	Manifestations	Therapy	PFS in weeks
1) CR after TKI therapy					
1	55	Clear cell	Adrenal, Bone	Sunitinib	106
2	74	Clear cell	Pulmonary	Sunitinib	308
3	62	Clear cell	Pulmonary	Sorafenib	472
4	63	Clear cell	Pulmonary, Bone	Sunitinib	100
5	69	Clear cell	Pulmonary	Sunitinib	60
6	64	Clear cell	Pulmonary, Lymphogenic	Sunitinib	316
2) Aggressive disease/refractory to therapy					
1	41	Sarcomatoid/chromophob	Pulmonary, hepatic	None	8
2	54	Clear cell	Bone	Sorafenib	5
3	61	Sarcomatoid	Pulmonary, hepatic, lymphogenic	Temsirolimus	12
3) Cytokine treatment					
1	58	Clear cell	Pulmonary, Lymphogenic	Cytokines	33*
2	40	Papillary	Pulmonary, Adrenal, Spleen, Muscle, Local recurrence	Cytokines	4*
3	59	Not classified	Bone, Adrenal, Lymphogenic	Cytokines	30*
4	59	Clear cell	Pulmonary, Bone	Cytokines	109
5	62	Chromophob	Pulmonary, Lymphogenic	Cytokines	23
6	60	Clear cell	Pulmonary	Cytokines	79
7	47	Clear cell	Pulmonary	Cytokines	23

Patients (n = 16) included the following subgroups: 1) Complete remission (CR) after tyrosine kinase inhibitor (TKI) therapy: n = 6. 2) Aggressive disease/refractory to therapy: n = 3. 3) Cytokine treatment (IL-2/IFN- α /5-FU/GM-CSF): n = 7. *lost to follow-up after the weeks stated.

vance of PD-L1 expression of tumor infiltrating lymphocytes remains interesting, particularly in the context of other immunomodulatory factors in the tumor microenvironment [16, 24]. Interestingly, in mRCC PD-L1 expression on tumor cells and lymphocytes has been more clearly correlated with an adverse outcome [26-31]. To further elucidate the underlying mechanisms of immunosuppression and response to therapy, we analyzed the expression of immunomodulatory molecules on tumor tissue samples within different subgroups of mRCC patients: patients with aggressive or TKI-refractory disease, patients with TKI-responsive disease, and patients treated with cytokines, in whom a negative correlation between CD80/CD86 expression on T cells and prognosis had previously been described [32]. Different molecules associated with tumor cell proliferation (VEGFR-1, HIF-1- α) or immunomodulatory functions (CD80, CD86, PD-1, PD-L1, TGF- β 1, and FOXP3) [14, 33] were included in our histological analyses.

Material and methods

Patients

In our retrospective analysis, tissue samples (n = 18) from different subgroups of mRCC patients (n = 16) were included: 6 patients had been highly responsive to TKI therapy (complete remission under TKI therapy +/- metastasectomy), 3 patients had been refractory to TKI-therapy, and 7 patients had been treated with cytokines [32]. Clinical information as well as follow-up reports were obtained through medical record review. The following parameters were evaluated: histological subtype of the tumor, age at study entry, tumor manifestations, therapy regimen and time of progression-free survival (see **Table 1**). The study was performed in accordance with local ethical guidelines.

Immunohistological analysis

Immunohistological analyses were performed on 18 tumor tissue samples of 16 mRCC patients. Samples included nephrectomy samples (n = 13) and metastasectomy samples (n = 5). Four-micrometer-thick sections from paraffin-embedded samples were performed for immunohistological staining. The following antibodies were used:

