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RCC-Ma loss predicts poor survival and metastatic risk in clear cell renal cell carcinoma

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ABSTRACT

Background: With the increasing number of renal cell carcinoma subtypes and implications for prognosis and therapy, correct classification of renal masses remains a challenging issue. Clear cell renal cell carcinoma (ccRCC) is a tumor with an immunoprofile that often does not follow paradigmatic rules. Thus, the aim of this study was to analyze the heterogeneity of immunohistochemical staining patterns in ccRCC regarding patient prognosis.

Methods: The study cohort consisted of 727 ccRCC patients with surgical treatment between 1995 and 2006 and with comprehensive clinicopathological information and follow-up data. Only 1.6 % of patients received modern targeted therapy after surgery. The patients were stratified analogue to the Leibovich Risk Score (LRS). A tissue microarray was immunohistochemically stained for vimentin, CAIX, CD10, RCC-Ma, AMACR, CK7 and CD117. The expression in the tumor tissue was semiquantitatively scored and tested for association with clinicopathological tumor features and patient survival.

Results: Loss of RCC-Ma was an independent prognostic biomarker for disease specific survival ($p = 0.01$) and associated with a higher risk of developing metastasis in the intermediate risk group of the LRS as well as aggressive tumor features, such as higher tumor grade and stage, metastasis and necrosis. The other analyzed immunohistochemical biomarkers had no impact on patient prognosis.

Conclusion: As a predictor of poor survival and metastatic risk, RCC-Ma is likely to be a valuable contributor to the risk stratification in ccRCC patients. Moreover, this study cohort provides a valuable resource for investigations on the natural, therapy-naive clinical course of the disease and can serve as a reference for other study collectives, including patients treated with up-to-date targeted therapies.

1. Introduction

Renal cell carcinoma (RCC) accounts for 2 % of newly diagnosed malignant tumors worldwide and 2 % of cancer-related deaths [1]. The most common subtype is clear cell renal cell carcinoma (ccRCC), followed by papillary RCC (pRCC) and chromophobe RCC (chRCC). The correct diagnosis of ccRCC is usually straightforward with the typical morphology of a highly vascularized tumor consisting of sheets of neoplastic cells with a round to oval nucleus and a water-clear cytoplasm. However, the number of RCC subtypes has increased and the precise classification of a renal mass can be challenging. Moreover, ccRCC displays broad morphological heterogeneity, which can render the discrimination of ccRCC from other RCC subtypes difficult. Differential diagnoses of ccRCC include pRCC, chRCC, microphthalmia-associated transcription factor translocation RCC (MITF-RCC), clear cell renal cancers with leiomyomatous stroma or clear cell papillary renal cell tumors (ccpRCT). The issue of the correct classification of a renal tumor as ccRCC remains critical since ccRCC is

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Table 1
Patient Cohort.

	N	%
Tumor Subtype		
ccRCC	727	100.0
Tumor Grading		
G1	40	5.5
G2	502	69.1
G3	165	22.7
G4	20	2.8
Tumor Stage		
pT1	450	61.9
pT2	85	11.7
pT3	183	25.2
pT4	9	1.2
Lymph Node Metastasis		
N0/pN0	698	96.0
N1/pN1	29	4.0
Distant Metastasis		
M0/pM0	693	95.3
M1/pM1	34	4.7
Lymph Vessel Invasion		
L0	714	98.2
L1	13	1.8
Blood Vessel Invasion		
V0	594	81.7
V1	133	18.3
Resection Status		
R0	677	93.1
R1/R2	23	3.2
Rx	27	3.7
Sex		
male	470	64.6
female	257	35.4
Age at Surgery		
< 65 years	391	53.8
> 65 years	336	46.2
Tumor Necrosis		
no	561	77.2
yes	166	22.8
Follow-up Information		
yes	562	77.3
no	165	22.7
Survival Status		
alive	249	44.3
deceased	313	55.7
death by RCC	93	16.5

associated with an unfavorable clinical course compared to other subtypes [2]. To increase the diagnostic precision, immunohistochemical stainings are usually performed for further characterization of the RCC. ccRCC is considered to be positive for vimentin, CD10, renal cell carcinoma marker (RCC-Ma) and carboanhydrase 9 (CAIX), variably positive for alpha-methylacyl-CoA racemase (AMACR) and negative for CD117 [3]. The latter are expected to be positive in pRCC or chRCC, respectively. In ccRCC, CK7 is mostly negative or expressed in single tumor cells. However, staining quantity and quality are highly variable between individual tumors and different biomarkers, respectively. This issue might lead to difficulties in the interpretation of immunohistochemical staining patterns and possibly to misdiagnosis, which are more impaired through overlapping immunohistochemical profiles [4,5].

To date, histopathological evaluation with information on RCC subtype, tumor grading, sarcomatoid dedifferentiation or presence of necrotic tumor areas contributes to the risk assessment of patients with RCC, next to clinical staging algorithms or prognosis scores. In the light of an increasing number of in part molecularly defined RCC subtypes [6] and the introduction of immune checkpoint inhibitors, there is an ongoing need for prognostic and predictive biomarkers. Potential biomarker candidates are investigated using histological, immunohistochemical and molecular techniques comprising either broad transcriptomics or single cell analysis. Lately, deep learning approaches using artificial intelligence complement the plethora of techniques. However, no biomarker has been integrated in the clinical management of patients with RCC so far [7]. One of the major hurdles is that potential biomarkers must be broadly available and cost-effective. Immunohistochemical markers used for RCC subtyping meet the latter requirements at least and bear the potential to provide additional prognostic information if differentially expressed.

Thus, the aim of this study was to introduce a large single-center study cohort of ccRCC with consecutive analysis of the prognostic value of a standard diagnostic immunohistochemical marker panel. To this end, all tumors were first re-classified according to the current WHO classification and the variability of a immunohistochemical marker panel characterized and further analyzed with regard to patient prognosis.

