

Prognostic value of B7-H1, B7-H3 and the stage, size, grade and necrosis (SSIGN) score in metastatic clear cell renal cell carcinoma

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Introduction We compared the potential prognostic impact of B7-H1 and B7-H3 glycoprotein expressions with the Mayo Clinic Stage, Size, Grade, and Necrosis (SSIGN) score in metastatic clear cell renal cell carcinoma (mccRCC) during a long term follow-up.

Material and methods We investigated 44 mccRCC patients, who underwent radical nephrectomy between 1995 and 2006 at a single tertiary academic center and received interferon therapy (IFNT) for at least three months. The SSIGN score was applied as a validated prediction outcome model. Representative tumor sections were immunostained with anti-B7-H3 and anti-B7-H1 antibodies. Hereafter, positive antigen-antibody reactions were measured using the Positive-Pixel-Count Algorithm of the Aperio-Technology Image Scope software.

Results In total, 48% of patients were treated with cytoreductive nephrectomy and postoperative IFNT due to synchronous mccRCC, whereas 52% received IFNT after developing metachronous mccRCC. The SSIGN score was independently associated with a higher mortality risk. Patients with a SSIGN score ≤ 9 showed an extended 'nephrectomy to start of INFT'-interval ($p = 0.02$), less synchronous clinical metastases ($p = 0.0002$), as well as an increased median overall – (OS) or cancer-specific survival (CSS) ($p = 0.01$), respectively. Furthermore, B7-H3 expression levels of $\leq 16\%$ were associated with an improved OS or CSS and correlated with a more frequent pathologic grade 1–2, as well as a longer 'nephrectomy to start of IFNT'-interval, respectively. B7-H1 expression patterns did not correlate with survival.

Conclusions The SSIGN score demonstrated the best prognostic performance. In contrast, B7-H3 expression patterns showed a low association with histopathological parameters, but predicted the cut-off-dependent impaired survival and in the future may define a cut-off to indicate checkpoint-inhibitor treatment.

Key Words: B7-H1 ↔ B7-H3 ↔ SSIGN score ↔ metastatic renal cell carcinoma
↔ Positive-Pixel-Count Algorithm

INTRODUCTION

Metastatic renal cell carcinoma (mRCC) represents about 30% of all RCC cases [1], whereas, 20% of patients who undergo surgical management for localized RCC show relapse [2]. In the pre-targeted thera-

py era, mRCC was associated with a median survival of approximately 7 months [3] and cytokines represented the standard of care from the 1980s until the early 2000s [4].

In 2004, a combined analysis demonstrated a significant median overall survival (OS) benefit of 5.8

months for the combination of nephrectomy plus interferon therapy (IFNT) in mRCC [3]. Nevertheless, IFNT has been replaced by targeted therapies inhibiting the vascular endothelial growth factor receptor (VEGFR) and mammalian target of rapamycin (mTOR) signaling pathways [5].

The immunologic treatment approach has always been in the focus of research and the co-stimulatory glycoprotein B7-H1 (PD-L1, CD 274) has been implicated as a potent inhibitor of T-cell-mediated antitumoral immunity and high expression levels were significantly associated with death in predominantly localized RCC [6]. Recently, the COMPARZ [7] and Checkmate 025 [8] trial observed an association between elevated PD-L1 expression and worse OS in clear cell (cc) mRCC, despite VEGFR- or checkpoint-inhibitor treatment, respectively.

Furthermore, tumor cell or diffuse tumor vasculature expression of B7-H3 (CD 276) was shown to be significantly associated with multiple adverse clinical or pathologic features and with an increased risk of death from RCC [9].

In addition, PD-L1 expression was associated with aggressive features such as higher tumor-node-metastasis (TNM) stage, tumor size, or Fuhrman grade, and an increased risk of cancer-specific mortality in RCC patients [10]. Therefore, we assumed that immunologic checkpoints may be as predictive for oncologic outcomes as certain histopathologic data of nephrectomy specimens and applied the validated Mayo Clinic stage, size, grade, and necrosis (SSIGN) score [11] for outcome prediction in our current study. It was introduced in 2002 based on ccRCC patients treated with radical nephrectomy [12].

We retrospectively evaluated the prognostic relevance of B7-H1, B7-H3 expressions and the SSIGN Score after nephrectomy in 'synchronous or metachronous' (SM) mRCC patients who received IFNT. The subsequent administration of further systematic therapies hereafter was also noted during the follow-up until February 2018.

MATERIAL AND METHODS

Patient selection

This study was approved by the Institutional Review Board (20-279Ex08/09) of the Medical University of Graz (MUG). Clinico-pathological data and medical history from 250 ccRCC patients were evaluated who had undergone nephrectomy at the MUG from 1993 to 2006. All subjects included into analyses must have received IFNT for at least 3 months due to mRCC.