The mouse monoclonal antibody against PD-1 (clone MRQ-22), (Zytomed Systems GmbH, Berlin, Germany) was used in a dilution of 1:50. The antibodies against PD-L1, FOXP3, HIF-1- α , TGF- β 1, CD80 and CD86 were purchased from Abcam (Abcam plc 330 Cambridge, UK). For PD-L1 detection, a rabbit monoclonal antibody was used in a dilution of 1:300 (clone EPR1161 (2)), FOXP3 (clone 236A/E7) was chosen in a dilution of 1:100, HIF-1- α (mouse monoclonal antibody clone H1 α 67) in a dilution of 1:100, CD80 (clone EP1155Y) in a dilution of 1:2000, and CD86 (clone EP1158Y) in a dilution of 1:50. VEGFR-1 (polyclonal) was used in a dilution of 1:50 from Acris Antibodies (Acris Antibodies GmbH, Herford, Germany). All these stains were performed using the Bond-Max™ device (Leica Biosystems GmbH, Wetzlar, Germany). Antigen retrieval and visualization of bound antibodies were performed employing the manufacturer's protocols and reagents (Bond Polymer Refine, DAB; Leica). The sections were dewaxed and subjected to an antigen retrieval protocol within a BenchMark Ultra (Ventana) followed by incubation with the primary antibody. Bound antibodies were visualized using the streptavidin-biotin-peroxidase method and diaminobenzidine as chromogen (UltraviewKit, also obtained from Ventana). The expression level was quantified as follows: (-) = Negative/single cells, (+) \leq 25% expression, (++) = 25-50% expression, (+++) \geq 50% expression, (n.a.) = not available.

Flow cytometric analyses

For FACS analysis, fluorochrome-labeled antibodies against CD80, CD86, and isotype controls (all BD Biosciences, Heidelberg, Germany) were used according to the manufacturer's instructions. 60.000 cells were analyzed on a BD FACSCanto using BDFACSDiva software, (BD Biosciences). Analysis was performed using Flow Jo software version 10 (FLOWJO LLC, Asland, OR, USA). Flow cytometric analyses were performed on two renal carcinoma cell lines (CAKI1-HTB46 and CAKI2-HTB47), (ATCC/LGC Standards, Wesel, Germany).

Results

Expression of immunomodulatory molecules on renal cell carcinoma

Low-level expression of CD80 was shown in 89% (16/18) of tumor tissue samples (n = 18)