2. Methods and Materials

2.1. Patient cohort and tumor tissue collection

Samples of tumor tissue and corresponding normal renal tissue from 762 patients with ccRCC were collected. The exclusion criteria were either ccRCC with a date of diagnosis less than four years before the beginning of the study or nonclear cell renal cell carcinomas. This study protocol was designed in accordance with the Declaration of Helsinki. The use of excess tissue samples older than four years was approved and the need for written informed consent from the patients was waived by the Ethics Committee of the State Medical Association of Rhineland-Palatinate due to the retrospective design of the study (ethics approval number: 837.360.16 (10679)). The patients underwent surgery at the Department of Urology at the University Medical Center of the Johannes Gutenberg University from 1995 to 2006. Follow-up at the time of the collection of clinical data included office visits with physical and laboratory exams, abdominal computed tomography or ultrasound, chest radiography or computed tomography on average every 6 months for 3 years followed by annual visits thereafter. Some patients were followed by outpatient colleagues and data were obtained from clinical files or by telephone. The follow-up data were obtained in part from the Cancer Registry of Rhineland-Palatinate, Germany. All tumors were restaged according to the 8th edition of the TNM classification [8]. The four-tiered ISUP grading system was used to determine the tumor grade [9].

2.2. TMA construction

A tissue microarray (TMA) containing tumor tissue and corresponding nonneoplastic renal parenchyma was constructed as previously described [10]. Briefly, after choosing representative areas, two independent tissue cores with a diameter of 0.1 cm per tumor or normal kidney tissue were arranged on the recipient TMA block. Fourteen TMA slides each of tumor and normal tissue were generated. One slide contained up to 120 tissue samples from 60 patients.

2.3. Immunohistochemistry (IHC)

The TMA slides were immunohistochemically stained for vimentin (clone V9, ready to use, Dako, Glostrup, Denmark), CK7 (clone OV-TL, ready to use, Dako), CAIX (clone EP161, Cell Marque, Rocklin, USA), CD10 (clone 56C6, ready to use, Dako), RCC-Ma (clone SPM314, ready to use, Dako), AMACR (clone 14H4, ready to use, Dako), cathepsin K (clone 3F9, ready to use, Master Diagnostica, Sevilla, Spain), TFE3 (clone MD37/MRQ-37, ready to use, Master Diagnostica), MelanA (clone A103, 1:50, Dako), HMB45 (clone HMB45, ready to use, Dako) and CD117 (A4502, 1:100, Dako, Glostrup, Denmark). Antigen retrieval was performed at pH 9 for vimentin, CAIX, CK7, CD10, AMACR, cathepsin K, TFE3, MelanA, HMB45 and CD117 and at pH 6 for RCC-Ma.

Vimentin, CK7, RCC-Ma, CD10, AMACR and CD117 were stained with automatized immunostainers (autostainer plus, Dako) and