Histopathologic reevaluation and clinical features

Overall, 78 mRCC patients could be included into the study. Blinded for all other patient information, whole-tissue sections (WTS) from all specimens most representative of the tumor were reevaluated by one urologic pathologist (S.M.) and 44 patient sections were classified as exclusively ccRCC. Original classifications of pathologic tumor-stages and grades of the year of nephrectomy were adapted to the RCC TNM system of 1997 [13].

Mayo Clinic SSIGN Score

The SSIGN score was calculated from 0 (most favorable outcome) to 15 (worst outcome) as originally defined by Frank et al. [12].

Immunohistochemical staining

Formalin-fixed, paraffin-embedded (FFPE) specimens (5- μ m sections) were deparaffinized and rehydrated in a graded series of ethanol. Antigen retrieval was performed at 120°C for 10 min in the Dako Cytomation Target Retrieval Solution (pH 9.0), using a Decloaking Chamber (Biocare Medical). Subsequently, sections were washed three times in phosphate-buffered saline (PBS) for 10 min. Endogenous peroxidase activity was blocked by treatment with H₂O₂ in methanol for 20 min.

Immunostaining of B7-H3

All sections were incubated with anti-human B7-H3 antibody (goat, R&D Systems, Cat.no. AF1027; 1:20) for 30 min at room temperature, followed by anti-human B7-H3 antibody (rabbit anti goat horseradish peroxidase [HRP]-antibody; Dako, 1:150) for 30 min. All antibodies and antibody controls were diluted with the Dako antibody diluent (Dako) and the sections were rinsed in PBS between the incubation steps. Antibody binding was visualized by incubating in a mix of 20 μ l 3, 3'-diaminobenzidine and 1 ml substrate buffer. Goat immunoglobulin G (Linaris, 1:1000) was used as the negative control. The sections were counterstained with Mayer's hemalum.

Immunostaining of B7-H1

The sections were incubated with Ultra-V-Block (Kit-content) for 5 min, followed by Anti-B7-H1 rabbit antibody (Novus Biologicals, NBP1-03220; 1:200) for 30 min, primary antibody enhancer for 10 min and the HRP-polymer detection reagent (Ready-To-Use; Thermo scientific) for 30 min. Rabbit immunoglobu-

lin G (Linaris, 1:1000) was used as negative control. Sections were counterstained with Mayer's hemalum.

Automated analysis

Slides were scanned with the Scan Scope Model T3 (Aperio Technologies, Leicabiosystems; AT) and the AT Image Scope software was used to manually select the region of interest (ROI) containing the tumor tissue. Stained cell-free vascular lumina, fatty tissue and individual artifacts were excluded for analysis (Figure 1: A/D). Those locations of the ROI which exhibited a brown antigen-antibody reaction, were detected with the Positive-Pixel-Count Algorithm (PPCA) of the AT Image Scope software (Figure 1: B/E). The 'pseudo-color mark-up image' of the PPCA depicts weakly brown-positive pixels as yellow, brown-positive pixels as orange and strongly brown-positive pixels as red, respectively (Figure 1: C/F). Negative pixels were illustrated as blue. The percentage of positive antigen-antibody reactions defined as positive brown staining in the tumor tissue samples was calculated as a total-positive pixel staining divided by the total amount of pixels.

Statistical analysis

Associations of B7-H1 and B7-H3 expressions with clinical and pathological features were evaluated us-

ing χ^2 , Fisher's exact tests and correlation methods, respectively. To identify associations of the SSIGN score, B7-H1 or B7-H3 expression and potential cut-off points with survival, we performed cox-proportional hazards models. Kaplan–Meier analyses calculated for the survival probability. Progression-free survival (PFS) was defined as the time from initiation of IFNT to progression or death. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed with JMP Version 12.2.0 (SAS, Cary, NC, USA).

RESULTS

Cytoreductive nephrectomy (CN) and immediate IFNT due to synchronous systemic disease was performed in 48% of all patients and the median time from nephrectomy to IFNT was 54 days. In contrast, 52% of all patients received IFNT after developing metachronous metastatic disease in the median 32 months after nephrectomy. Median time from nephrectomy to the start of IFNT and the duration of IFNT was 346 days and 9.5 months, respectively. Metastasectomy was performed in 30% of patients, but was not significantly associated with PFS ($p = 0.82$), OS ($p = 0.68$), or cancer-specific survival (CSS) [$p = 0.62$] benefit, respectively. Patients' clinico-pathological data, treatments, follow-up and survival characteristics are demonstrated in Table 1. At least one further systemic treatment post-