CD80 and CD86 expression in RCC

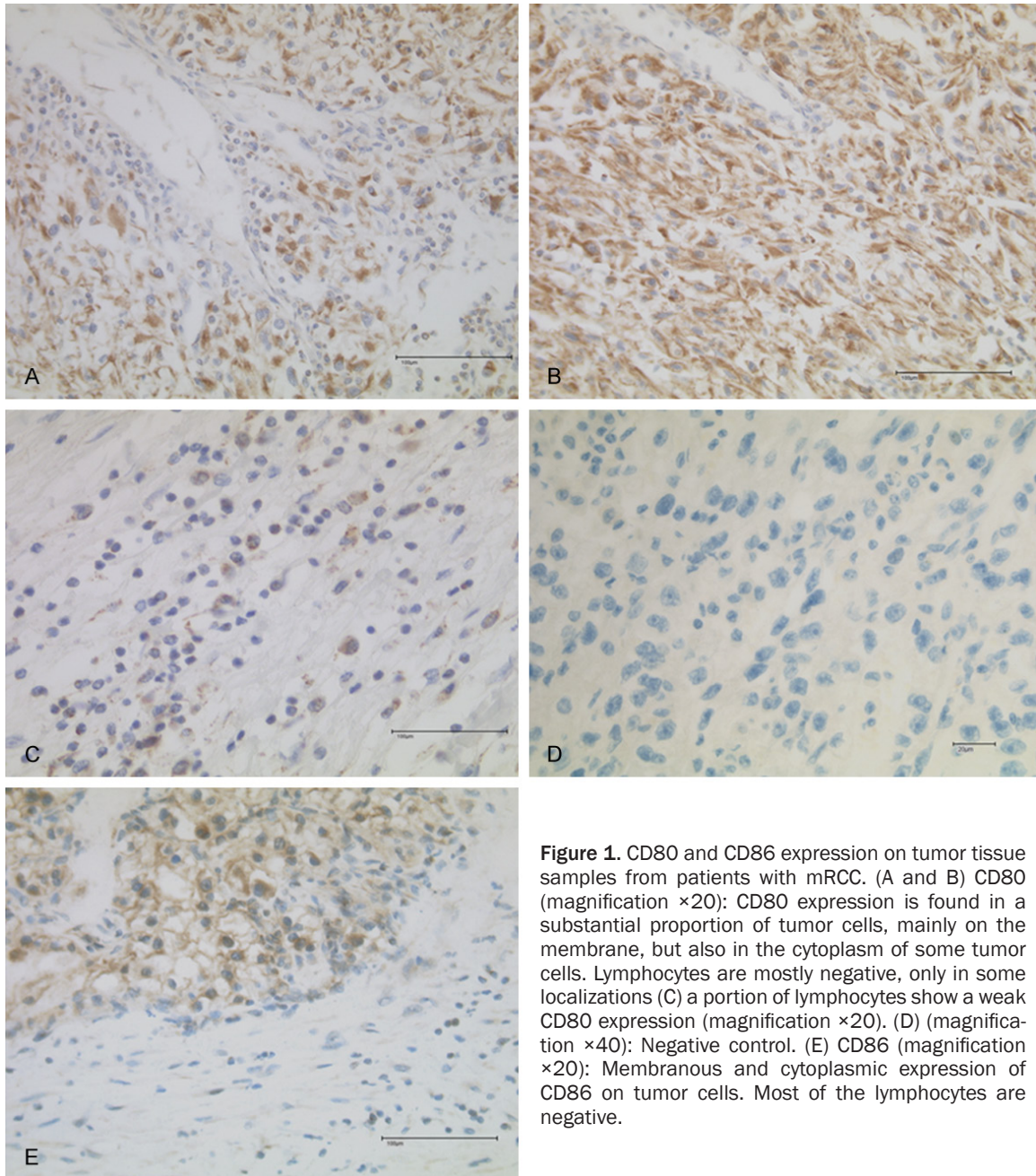


Figure 1. CD80 and CD86 expression on tumor tissue samples from patients with mRCC. (A and B) CD80 (magnification $\times 20$): CD80 expression is found in a substantial proportion of tumor cells, mainly on the membrane, but also in the cytoplasm of some tumor cells. Lymphocytes are mostly negative, only in some localizations (C) a portion of lymphocytes show a weak CD80 expression (magnification $\times 20$). (D) (magnification $\times 40$): Negative control. (E) CD86 (magnification $\times 20$): Membranous and cytoplasmic expression of CD86 on tumor cells. Most of the lymphocytes are negative.

of mRCC patients. CD80 was expressed in the cytoplasm as well as on the cell membrane of the tumor cells (**Figure 1A, 1B**). Tumor infiltrating lymphocytes only scarcely expressed CD80 (**Figure 1C, 1D**). CD86 expression could also be demonstrated in 89% (16/18) of patients' tumor tissue samples. For CD86, both cytoplasmic and membranous expression could be observed in the tumor cells. Tumor infiltrating lymphocytes were mostly negative (**Figure 1E**). In the different subgroups of mRCC patients, no significant differences were observed concerning CD86 or CD80 expression (**Table 2**).

To support the finding of CD80/CD86 expression on RCC, additional flow cytometric analyses were performed with renal carcinoma cell lines (CAKI1-HTB46 and CAKI2-HTB47). Low-level CD86 expression could be demonstrated on both cell lines, whereas CD80 was negative (**Figure 2**). Furthermore, PD-L1 expression was analyzed on the available tumor tissue samples within the different subgroups. Interestingly, 67% (2/3) of patients refractory to TKI-therapy showed PD-L1 expression on their tumors. In contrast, in the group of TKI-responsive patients, PD-L1 expression was only found in

CD80 and CD86 expression in RCC

Table 2. Expression of PD-1 and PD-L1 on samples of different subgroups of mRCC patients

Patient	CD86	CD80	PD-L1		PD-1	
	Tumor	Tumor	Tumor	Lymphocytes	Tumor	Lymphocytes
CR after TKI therapy						
1	+	++	-	-	-	-
2	+++	++	-	+	-	-
3	n.a.	++	n.a.	n.a.	n.a.	n.a.
4-1	++	++	-	-	-	-
4-2	+++	++	-	-	-	-
4-3	+++	+	-	-	-	-
5	+	+	-	-	n.a.	-
6	+++	++	-	-	-	-
Aggressive disease/refractory to therapy						
1	++	+	+	-	-	-
2	++	-	-	-	-	-
3	++	+	+	-	-	-
Cytokine treatment						
1	-	++	-	-	-	-
2	+	+	-	-	-	+
3	-	-	-	-	-	-
4	++	+	-	-	-	+
5	++	+	-	-	-	-
6	++	++	-	-	-	-
7	++	++	-	-	-	-

Expression of immunomodulatory markers was observed in tissue samples of different subgroups of mRCC patients and was quantified as follows: (-) = Negative / single cells, (+) ≤ 25% expression, (++) = 25-50% expression, (+++) ≥ 50% expression, (n.a.) = not available. The group contains three samples from one patient (patient 4). PD-1 = programmed cell death protein 1, PD-L1 = programmed death protein ligand 1.