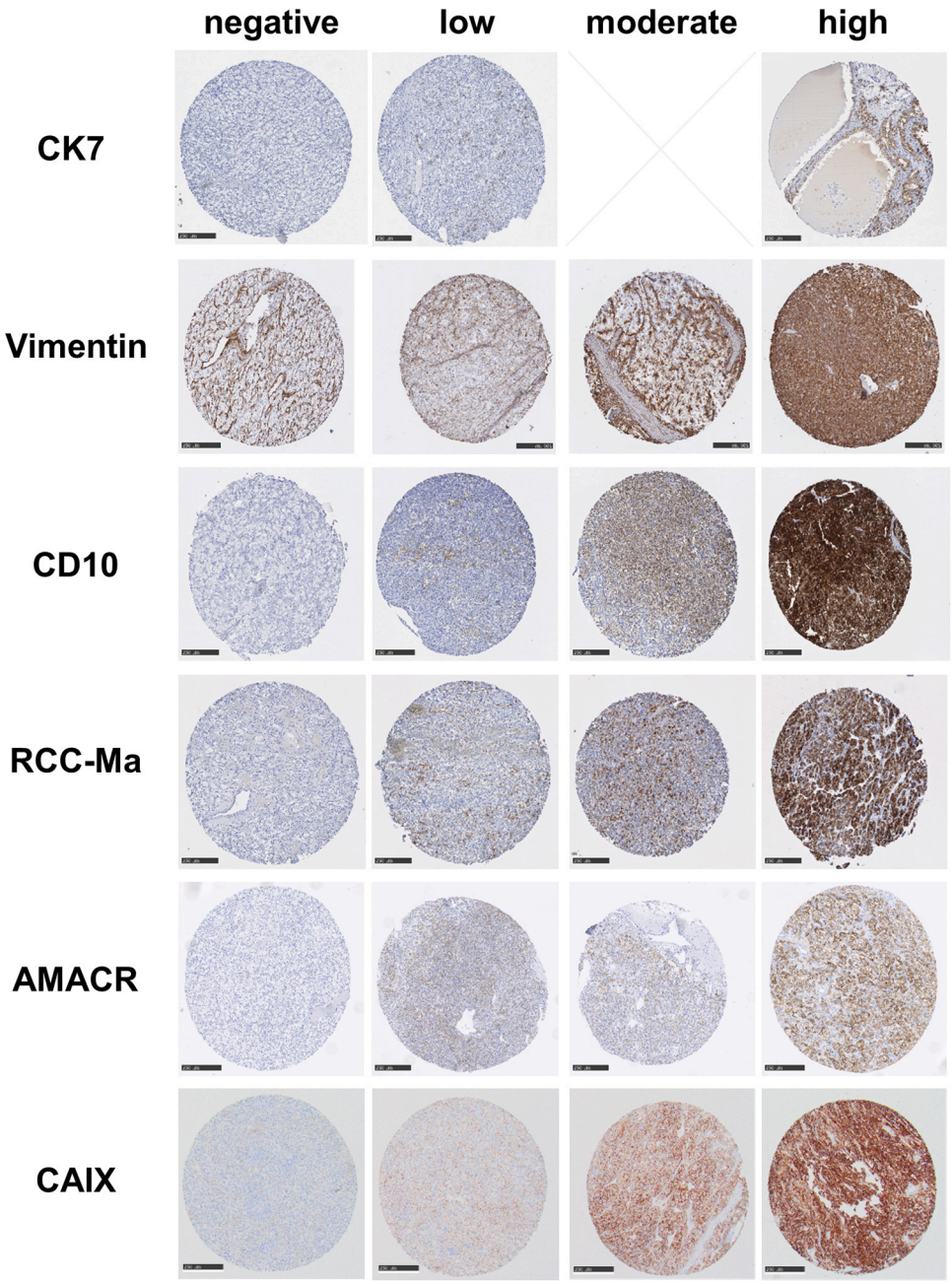

Fig. 1. Immunohistochemical staining variability in clear cell renal cell carcinoma (ccRCC). The tissue microarray was stained for CK7, vimentin, CD10, RCC-Ma, AMACR and CAIX. CK7 expression was scored as low in tumors with single positive tumor cells and as high when larger groups of tumor cells were positive. All other markers were semiquantitatively scored to form an immunoreactive score (IRS) and further subgrouped into negative (IRS: 0), low (IRS: >0–4), moderate (IRS: >4–8) or high (IRS: >8–12) positivity.

Cathepsin K, TFE3, MelanA and HMB45 with the Dako Omnis automated staining platform.

2.4. Semiquantitative scoring

The staining of vimentin, CAIX, CD10, RCC-Ma, CD117 and AMACR was analyzed using a semiquantitative system: Based on the intensity the staining quality was classified as negative (0 points), weak (1 point), moderate (2 points) or strong intensity (3 points). The proportion of stained tumor cells was assessed and the distribution of the values visualized using bar plots. Based on these distribution, the quantity of positive tumor cells was further subgrouped in < 1 % (0 points), 1–10 % (1 point), 10–50 % (2 points), 50–80 % (3 points) or > 80 % (4 points). Quantity and quality were multiplied to calculate an immunoreactive score (IRS) ranging from 0 to 12 points. Score values from 1-4 were considered weak, 5–8 moderate and 9–12 strong. CK7 expression was low in tumors with single positive tumor cells and high when larger groups of tumor cells were positive. Cathepsin K, TFE3, Melan A and HMB45 were classified as either negative or positive.

2.5. Tumor reclassification

All tumors were critically re-evaluated regarding the RCC subtype. The exclusion criteria were as follows:

Table 2
Immunoprofile of ccRCC.

Biomarker	IRS		neg		low pos		mod pos		high pos			total pos		NA
	mean	STD	N	%	N	%	N	%	N	%	p value	N	%	N
CK7	NA	NA	602	83.4	87	12.0	NA	NA	29	4.0	**< 0.001**	116	16.1	9
Vimentin	7.2	3.9	4	0.6	198	27.6	232	32.4	283	39.5	**< 0.001**	713	99.4	10
RCC-Ma	4.9	3.5	85	11.9	256	35.8	268	37.4	107	14.9	**< 0.001**	631	88.1	11
AMACR	1.0	2.2	481	66.9	173	24.1	50	7.0	15	2.1	**< 0.001**	238	33.1	8
CD10	5.7	3.6	55	7.8	214	30.2	297	41.9	143	20.2	**< 0.001**	654	92.2	18
CD117	0	0	719	100.0	0	0	0	0	0	0	NA	0	0	8
CAIX	6.4	3.1	19	2.6	196	27.3	345	48.0	159	22.1	**< 0.001**	700	97.4	8
Cathepsin K	NA	NA	718	100.0	NA	NA	NA	NA	NA	NA	NA	0	0.0	9
MelanA	NA	NA	719	100.0	NA	NA	NA	NA	NA	NA	NA	0	0.0	8
HMB45	NA	NA	717	100.0	NA	NA	NA	NA	NA	NA	NA	0	0.0	10
TFE3	NA	NA	717	100.0	NA	NA	NA	NA	NA	NA	NA	0	0.0	10

IRS: immunoreactive score; STD: standard deviation; neg: negative; pos: positive; mod: moderate; NA: not analyzed

1. pRCC: predominant papillary growth pattern with fibrovascular cores and the presence of associated foamy macrophages. There was strong immunohistochemical positivity for AMACR.
2. chRCC: solid sheets of tumor cells with raisinoid nuclei and perinuclear halos and immunohistochemical positivity for CK7 and CD117.
3. ccpRCT: predominant tubulopapillary growth pattern with apically orientated low-grade nuclei, strong immunohistochemical positivity for CK7 and characteristic cup-shaped staining pattern for CAIX. Negativity for AMACR.
4. MITF-RCC: predominant papillary growth pattern, tumor cells with voluminous cytoplasm and high-grade nuclei, focal psammomatous calcifications. Positivity for Cathepsin K, Melan A or HMB45 and TFE3 for further characterization. Additional molecular analyses for further distinction between TFE3-translocated and TFEB-altered RCC were not performed due to the low number of cases.
5. Clear cell renal cancers with leiomyomatous stroma (RCCLS): (partial) encapsulation with prominent fibrotic stroma and clear cell tumor cells with voluminous cytoplasm and low-grade morphology.
6. Multilocular cystic renal neoplasm with low malignant potential (MLCNLMP): cystic tumors with a lining of tumor cells with clear cytoplasm and low-grade nuclei, no major regressive changes, and no invasive tumor foci.

Sarcomatoid or rhabdoid RCC without differentiated tumor components were classified as RCC not otherwise specified (NOS).

2.6. Tumor necrosis

Information on the presence of tumor necrosis and the proportion relative to the total tumor area was collected for all tumors via morphological examination using a light microscope.