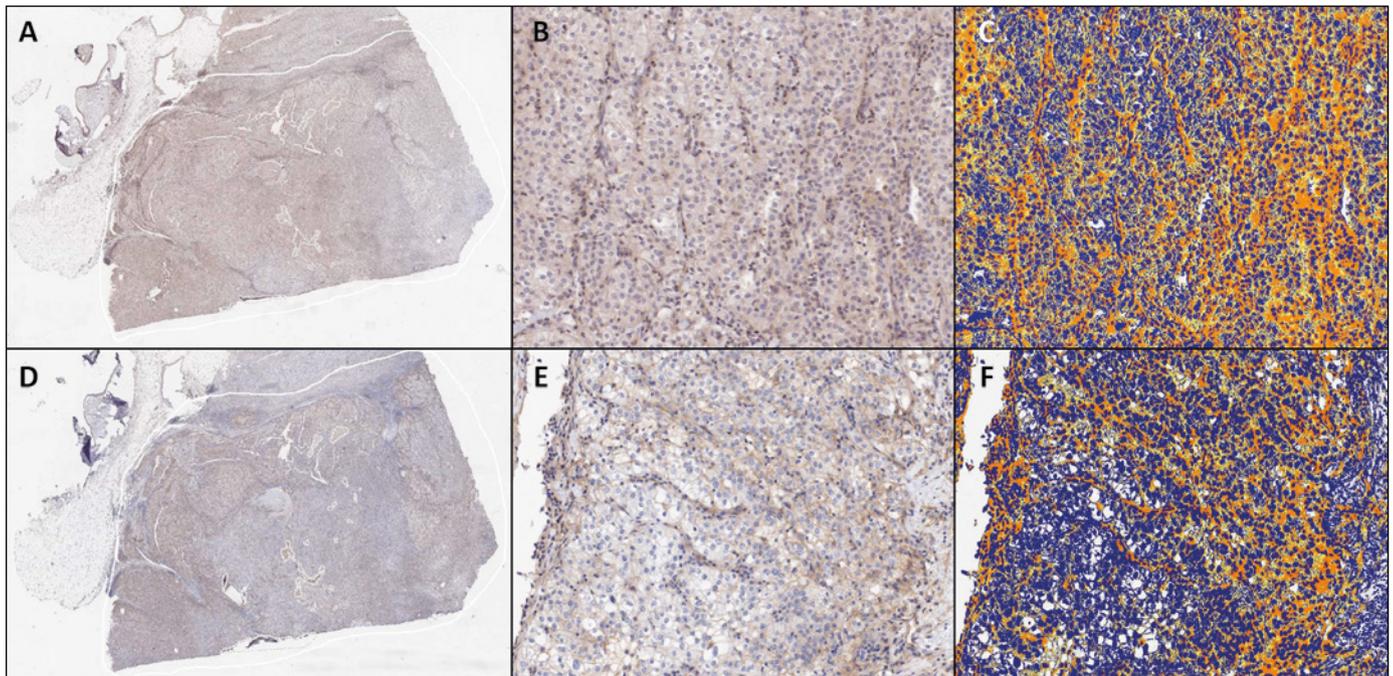


Figure 1A. Scanned whole tissue section after B7-H1 staining and depicted tumor area (white line); **B:** 20-fold magnification; **C:** Pseudo-Color Mark-up Image of the Positive-Pixel-Count Algorithm of B. **D:** Scanned whole tissue section after B7-H3 staining and depicted tumor area (white line); **E:** 20-fold magnification; **F:** Pseudo-Color Mark-up Image of the Positive-Pixel-Count Algorithm of E.

Table 1. Patients' pathologic, clinical, therapeutic, follow-up and survival characteristics

Characteristics	Results
Total number	44
Age (median, IQR)	59 (55–64)
Men (n, %)	30, 68
Women (n, %)	14, 32
pT1 (n, %)	5 (11.4)
pT2 (n, %)	6 (13.6)
pT3a (n, %)	7 (15.9)
pT3b (n, %)	26 (59.1)
Tumor size (cm, median, IQR)	7 (4.6–9.8)
G1-2 (n, %)	27 (62.8)
G3-4 (n, %)	16 (37.2)
Tumor necrosis (n, %)	19 (43)
SSIGN Score (median, IQR, min.–max.)	6 (4–9, 0–13)
B7-H1 Expression (%: median, IQR, min.–max.)	20 (13–29, 0.35–63)
B7-H3 Expression (%: median, IQR, min.–max.)	16 (12–21, 4–32)
Synchron pathologic Lymphnode positivity (n, %)	4 (9)
Clinical metastasis at surgery (n, %)	21 (47.7)
Metastasectomy (n, %)	13 (29.5)
Free of metastasis after metastasectomy (n, %)	5 (38.4)
Sites affected with metastasis at IFNT-start (median, IQR; min.-max.)	1; 1–2; 1–3
Lung (n, %)	33 (75)
Bone (n, %)	9 (20.4)
Lymphnodes (n, %)	7 (16)
Liver (n, %)	6 (13.6)
Brain (n, %)	1 (2)
Pancreas (n, %)	3 (7)
Adrenal Gland (n, %)	6 (13.6)
Vascular wall invasion V.iliaca ext/V.lumbalis (n, %)	1 (2.3)
Time from Nephrectomy to IFNT in days (median, IQR)	394 (58–1270)
Interferon alfa-2a (Roferon), (%)	7
2b (Intron-A), (%)	48
Roferon and gamma-1b (Imukin), (%)	45
IFNT frequency and dosing	3–5 x/ week, 3–5 million I.E.
ECOG 0/1/2 at INFT-start (n, %)	16 (36.4)/20 (45.5)/8 (18.2)
Duration of INFT in months (median, IQR)	9.5 (5–17)
Total number of post INF adjuvant therapies (median, IQR; min.–max.)	0; 0–1; 0–6
1 subsequent systemic therapy (n, %)	16 (36.4)