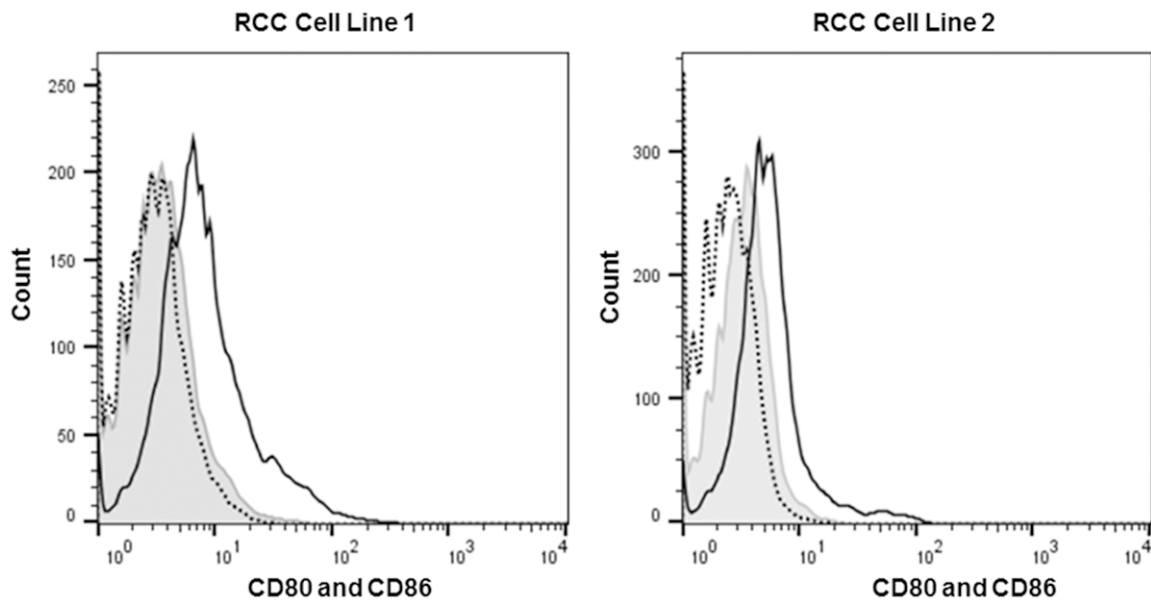


Figure 2. CD86 and CD80 expression on renal carcinoma cell lines. Flow cytometric analyses demonstrated weak CD86 expression on both RCC cell lines, whereas CD80 is not expressed (RCC cell line 1 = CAKI1-HTB46 and RCC cell line 2 = CAKI2-HTB47). (—) = CD86, (....) = CD80, (●) = Isotype control.

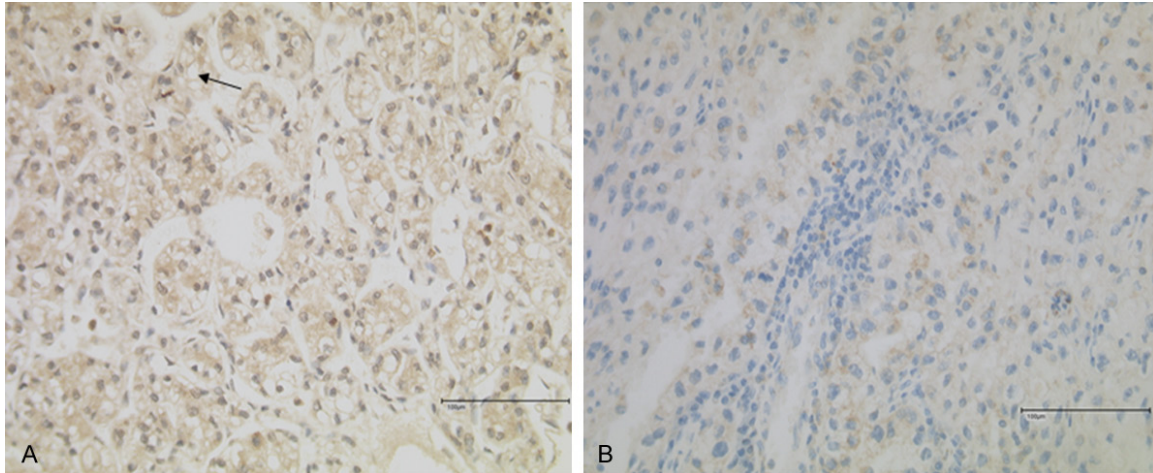


Figure 3. Expression of immunomodulatory markers on samples of different subgroups of mRCC patients. A: TGF- β 1 (magnification $\times 10$): TGF- β 1 is mostly expressed in the cytoplasm of the tumor cells but also a membranous staining could be observed (arrow). B: VEGFR-1 (magnification $\times 10$): Weak cytoplasmic expression of VEGFR-1 was observed in a portion of tumor cells. TGF- β 1 = transforming growth factor beta 1, VEGFR-1 = vascular endothelial growth factor receptor 1.