2.7. Statistics

The free software package R (version 4.2.0) and the RStudio platform were used for data analysis and plot generation. For comparison of categorical data, the chi-squared test was used, and the Wilcoxon test was used for count data. Disease specific survival (DSS) was calculated as the time from the day of surgery to ccRCC-dependent death and overall survival (OS) was defined as the time from the day of surgery to death from any cause. Metastasis free survival was calculated as the time from the day of surgery to the development of the first distant metastasis. Survival curves were generated with the Kaplan—Meier method and statistically compared with the log rank test. The Cox proportional hazards regression model was used for univariate and multivariate data analyses. Comparisons between groups were performed using the Wilcoxon test, Student's t test, chi-squared test or Chi-square Goodness-of-Fit test as appropriate. Correlation analysis was performed calculating the correlation coefficient of either Pearson or Spearman, depending on the results of the pre-test for normal distribution assessed with the Shapiro-Wilk-test. Differences with error probabilities of $p < 0.05$ were considered significant.

3. Results

3.1. Tumor reclassification

All 764 tumors, initially classified as ccRCC, were critically re-evaluated regarding the subtype in consideration of morphological and immunohistochemical characteristics. The diagnosis of ccRCC was confirmed in 727 patients. Three tumors were reclassified as MLCNLMP, four each as ccpRCT, chRCC or RCC NOS, five as RCCLS, six as pRCC and eleven as MITF-RCC. Critical cases regarding the diagnosis were discussed between PS and SP with substantial agreement (Cohen's Kappa: 0.779, $p < 0.001$).

3.2. Clinicopathological characteristics of the ccRCC patient cohort

In the study cohort of patients with ccRCC, clinical follow-up data were available for 562 patients (77.3 %). In this subset, 142 (25.3 %) patients in total experienced, in part multiple types of disease progression; therefore, patients may be counted in more than one category: 18 (3.2 %) experienced local recurrence, 20 (3.6 %) experienced contralateral ccRCC, and 123 (21.9 %) experienced metachroneous relapse at distant sites. In total, 313 patients (55.7 %) died during the follow-up, 93 patients (16.5 %) died due to ccRCC. The minimum follow-up time was zero days due to death on the day of surgery, and the maximum follow-up time was 317.1 months (mean 104.2 months, median 87.2 months). Fifteen patients with available clinical follow-up data received drug-based therapy after surgery: three patients received chemotherapy, five patients received interferon or interleukin-based immunotherapy, three patients received mammalian target of rapamycin (mTOR) inhibitors, nine patients received tyrosine kinase inhibitors (TKIs), and one patient received an immune checkpoint inhibitor (ICI). A subset of these patients received multiple therapies. Most ccRCCs were moderately (G2) or poorly (G3) differentiated, staged as pT1, completely resected (R0) and had no synchronous lymph node or distant metastasis (Table 1). Approximately 25 % of the tumors exhibited partial tumor cell necrosis (Table 1). For summaries of the non-ccRCC tumor entities see Supplementary Table 1.

3.3. Immunohistochemistry of ccRCC

Vimentin, CD10, RCC-Ma, CAIX and AMACR showed a broad range of quantities and qualities from completely negative to strongly positive staining patterns (Fig. 1). The variability was described through the IRS (Table 2): 99.4 % of ccRCC were positive for vimentin, 88.1 % for RCC-