Table 1. Continued

Characteristics	Results
Sorafenib (n, %)	14 (31.8)
Sunitinib (n, %)	8 (18.2)
Pazopanib (n, %)	4 (9.1)
Axitinib (n, %)	2 (6.3)
Everolimus (n, %)	3 (6.8)
Temsirolimus (n, %)	4 (9.1)
Bevacizumab (n, %)	1 (2.3)
Gemcitabine (n, %)	3 (6.8)
Interleukin-2 (n, %)	2 (4.5)
Tamoxifen (n, %)	1 (2.3)
Follow up in years (median; IQR, min.–max.)	4.7; 1.8–12.3, 0.5–22.5
Cancer specific deaths (n, %)	36, 82
Total Mortality (n, %)	38, 86.4
Progression free survival (a, median; 95% CI low-high)	1.1; 0.7–1.4
Overall survival (a, median; 95% CI low-high)	4.7; 2–7.8
Cancer specific survival (a, median; 95% CI low-high)	4.7; 2–7.8

n – number; IQR – Inter Quartile Range; SSIGN – Mayo Clinic Stage; Size, Grade and Necrosis; IFNT – Score, Interferon Therapy; ECOG – Eastern Cooperative Oncology Group performance status

IFNT was added in 36% of all patients. We observed no difference between SM mcrRCC patients in the number of post INFT systemic therapies. At least one post IFNT systematic therapy resulted in a significant OS ($p = 0.003$) and CSS ($p = 0.002$) advantage.

Median follow-up was 4.7 years (IQR: 1.8–12.3) with a median PFS of 1.1 (95% CI low-high: 0.7–1.4) years and OS or CSS of 4.7 (95% CI low-high: 2–7.8) years each. In total, 86% of patients in this cohort died, whereby 82% of the observed deaths were cancer-specific.

The median SSIGN score, B7-H1 and B7-H3 expressions were 6 (IQR: 4–9, min.-max.: 0–13), 20% (IQR: 13–30, min.–max.: 0.35–63), and 16% (IQR: 12–21, min.-max.: 4–32), respectively. All univariate and multivariate proportional hazards models for the applied clinical parameters are demonstrated in Table 2. The SSIGN scores demonstrated significant differences according to OS and CSS analysis (both $p = 0.01$) and tended to differ in association with PFS ($p = 0.06$). Increased SSIGN scores correlated significantly with a higher tumor stage ($p = 0.005$), -size ($p = 0.002$), -grade ($p < 0.0001$), -histological tumor necrosis ($p = 0.004$), nodal, as well as clinical metastases, (both $p \leq 0.0001$), a shorter 'nephrectomy to start of INFT'-interval ($p < 0.0001$) and worse Eastern Cooperative Oncology Group (ECOG) performance

status at the start of IFNT ($p = 0.04$). A uni- and multivariate cox regression analysis of the SSIGN score demonstrated a higher overall – ($p = 0.01$ and $p = 0.03$), as well as cancer-specific risk ($p = 0.01$ and $p = 0.04$) for mortality per increasing SSIGN

score unit, respectively. Moreover, patients with a SSIGN score ≤ 9 showed equivalent PFS ($p = 0.1$; data not shown) compared to a SSIGN score >9 , but an improved median OS or CSS (both $p = 0.01$) [Figure 2; G/J], decreased synchronous clinical metasta-

Table 2. Univariate and multivariate lifetime proportional hazards analysis for clinical and biological factors. Significant p -values are demonstrated in bold font