12.5% (1/8 samples) (**Table 2**). In our cohort of patients, only scarce lymphocyte infiltrations could be demonstrated in tumor tissue. Therefore, no significant PD-1 expression could be observed on tumor-infiltrating lymphocytes (TIL) in the different subgroups ($n = 18$).

Since molecules influencing tumor growth or immunological responses may be expressed both on tumor cells themselves and in the tumor microenvironment, we have also studied the expression of different other molecules that are associated with tumor progression and/or immunological tolerance. TGF- β 1 was only weakly expressed on tumor cells in samples of TKI-responsive patients (62.5% = 5 of 8 samples with expression on < 25% of tumor cells), whereas the expression level was markedly higher in patients refractory to therapy (67% = 2 of 3 cases with expression on 25-50% of tumor cells, 33% = 1 of 3 cases with expression on > 50% of tumor cells) (**Figures 3A and 4A**). The same distribution was observed for FOXP3: TKI-responsive patients showed only weak expression (in 100% = 8 of 8 cases with expression in only single lymphocytes or negative expression pattern), whereas higher expression could be observed in TKI-refractory patients (33% = 1 of 3 cases with expression on 25-50% of lymphocytes, 67% = 2 of 3 cases with expression in only a few cells) (**Figure 4B**). Surprisingly, a different pattern was observed for HIF-1- α on tumor cells: in TKI-responsive

patients, a higher expression was found (62.5% = 3 of 8 cases with expression on > 50% of tumor cells, 12.5% = 1 of 8 cases with expression on 25-50% of tumor cells, 25% = 2 of 8 cases with expression on < 25% of tumor cells). In contrast, TKI-refractory patients showed a lower expression (100% = 3 of 3 cases with expression on only a few tumor cells or negative expression pattern) (**Figure 4C**). The expression pattern for VEGFR-1 was not substantially different: in TKI-responsive patients only a weak expression was found (62.5% = 5 of 8 cases with expression on some tumor cells or negative expression, 25% = 2 of 8 cases with expression on < 25% of tumor cells, and 12.5% = 1 of 8 cases expression on 25-50% of tumor cells). TKI-refractory patients also showed weak expression (100% = 3 of 3 cases with expression on < 25% of tumor cells) (**Figures 3B and 4D**).

Discussion

In patients with mRCC, targeted therapies have led to improvement in progression-free survival and (partially) overall survival. These therapies mainly mediate tumor cell death, however, multiple additional immunomodulatory mechanisms have been described in RCC [34-40]. To further identify the underlying mechanisms of tumor-induced immune suppression and its correlation with response to therapy, we examined the expression pattern of molecules that