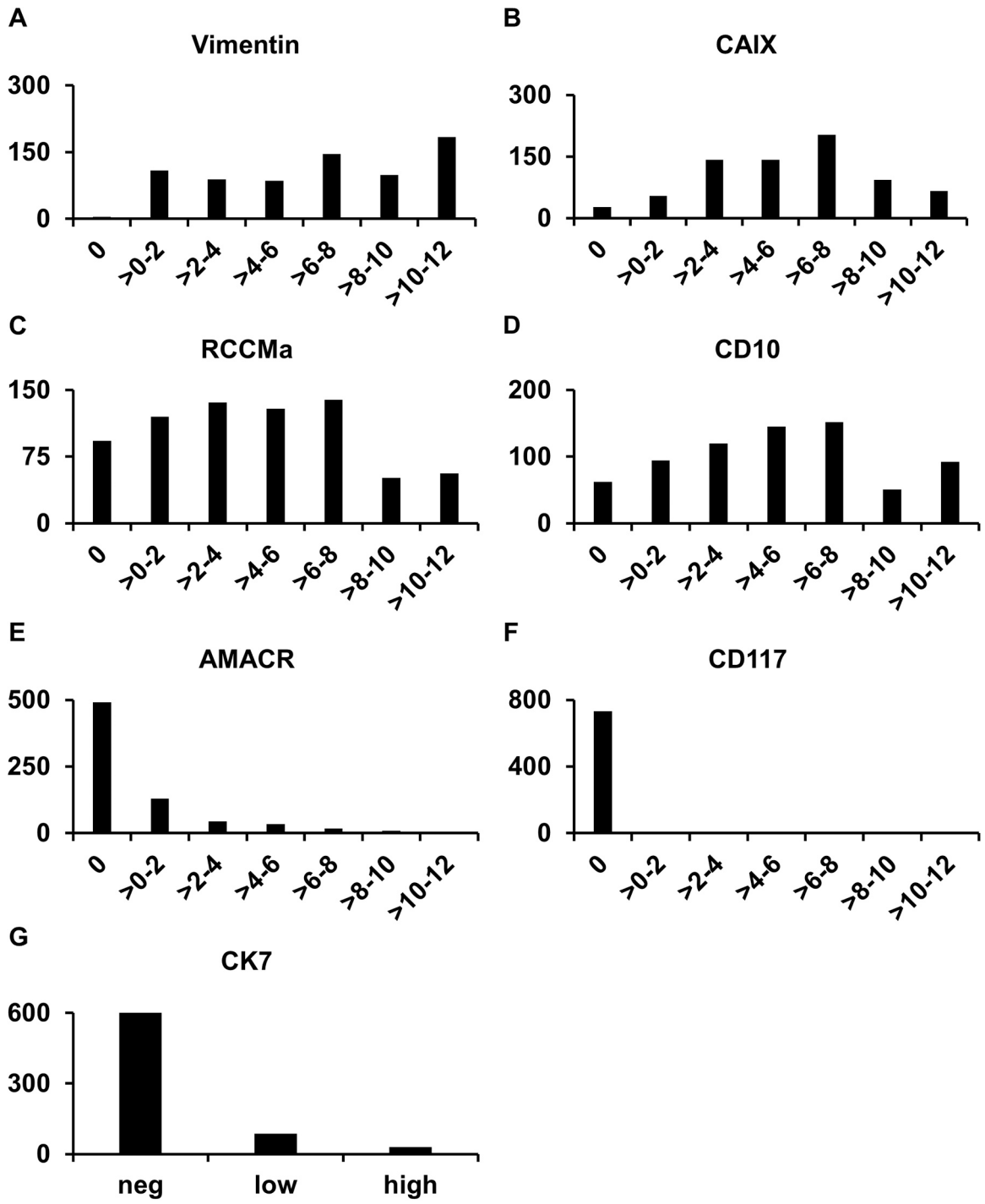

Fig. 2. Distribution of immunohistochemical staining scores in clear cell renal cell carcinoma (ccRCC). Vimentin, carbonic anhydrase 9 (CAIX), renal cell carcinoma marker (RCC-Ma), CD10, α-methylacyl-CoA racemase (AMACR) and CD117 were semiquantitatively analyzed to calculate an immunoreactive score with respect to staining quality and quantity (0: negative; 12: strongly positive). Cytokeratin 7 (CK7) was scored according to the following staining patterns: negative (neg), single-cell positive (low) and positive sheets of tumor cells (high).

Ma, 92.2 % for CD10 and 97.4 % for CAIX. CAIX-negative cases on the TMA were mostly the sarcomatoid or rhabdoid component of a partially dedifferentiated ccRCC. AMACR staining was detected in 33.1 % of the patients, and CK7 was detected in 16.1 % of the patients. Based on the IRS values, the ccRCC were classified into negative tumors or tumors with low, moderate or high positivity. The Chi-square Goodness-of-Fit test was significant, rejecting an equal distribution between the subgroups for each biomarker (Table 2). CD117, cathepsin K, MelanA, HMB45 and TFE3 were negative in all analyzed ccRCC samples. The distribution of the IRS values was approximately normal for CAIX, RCC-Ma and CD10 (Fig. 2). Most ccRCCs were moderately to strongly positive for vimentin, whereas the number of AMACR-positive ccRCCs decreased exponentially with increasing IRS (Fig. 2). Only a minority of patients showed stronger positivity for CK7 than for CK7 in single tumor cells (Fig. 2).

Table 3
Univariate Survival Analysis.

	DSS			OS		
	HR	95 % CI	global p-value	HR	95 % CI	global p-value
Tumor Grading						
G1	ref			ref		
G2	2.1	0.5–8.5	0.3	1.4	0.8–2.4	0.2
G3	6.9	1.7–28.7	**0.010**	2.4	1.4–4.1	**0.002**
G4	69.9	14.6–333.8	**< 0.001**	25.8	11.4–58.6	**< 0.001**
Tumor Stage						
pT1	ref			ref		
pT2	4.2	2.4–7.4	**< 0.001**	1.6	1.1–2.2	**0.006**
pT3	5.4	3.3–8.9	**< 0.001**	2.5	1.9–3.2	**< 0.001**
pT4	18.2	6.3–52.7	**< 0.001**	5.8	2.3–14.1	**< 0.001**
Lymph Node Metastasis						
N0/pN0	ref			ref		
N1/pN1	13.8	7.4–25.4	**< 0.001**	8.5	5.0–14.4	**< 0.001**
Distant Metastasis						
M0/pM0	ref			ref		
M1/pM1	14.6	9.0–23.9	**< 0.001**	5.6	3.7–8.5	**< 0.001**
Lymph Vessel Invasion						
L0	ref			ref		
L1	10.7	4.3–26.8	**< 0.001**	7.7	3.6–16.6	**< 0.001**
Blood Vessel Invasion						
V0	ref			ref		
V1	3.9	2.5–6.0	**< 0.001**	2.4	1.8–3.1	**< 0.001**
Resection Status						
R0	ref			ref		
R1/R2	6.7	3.4–13.0	**< 0.001**	4.1	2.5–6.8	**< 0.001**
Rx	1.8	0.7–4.4	0.2	1.7	1.0–2.9	**0.04**
Sex						
male	ref			ref		
female	1.2	0.8–1.9	0.3	1.0	0.8–1.2	0.9
Age at Surgery						
< 65 years	ref			ref		
> 65 years	1.1	0.7–1.6	0.7	2.2	1.8–2.8	**< 0.001**
Tumor Necrosis						
no	ref			ref		
yes	3.3	2.2–5.0	**< 0.001**	1.7	1.3–2.1	**< 0.001**
CK7						
negative	ref			ref		
low	0.8	0.4–1.5	0.5	0.8	0.6–1.2	0.2
high	0.6	0.2–2.0	0.4	0.7	0.4–1.2	0.2
Vimentin						
negative/ low	ref			ref		
moderate	0.6	0.4–1.1	0.07	0.6	0.5–0.9	**0.002**
high	0.8	0.4–1.2	0.2	0.8	0.6–1.1	0.1
RCC-Ma						
negative	ref			ref		
low	0.3	0.2–0.5	**< 0.001**	0.8	0.6–1.2	0.2
moderate	0.4	0.2–0.6	**< 0.001**	0.7	0.5–1.0	0.1
high	0.4	0.2–0.7	**0.004**	0.8	0.5–1.2	0.4
AMACR						
negative	ref			ref		
low	0.7	0.4–1.2	0.2	0.9	0.7–1.2	0.4
moderate	1.1	0.5–2.4	0.8	1.3	0.8–2.0	0.3
high	1.6	0.5–5.2	0.4	1.3	0.6–2.9	0.4
CD10						
negative	ref			ref		
low	0.4	0.2–1.0	0.06	0.9	0.6–1.3	0.5
moderate	1.1	0.5–2.3	0.8	1.1	0.7–1.6	0.8
high	1.2	0.6–2.7	0.6	1.4	0.9–2.2	0.1
CAIX						
negative	ref			ref		
low	0.8	0.3–2.3	0.7	1.0	0.5–2.2	0.9
moderate	0.4	0.2–1.2	0.1	1.0	0.5–2.1	0.9
high	0.6	0.2–1.7	0.3	1.1	0.5–2.2	0.9