Lifetime Proportional Hazards Analysis	PFS UV (p)	RR	OS UV (p)	RR	CSS UV (p)	RR	PFS MV (p)	RR	OS MV (p)	RR	CSS MV (p)	RR
Age	0.45		0.23		0.25		0.33		0.8		0.9	
B7-H1 Expression	0.54		0.31		0.44		0.79		0.3		0.5	
B7-H3 Expression	0.95		0.19		0.24		0.22		0.43		0.5	
SSIGN-Score	0.14		0.01	1.15 (95%CI: 1–1.3)	0.02	1.14 (95%CI: 1–1.3)	0.27		0.02	1.2 (95%CI: 1–1.5)	0.04	1.2 (95%CI: 1–1.5)
Total number of organs with metastasis	0.006	2 (95%CI: 1.2–3.3)	0.002	2.2 (95%CI: 1.4–3.5)	0.001	2.3 (95%CI: 1.4–3.6)	0.03	1.8 (95%CI: 1–3.2)	0.01	2.1 (95%CI: 1.2–4)	0.01	2.2 (95%CI: 1.2–4)
Time from nephrectomy to IFNT (yrs)	0.46		0.048	0.88 (95%CI: 0.8–1)	0.07		0.55		0.9		0.9	
Length of IFNT	0.01	0.97 (95%CI: 0.94–0.99)	0.23		0.31		0.0001	0.95 (95%CI: 0.9–1)	0.7		0.7	
Total number of post IFNT adjuvant therapies	0.68		0.002	0.72 (95%CI: 0.6–0.9)	0.002	0.71 (95%CI: 0.5–0.9)	0.25		0.04	0.8 (95%CI: 0.6–1)	0.03	0.7 (95%CI: 0.5–1)

PFS – progression free survival; OS – Overall Survival; UV – Univariate; MV – Multivariate; CSS – Cancer Specific Survival; RR – Relative Risk per unit change in regressor; calculated only for significant results, IFNT – Interferon Therapy; CI – Confidence Interval

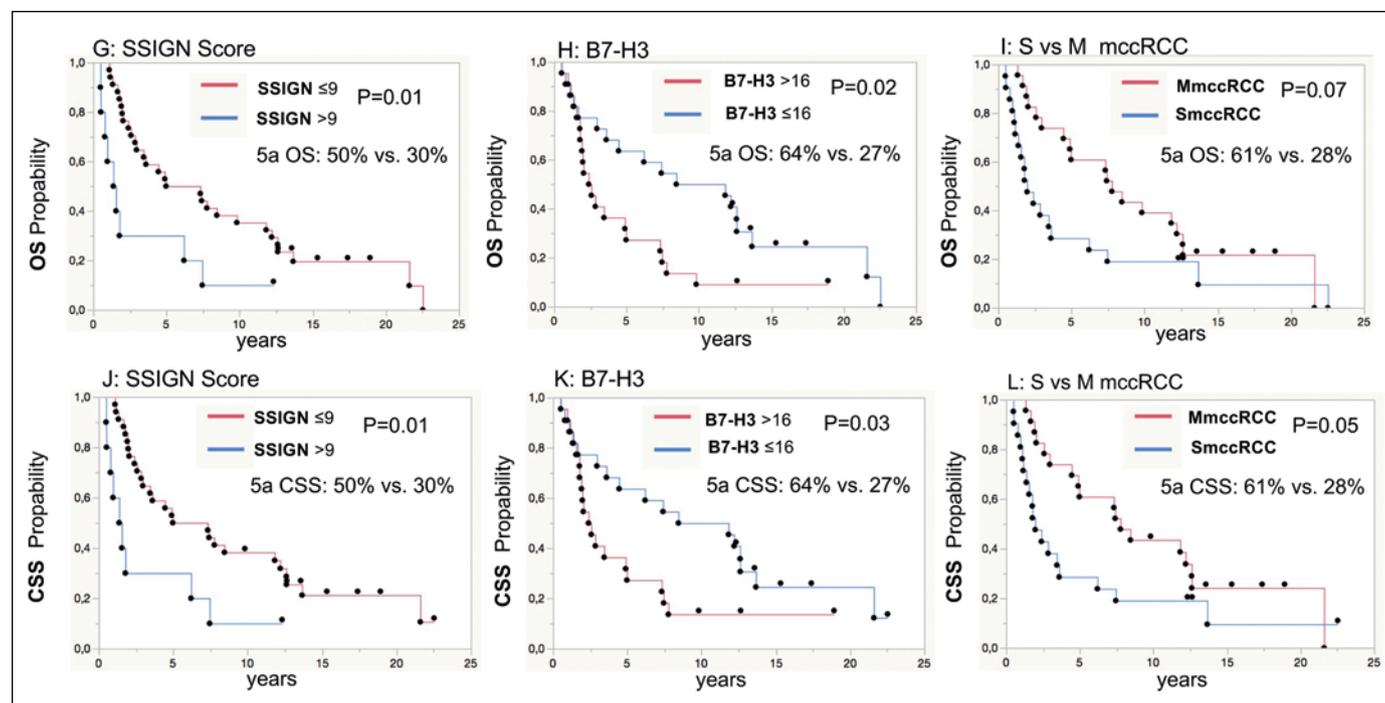


Figure 2. Kaplan-Meyer curves G, H, I and J, K, L demonstrate Overall Survival (OS) and Cancer Specific Survival (CSS) differences of the Mayo Clinic Stage, Size, Grade and Necrosis (SSIGN) Score cut-off ≤ 9 vs. >9 , B7-H3 expression cut-off ≤ 16 vs. >16 and Metastatic (M) vs. Synchronous (S) metastatic clear cell Renal Cell Carcinoma (mccRCC), respectively.