CD80 and CD86 expression in RCC

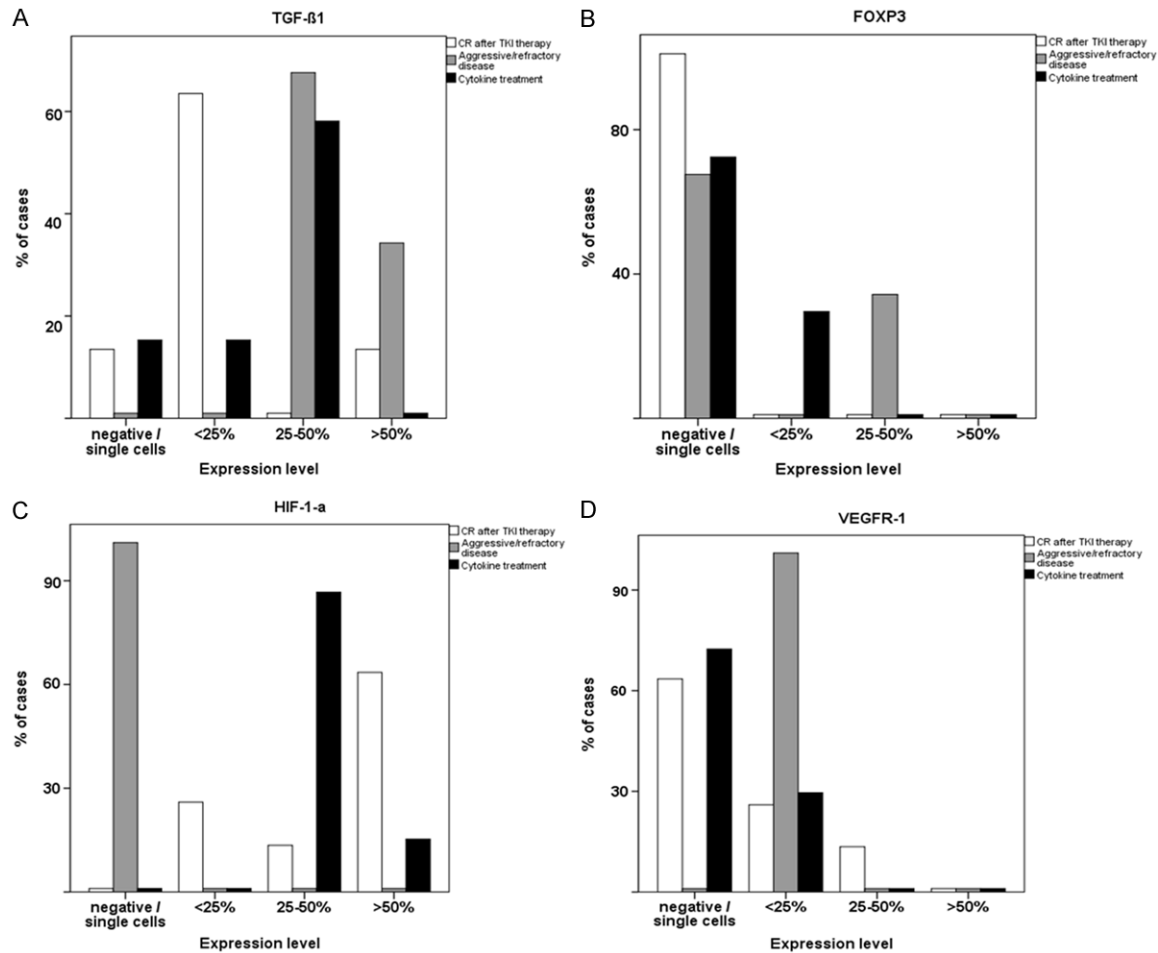


Figure 4. Expression of immunomodulatory markers on samples of different subgroups of mRCC patients. Expression of immunomodulatory markers was observed in tissue samples of different subgroups of mRCC patients and was quantified as seen above. Expression in tumor infiltrating lymphocytes (TIL): FOXP3 = forkhead box P3, Expression in tumor cells: HIF-1- α = hypoxia inducible factor 1-alpha, TGF- β 1 = transforming growth factor beta 1, VEGFR-1 = vascular endothelial growth factor receptor 1.

have been implicated in both tumor progression and immune responses in tumor tissue of patients with mRCC.

HIF-1- α expression was particularly found in the subgroup of patients responding to TKI therapy. HIF-1- α is a driver of several pathways associated with disease progression in RCC [41]. Since some of these pathways are targeted by TKI therapy, it could be hypothesized that high HIF-1- α expression is predictive for response to TKI therapy. However, this issue remains controversial, since no correlation between HIF-1- α expression and response to VEGF-directed targeted therapies has been established so far [41, 42].

The most interesting finding was that CD86 and CD80 are expressed in mRCC. CD80/86

expression could be demonstrated throughout the different subgroups of mRCC patients, however, no clear correlation with clinical outcome could be established. This finding was supported by CD86 expression on RCC tumor cell lines, which could be detected by flow cytometry. PD-L1 expression was associated with aggressive/refractory disease. This is in line with previous reports describing a correlation between PD-L1 expression and an adverse outcome [26-30]. Additionally, further immunosuppressive molecules such as TGF- β 1 and FOXP3 were predominantly found in patients with aggressive/refractory disease. Since CD80/CD86 expression was repeatedly demonstrated on tumor samples within different subgroups of mRCC patients and- in the case of CD86- also on renal carcinoma cell lines, we

can exclude technical artifacts leading to non-specific staining.