DSS: disease specific survival; DFS: disease free survival; OS: overall survival; HR: hazard's ratio; CI: confidence interval; ref: reference

3.4. Survival analysis of the study cohort

Next, the prognostic value of the biomarkers with staining variability was assessed in addition to standard prognostic parameters. In the univariate survival analysis using the Cox proportional hazards model, the established prognostic factors tumor grade and stage, lymph node and distant metastasis, blood and lymph vessel invasion, resection status and tumor necrosis were significantly associated with patient survival (Table 3). Compared with RCC-Ma-negative tumors, patients with RCC-Ma-positive tumors had significantly better DSS, but not OS (Table 3, Figs. 3A and 3B). A moderate positivity for Vimentin was significantly associated with a favorable OS compared to ccRCC with negativity or low positivity (Table 3). CD10, CAIX, AMACR and CK7 had no prognostic impact on DSS or OS (Table 3). In the multivariate survival analysis with respect to tumor grade and stage, lymph node and distant metastasis, resection status and tumor necrosis, RCC-Ma positivity was significantly associated with favorable DSS, but not OS, compared to RCC-Ma-negative ccRCC (Table 4). Consequently, loss of RCC-Ma was significantly associated with higher tumor grade, advanced tumor stage, presence of lymph node and distant metastases, invasion of lymph and blood vessels, positive resection margins and the presence of tumor cell necrosis (Table 5).

3.5. Prognostic relevance of RCC-Ma for disease progression

Since RCC-Ma expression was significantly associated with DSS, but not OS, the prognostic relevance of RCC-Ma for disease progression was assessed. At first, all non-metastasized ccRCC were dichotomized into a RCC-Ma-negative and a RCC-Ma-positive subset. Then, the time from surgery to the first metachronous distant metastasis was compared between the two subsets. In the RCC-Ma-negative subset, the mean time to metastasis (15.5 ± 12.0 months) was significantly shorter than in the RCC-Ma-positive subset (63.3 ± 57.8 months) ($p = 0.002$). There were no significant differences in the distribution of the metastatic sites between the two subsets (Supplemental Table 2). Next, the Leibovich risk score (LRS) [11] was utilized for further investigation, which estimates the risk for the development of distant metastasis of primarily non-metastasized ccRCC. Stratification of the patient cohort into low, intermediate and high risk groups showed three distinctly separated survival curves in the Kaplan-Meyer analysis as expected (Fig. 3C). Then, the three risk groups were dichotomized further based on the RCC-Ma expression (negative vs. positive). This modification of the LRS showed, that the curve of patients in the intermediate risk group with RCC-Ma-negative ccRCC was closer to the high risk group compared to the ones with RCC-Ma-positive tumors (Fig. 3D).

3.6. Correlation of the RCC-Ma stainings of TMA and whole slide images

A heterogeneous staining pattern of RCC-Ma has been described previously, when assessed on whole slide images (WSI) of ccRCC [12]. Therefore, the correlation of the RCC-Ma staining results between the TMA and corresponding WSI was investigated in a subset of the patient cohort. Eight cases from the each RCC-Ma subgroup (negative, low, moderate, high positivity) were selected and the corresponding WSI stained for RCC-Ma. For better comparability, the WSI stainings were scored using the IRS and further classified as negative, low, moderate or strong positive as described. The IRS values of the WSI and TMA were correlated significantly (Spearman correlation coefficient r_s: 0.8; $p < 0.001$). Comparison of the categorical staining results showed a significant relationship between WSI and TMA, too (Supplemental Table 3). Representative cases are shown in Fig. 4.