ses ($p = 0.0002$) and an extended 'nephrectomy to the start of INFT'-interval ($p = 0.02$), respectively. Patients without metastatic disease at the time of nephrectomy demonstrated lower SSIGN scores ($p < 0.001$), a longer period from nephrectomy to start of IFNT ($p = 0.005$), a more favorable ECOG performance status at the start of IFNT ($p = 0.04$), no difference in PFS and only tended to have an improved OS ($p = 0.07$) and CSS ($p = 0.05$) [Figure 2; I/L] compared to patients with synchronous metastases (Table 3). In contrast, median PFS of patients with one, two or three metastatic sites was 1.4, 1.1 or 0.5 years, respectively ($p = 0.007$). In addition, an increasing number of organs affected by metastases was significantly associated with a two-fold higher risk of disease progression and overall- or cancer-specific mortality in univariate and multivariate proportional hazards models. Higher B7-H1 expression levels were exclusively associated with metastasectomy ($p = 0.02$) and less

metastatic disease at IFNT start ($p = 0.02$). Higher B7-H3 expressions showed an increased rate of histological tumor necrosis ($p = 0.02$), shortened IFNT periods ($p = 0.01$) and tended to correlate with tumor grades 3–4 ($p = 0.05$). Although proportional hazards models of B7-H1 and B7-H3 expressions were not significantly associated with the patient's survival, an improved median OS and CSS was observed in patients with a B7-H3 expression $\leq 16\%$ (both: $p = 0.02$) [Figure 2; H/K]. This cut-off was not associated with PFS. However, a B7-H3 expression $> 16\%$ correlated with a more frequent tumor grade 3–4 ($p = 0.02$) and a shorter 'nephrectomy to the start of IFNT'-interval ($p = 0.04$).

DISCUSSION

To the best of our knowledge, this is the first study to investigate the prognostic relevance of the B7-H1 and B7-H3 glycoprotein expression in mcrRCC pa-

Table 3. Association of pathologic, clinical, therapeutic and survival characteristics with Synchronous (S) and Metachronous (M), metastatic (m), clear cell (cc). Renal Cell Carcinoma (RCC). Number (n), Inter Quartile Range (IQR), a (years), Stage, Size, Grade, Necrosis (SSIGN) Score, Interferon Therapy (IFNT). Significant p-values are demonstrated in bold font

	S mRCC	M mRCC	p
Total number n (%)	21 (48)	23 (52)	
Age (a, median, IQR)	59, 56–65	58, 55–64	0.82
Sex (men/women)	16/5	14/9	0.28
SSIGN Score (median, IQR)	9, 7.5–11	4, 3–5	<0.001
B7-H1 Expression (%: median, IQR)	24.4, 14.9–32.9	19.5, 11.7–27.1	0.29
B7-H3 Expression (%: median, IQR)	16.8, 12.5–20.4	15.2, 10.8–21.6	0.97
B7-H3 Expression $\leq 16\%$ vs. $> 16\%$ (n)	9 vs. 12	13 vs. 10	0.36
Synchron pathologic Lymphnode positivity n (%)	4 (19)	0 (0)	0.03
Metastasectomy n (%)	8 (38)	5 (22)	0.23
Free of metastasis after metastasectomy (yes/no)	3/5	2/3	0.93
Sites affected with metastasis at INFT-start (median, IQR; min.–max)	1, 1–2, 1–3	1, 1–2, 1–3	0.83
Lung n (%)	16 (76)	17 (74)	0.86
Bone n (%)	5 (24)	4 (17)	0.6
Lymphnodes n (%)	3 (14)	4 (17)	0.78
Liver n (%)	4 (19)	2 (9)	0.32
Brain n (%)	0 (0)	1 (4)	0.33
Pancreas n (%)	0 (0)	3 (13)	0.09
Adrenal Gland n (%)	4 (19)	2 (9)	0.31
Non resectable tumor thrombus with vascular wall invasion external iliac vein and lumbal vein, n (%)	0 (0)	1 (4)	0.33
Time from Nephrectomy to INFT (a, median, IQR)	0.15, 0.03–1	2.7, 1–4.9	0.005
ECOG 0/1/2 at INFT-start, n	12/8/3	4/12/5	0.04
Duration of INF (months, median, IQR)	9, 4–15.5	10, 5–19	0.92
Total number of post INF adjuvant therapies (n, median, IQR, min.–max)	0, 0–1, 0–5	0, 0–2, 0–6	0.21
1 subsequent post IFN systemic therapy, n (%)	6 (29)	10 (43)	0.3
CS Deaths, n (%)	18 (86)	18 (78)	0.52
Total Mortality, n (%)	19 (90)	19 (82)	0.45
Overall survival (a, median; 95% CI low-high)	2 (1.1–3.6)	7.8 (4.5–12.2)	0.07
Cancer specific survival (a, median; 95% CI low-high)	2 (1.1–3.6)	7.7 (4.5–12.6)	0.05
Progression free survival (a, median; 95% CI low-high)	0.9 (0.1–4.6)	1.3 (1–4.4)	0.71