In the literature, CD80 and CD86 expression have been described on both tumor cells and tumor infiltrating lymphocytes (TIL).

In nasopharyngeal carcinoma [43], CD80 and CD86 expression was associated with improved clinical outcome [44]. In contrast, CD86 expression on myeloma cells and acute myeloid leukemia has been linked to an adverse prognosis [45-47]. Expression on melanoma cells could not be correlated with clinical outcome [48, 49]. Additionally, expression of CD80 in various murine cancer cell lines has previously been demonstrated [50]. To the best of our knowledge, this is the first report demonstrating expression of CD80 and CD86 in human renal cell cancer.

Additionally, there are reports on CD80-expressing TIL in renal cell carcinoma. It has been hypothesized that these CD80+ TIL pose an example of self-co-stimulation between T cells, which may finally lead to unresponsiveness [51]. Furthermore, there are conflicting results on the relevance and prognostic significance of TIL in RCC [52]. Some recent observations demonstrate that TIL in RCC have very heterogeneous profiles with regard to the expression of immune checkpoint molecules and the presence of mature DC within the tumor, leading to a different clinical outcome [52, 53]. It has been reported that particularly tumor infiltration with CD8+ T cells expressing high levels of PD-1 in the absence of mature DC may lead to immunosuppression [53]. Ultimately, we have previously reported on CD80/CD86+ T cells in peripheral blood of renal cell carcinoma patients under cytokine therapy. These CD80/CD86+ T cells were associated with an adverse outcome, possibly reflecting an inhibitory function. These different studies show that CD80/CD86 expression may mediate different immunological functions, depending on the immunological environment [32].

Thus, it seems very likely that the functional consequences of CD80 and CD86 expression on different cell types are not clear-cut with respect to stimulation or inhibition. It seems much more reasonable that they rather depend on the particular immunological context. Therefore, CD80/86 expression on RCC cells

might balance both stimulatory and inhibitory signals, e.g. via CTLA-4 and CD28 on T cells.

Interestingly, expression of CD80 and PD-L1 on T cells has recently been linked and it has been shown that they may lead to impaired T cell proliferation and decreased cytokine production. It has also been suggested, that CD80 may have a higher affinity for PD-L1 than for CD28, resulting in a predominantly inhibitory signal in the presence of PD-L1 [54]. Furthermore, tumor-infiltrating immune effector cells selectively expressing CTLA-4 (and not CD28) have been observed [55] and CTLA-4+ T cells have been described particularly in undifferentiated RCC with an adverse prognosis [56]. Thus, CD80/86 expression on the tumor might- at the same time-prevent T cell activation via CTLA-4 and inhibit effector T cells via PD-L1 interaction [14]. This may even occur in T cells, that express both CTLA-4 and CD28 since the binding affinity of CD80/CD86 for CTLA-4 is higher than for CD28 [57, 58], with CD80 being the dominant CTLA-4-ligand and CD86 the dominant ligand of CD28 [59]. Therefore, it seems reasonable that expression of CD80 on RCC cells is a mechanism to prevent T cell activation within the tumor. These immunosuppressive interactions via CD80/CD86-CTLA-4 and CD80-PD-L1 might outweigh immunostimulatory interactions via CD80/CD86-CD28. CD86 expression on tumor cells is an additional mechanism contributing to immune evasion since CD86 may lead to proliferation and homeostasis of Treg [60]. Additionally, it has been shown that co-stimulation via CD86 results in a TH2-polarization of T cells with predominant secretion of IL-4 and IL-10 which inhibit cytotoxic lymphocyte (CTL) responses [61, 62].

Interestingly, low-level CD80-expression was also observed in a murine colon carcinoma mouse model. In this mouse tumor model, low-level CD80 expression- in contrast to high-level CD80-expression- was associated with immune escape due to high affinity binding to CTLA-4 [50]. Therefore, the expression level of CD80/CD86 within the tumor might be a further determinant of immunological outcome.

In summary, we have demonstrated CD80 and CD86 expression in human renal cell carcinoma. In the context of recent literature, it seems very likely that these molecules are part of a network that may protect the tumor from being

attacked by T cells. It was beyond the scope of this study to unravel the exact mechanism, however, and to the best of our knowledge, this is the first report demonstrating CD80 and CD86 expression in human renal cell cancer. Our finding might be relevant for future immunotherapeutic strategies.

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Disclosure of conflict of interest

None.

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