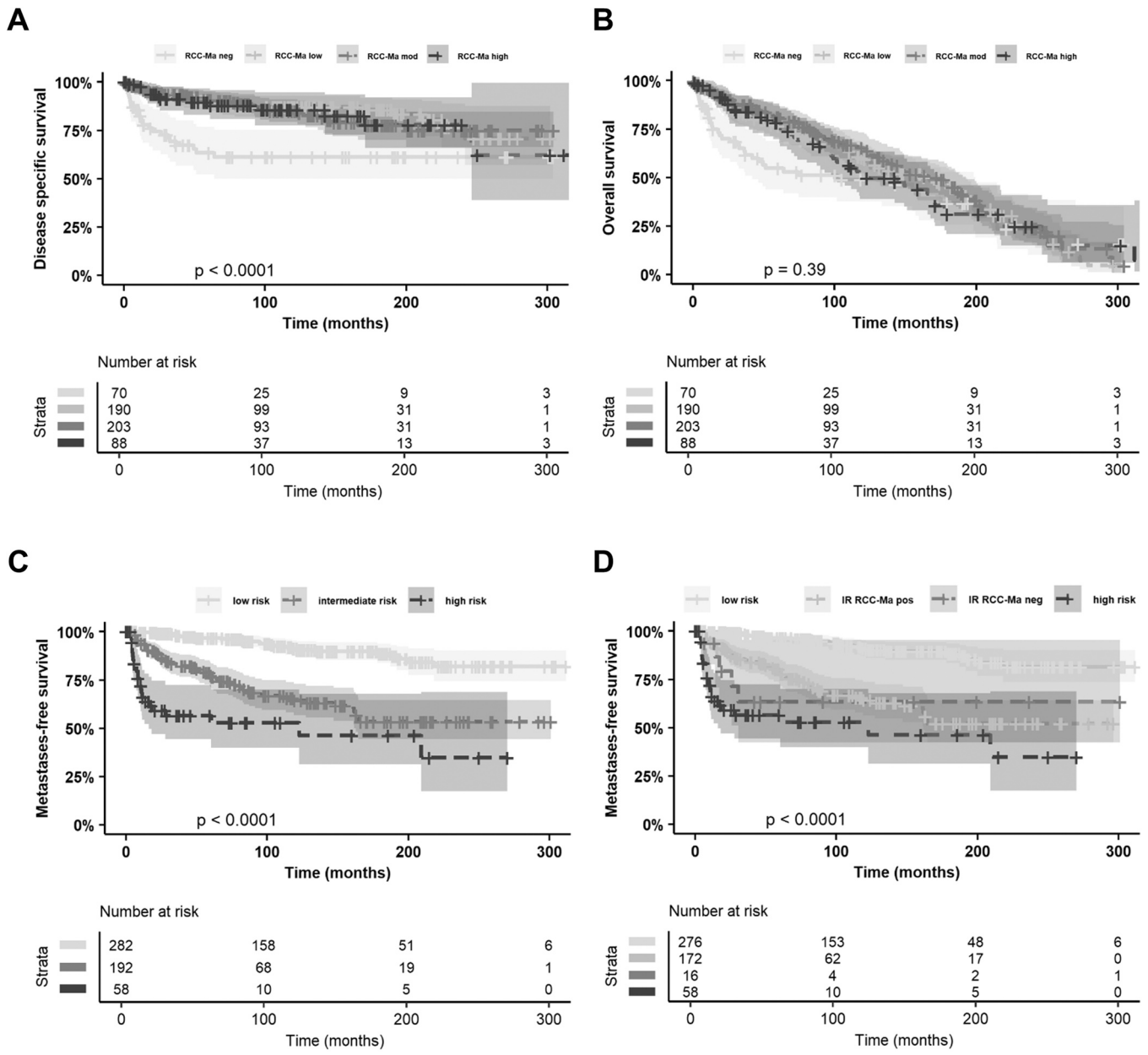

Fig. 3. Association of survival with the expression of renal cell carcinoma marker (RCC-Ma) in clear cell renal cell carcinoma (ccRCC). The expression of RCC-Ma in ccRCC tumors was semiquantitatively analyzed using an immunoreactive score. The study cohort was then subdivided into tumors with no (negative=neg), low, moderate (mod) and high positivity. Kaplan-Meier survival curves for **(A)** disease specific survival and **(B)** overall survival were plotted. Next, the study cohort was subdivided into the three risk groups of the Leibovich Risk Score **(C)**. The intermediate risk group was further subdivided into RCC-Ma positive and RCC-Ma-negative tumors **(D)**. The log-rank test was used for statistical comparison of the groups. Differences with error probabilities of $p < 0.05$ were considered significant.

4. Discussion

The aim of this study was to establish and characterize a ccRCC cohort and to analyze the staining variability of a panel of markers used in diagnostic routine for RCC subtyping to predict patient risk for death and disease progression. The diagnosis ccRCC was confirmed in a total of 727 patients. The number of cases ranged from 1995 to 2006, and only a minority of long-term survivors received modern targeted therapies such as tyrosine kinase inhibitors or immune checkpoint inhibitors. Thus, this ccRCC cohort reflects the natural clinical course after surgery. Approximately 25 % of patients experienced disease progression, which is in line with the literature [13]. Despite the fact that there is currently a plethora of therapeutic options for ccRCC, the results obtained with this study cohort are still in part transferable to today since targeted therapies are mostly administered for patients after disease progression. Therefore, the present study cohort is a valuable reference for patient cohorts receiving up-to-date drug-based therapies after surgery. For use in routine diagnostics, the results show the average staining patterns of the most common renal carcinoma subtype ccRCC. These findings can be used for better discrimination between ccRCC and nonccRCC tumors.

In this study, 88.1 % of ccRCC were positive for RCC-Ma, which is comparable to reported positivity rates between 84 % and 93 % the literature [12,14,15]. The diagnostic value of RCC-Ma for renal neoplasms is limited, because it is considered non-specific for discrimination of RCC subtypes and of primary renal neoplasms vs. neoplasms of other origin [16,17]. In the brain however, RCC-Ma is a helpful marker for for discriminating RCC metastasis from hemangioblastoma [18]. The major results of this study was that loss of RCC-Ma is an independent

Table 4
Multivariate Survival Analysis.

	DSS			OS		
	HR	95 % CI	global p-value	HR	95 % CI	global p-value
Tumor Grading						
G3/G4 vs. G1/G2	1.6	0.9–2.7	0.1	1.4	1.0–1.9	**0.03**
Tumor Stage						
pT3/pT4 vs. pT1/pT2	1.8	1.1–3.0	**0.02**	1.3	0.9–1.8	0.06
Lymph Node Metastasis						
N1/pN1 vs. N0/pN0	3.3	1.6–6.8	**0.001**	3.0	1.6–5.4	**< 0.001**
Distant Metastasis						
M1 vs. M0	5.8	3.3–10.1	**< 0.001**	3.5	2.1–5.6	**< 0.001**
Resection Status						
R1/R2/Rx vs. R0	2.2	1.2–4.0	**0.01**	1.9	1.2–2.8	**0.002**
Age at Surgery						
> 65 years vs. < 65 years	1.0	0.7–1.5	0.9	2.2	1.7–2.8	**< 0.001**
Tumor Necrosis						
yes vs. no	1.8	1.1–3.0	**0.03**	1.2	0.9–1.6	0.1
RCC-Ma						
pos. vs. neg.	0.5	0.3–0.9	**0.01**	1.1	0.7–1.5	0.8

DSS: disease specific survival; DFS: disease free survival; OS: overall survival; HR: hazard's ratio; CI: confidence interval; pos.: positive; neg.: negative

Table 5
Correlation of RCC-Ma with clinicopathological features.