tients after IFNT adjusted for the validated SSIGN score, metastatic sites and further systemic treatments in the long term. All the patients of our study cohort had the same therapeutic precondition reflecting a state-of-the-art mRCC treatment regarding the observational study period [3].

Since 2006 targeting agents have replaced cytokines in the treatment of mRCC albeit the rate of complete remissions has not increased in mRCC [5].

32% (n = 14) of all patients in our study received agents targeting VEGFR and mTOR post-IFNT. In addition, we observed a significant survival advantage for \geq one systemic treatment post IFNT. Interestingly, we found neither a difference in the number of post-IFNT-systemic therapies, nor a OS (p = 0.33) or CSS (p = 0.25) difference after \geq one systemic post-IFNT between SM mRCC patients.

Regardless, median PFS of 1.1 years in our cohort was twice as long as previously described for mRCC patients after nephrectomy and IFNT combination therapy [3]. This seemed to be associated with mainly solitary site affection in our cohort at the time of IFNT initiation. Nevertheless, mRCC has also demonstrated response durations to IFNT of up to 12.2 months in small prospective trials of the early nineties [14].

Patients with metachronous mRCC received IFNT 2.7 years (median) after nephrectomy. They demonstrated a relatively long median OS of 7.8 years, which is comparable with previous data showing a median OS of 6.1 years from the time of recurrence after nephrectomy for localized RCC [15]. Median OS of synchronous mRCC patients in our cohort was 2 years, which is marginally longer than the reported 20.6–22.5 months (median) for patients with previous CN and synchronous mRCC [16, 17, 18].

The SSIGN score was recently applied to contemporary radical and partial nephrectomy RCC patients and retained strong predictive ability. It is now being utilized to stratify patients for therapeutic clinical trials and assess the role of biomarkers in predicting survival for RCC patients [11]. In our cohort the SSIGN score approved its prognostic power by predicting the cut-off-independent mortality risk and impaired survival at a cut off of >9 . Interestingly, the checkmate 025 study emphasized the impact of tumor type and histologic class on oncologic outcomes especially after treatment with nivolumab, irrespective of the MSKCC prognostic score, number of previous antiangiogenic therapies, or region.

Incremental understanding of the complex interaction between cancer and host cells regained focus on co-signaling cell surface glycoproteins of the B7-CD28 family. B7-H1 expression seems restricted to a fraction of macrophage-lineage cells or activated T-cells and is not present in healthy human

kidney tissue. In contrast, RCC aberrantly expresses B7-H1 and binding to the T-cell PD-1 receptor results in impaired cytokine production or apoptosis of activated T cells that allows RCC to escape immune detection and destruction [6]. As a result, anti-PD-1 human monoclonal antibodies were investigated in the Checkmate 025 and 214 trial, demonstrating an improved OS in mRCC [19]. Interestingly, we observed that a high B7-H1 expression was associated with metastasectomy and less metastases at the start of IFNT, but did not correlate with other parameters, survival or differ between SM mRCC. Thompson et al. stained RCC paraffin specimens with anti-B7-H1 (clone 5H1) and the percentages of B7-H1 positive stained tumor cells were reviewed independently by two urologic pathologists ($<5\%$ tumor staining were considered negative). They observed that an aberrant tumor B7-H1 expression correlated with the development of metachronous mRCC [20], but this may be in part due to the comparison of true localized cases with subsequently metastasized ones. In contrast, the COMPARZ, Checkmate 025 and 214 trial focused on mRCC patients and demonstrated that elevated PD-L1 expression correlated with poorer survival. The last two trials used the Dako PD-L1 IHC 28-8 pharmDx test (Monoclonal Rabbit Anti-PD-L1, Clone 28–8) for the detection of PD-L1 protein in FFPE RCC tissue sections. The cut-off dependent ($\geq 1\%$ vs. $< 1\%$) tumor PD-L1 membrane expression was assessed in sections that had at least 100 evaluable PD-L1 positive tumor cells but no total or median expression numbers were described. As far as we know, B7-H1 expression rates in RCC in association with the anti B7-H1 antibody NBP1-03220 have not yet been published. Furthermore, in our study we used an automated quantification algorithm for positive brown stainings, but no expression cut offs for B7-H1 positivity. These differences may have led to relatively high B7-H1 expression rates, but no chosen cut-off demonstrated an impact on oncologic outcome. Furthermore, we observed only one patient specimen with a B7-H1 expression rate of $< 1\%$ or $\leq 5\%$, respectively. Therefore, we could not apply the B7-H1 cut off strategy for positive stainings according to the COMPARZ, Checkmate 025 or 214 trial.

The true value of the B7-H1 expression as a biomarker is limited by the lack of standard detection antibodies, IHC cut-off points to define positivity [21] and an uncertainty, whether the B7-H1 expression should be ideally assessed in tumor cells, tumor infiltrating cells or both [22]. In accordance with previous reports [23, 24, 25] we suppose, that the application of automated measurement and quantification of positive antigen-antibody reactions within

the ROI might be more representative and reduce analytical variability or even false results. Nevertheless, current mRCC therapy selection based on the PD-L1 biomarker is not recommended [26].

The B7-H3 expression on tumor cells has been implicated as a potent inhibitor of T-cell activity to escape the anti-tumor immune response [27]. Furthermore, B7-H3 is expressed at high levels in the vascular endothelium of various malignancies, whereas no expression was found in normal glomeruli, renal cortical vessels [28] or non-tumorous renal parenchyma adjacent to ccRCC [9]. In 2008, Crispen et al. described that B7-H3 expression in ccRCC by tumor cells or tumor vasculature correlated with adverse clinical and pathologic features and was associated with progression and cancer-specific death [9]. They defined B7-H3 expression in $\geq 10\%$ of tumor cells as a random cut-off for positivity and showed a median level of the B7-H3 expression of 30% in mainly localized ccRCC.

Quin et al. observed a median B7-H3 expression of 25% in ccRCC. B7-H3 expression was detected in 19% of ccRCC specimens and 98% of tumor vasculature. A diffuse pattern of vascular B7-H3 expression was associated with multiple adverse clinical and pathologic features, but no significant association was observed between tumor cell B7-H3 expression, clinico-pathologic features or survival [29]. Zhang et al. showed that B7-H3 was overexpressed in ccRCC tissues, especially in the tumor vasculature (97.56%). Vascular B7-H3 expression correlated with ccRCC tumor grade, pathologic T-stage and lymph node metastases [28].

Comparable with these three reports we applied a B7-H3 antibody (R&D Systems) in our study but we did not define a cut-off value for B7-H3 positivity due to a missing rationale. Probably therefore, we observed an even lower median expression of 16%, despite the inclusion of exclusively mcrRCC patients in our study cohort. In addition, B7-H3 expression demonstrated low prognostic potential for histopathological parameters, but predicted responses to IFNT and impaired survival at a cut-off of $> 16\%$.

An interim analysis from a clinical trial with a specific anti-B7-H3 antibody indicates antitumor properties and an increased T-cell activity, with no dose-limiting toxicity and no severe immune-related side effects [30]. Therefore, targeting B7-H3 in cancer tissue may constitute a synergistic treatment approach besides established treatment modalities.

However, our study is not devoid of important limitations. First, the limited sample size of the study cohort restrains the validity of our statistical analyses. Unfortunately, we could not additionally apply the generally accepted and further developed Motzer-criteria [16] due to the long term retrospective study design and missing blood values of the pre-'targeted therapy'-era. According to the retrospective study design our patients received a different standard of care, staging and follow-up. Furthermore, we adjusted pT-2 RCC categories of 1993-1996 to the TNM classification system of 1997. However, only two patients were downgraded to pT-1 resulting in a one point SSIGN score reduction, but this had no significant influence on our results. Last, our automated quantification method for positive antigen-antibody reactions in mcrRCC specimens represents a further strategy of IHC processing, but no generally accepted standard and has to be validated in future studies.

CONCLUSIONS

The validated SSIGN score demonstrated the best prognostic performance to predict patient outcomes in mcrRCC. The B7-H3 expression in mcrRCC demonstrated low prognostic potential for histopathological parameters, but predicted responses to IFNT. In addition, B7-H3 expression levels of $> 16\%$ might serve as a prognostic biomarker for short term survival in mcrRCC patients and may define a cut-off to indicate a checkpoint-inhibitor treatment. Conversely, B7-H1 expression was not able to predict oncologic patient outcomes.

CONFLICTS OF INTEREST

None of the contributing authors have any conflicts of interest, including specific financial interests, personal relationships with other people and organizations or affiliations relevant to the subject matter or materials discussed in the manuscript.

ETHICAL CONSIDERATIONS

The authors declare that no funding or other financial support was received and that they have no potential conflicts of interest.

This study has been approved by the ethical committee of the Medical University of Graz and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

All patients gave their informed consent prior to their inclusion into the study.

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