	RCC-Ma				
	N neg.	%	N pos.	%	p-value
Tumor Grading					**< 0.001**
G1	5	0.7	32	4.5	
G2	33	4.6	463	64.7	
G3	35	4.9	128	17.9	
G4	12	1.7	8	1.1	
Tumor Stage					**< 0.001**
pT1	34	4.7	410	57.3	
pT2	14	2.0	68	9.5	
pT3	31	4.3	150	20.9	
pT4	6	0.8	3	0.4	
Lymph Node Metastasis					**0.01**
N0/pN0	77	10.8	611	85.3	
N1/pN1	8	1.1	20	2.8	
Distant Metastasis					**0.01**
M0/pM0	76	10.6	607	84.8	
M1/pM1	9	1.3	24	3.4	
Lymph Vessel Invasion					**0.01**
L0	80	11.2	623	87.0	
L1	5	0.7	8	1.1	
Blood Vessel Invasion					**0.02**
V0	61	8.5	523	73.0	
V1	24	3.4	108	15.1	
Resection Status					**< 0.001**
R0	71	9.9	595	83.1	
R1/R2/Rx	14	2.0	36	5.0	
Sex					0.9
male	54	7.5	410	57.3	
female	31	4.3	221	30.9	
Age at Surgery					1.0
< 65 years	45	6.3	339	47.3	
> 65 years	40	5.6	292	40.8	
Tumor Necrosis					**< 0.001**
no	44	6.1	507	70.8	
yes	41	5.7	124	17.3	

N: number; neg.: negative; pos.: positive

prognostic biomarker for poor DSS of patients with ccRCC (Table 4). In contrast, there was no prognostic association with OS (Table 4). Next to the factor age at surgery, the association of RCC-Ma with metastasis might as well explain the prognostic value for DSS and the lack thereof for OS. Modification of the LRS through inclusion of the RCC-Ma status showed, that RCC-Ma negative ccRCC of the intermediate risk group had a similar risk for distant metastasis as the high risk group (Fig. 3D). This effect was particularly pronounced in the time directly after tumor surgery. This is in line with the findings that risk assessment models for RCC were most predictive within the first two years after diagnosis [19]. The LRS is recommended by the European Association of Urology for risk assessment after surgery of patients with localized RCC: The higher the risk group, the shorter are the monitoring intervals [20]. However, patients of the intermediate risk group are at risk for overtreatment, so that there is a high need for further substratification [21]. To partially solve this issue, the present results suggest that patients in the intermediate risk group of the LRS with RCC-Ma negative ccRCC might profit from the follow-up protocol of the high risk group. The combination of histopathological and immunohistochemical factors, such as LRS and RCC-Ma, is broadly available and cost-effective as previously demanded [7]. In general, the combination of different prognostic factors, e.g. risk score and immunohistochemical biomarker as in this study, seems to provide superior information on patient outcomes compared to the individual factor alone. Other integrated models have proven this concept [22]. Thus, research on biomarkers for RCC should focus on this approach next to the identification of novel targets. In a small subset of ccRCC (n = 32 cases), the correlation between the RCC-Ma staining of the TMA cores and the corresponding WSI was significant. However, there was a certain overlap between the negative cases and cases with low positivity (Supplementary Table 3). Therefore, it is feasible to presume that the results obtained from the TMA are only partially transferable to WSI and that the threshold is in the low positive group when the immunohistochemical RCC-Ma staining is assessed on WSI.

The analyzed tumor entity of the present study cohort was ccRCC and this subtype is derived from cells of the proximal tubule [23]. RCC-Ma is an antibody directed against a glycoprotein of the brush border of the proximal tubule [12]. Mechanistically, loss of RCC-Ma expression seems to represent a dedifferentiation of tumor cells from the cells of origin with more aggressive behavior. In line with this observation, loss of CD15, which is another marker of the proximal tubule, is associated with adverse prognosis in ccRCC [24,25]. Decrease of CD10, a third marker of the proximal tubule, was associated with higher tumor grade, but with limited prognostic value [26]. So far, of the three markers RCC-Ma must be considered the strongest prognosticator for ccRCC. To date, the underlying biology of RCC-Ma has not yet been fully investigated. The association with metastasis indicates a role in tissue homeostasis and integrity, so that the loss of RCC-Ma putatively leads to increased tumor cell dissemination and consequently metastatic spread to other organs. On the other hand, a role as transepithelial transport glycoprotein or a secretory function were suspected in the initial description of RCC-Ma because of the membranous localization [12]. Indeed, the proximal tubule in the kidney is involved in reabsorption processes, e.g. glucose uptake. Therefore, loss of RCC-Ma could also be a surrogate for altered tumor cell metabolism due to altered uptake of metabolites. Further studies on RCC-Ma should focus on its biological role and predictive value for targeted therapies.

The other analyzed immunohistochemical biomarkers had no significant or only minor prognostic impact on survival of patients with ccRCC. Moderate, but not high vimentin expression was associated with favorable OS compared to tumors with no or low vimentin positivity (Table 3). They were consequently not included in the multivariate analysis (Table 4). In the literature, high vimentin expression was associated with an immunosuppressive tumor microenvironment in metastatic renal cell carcinomas [27]. There were few patients with vimentin-negative ccRCC in this cohort (Table 2). Vimentin expression is considered a hallmark of this tumor entity; however, ccRCC without

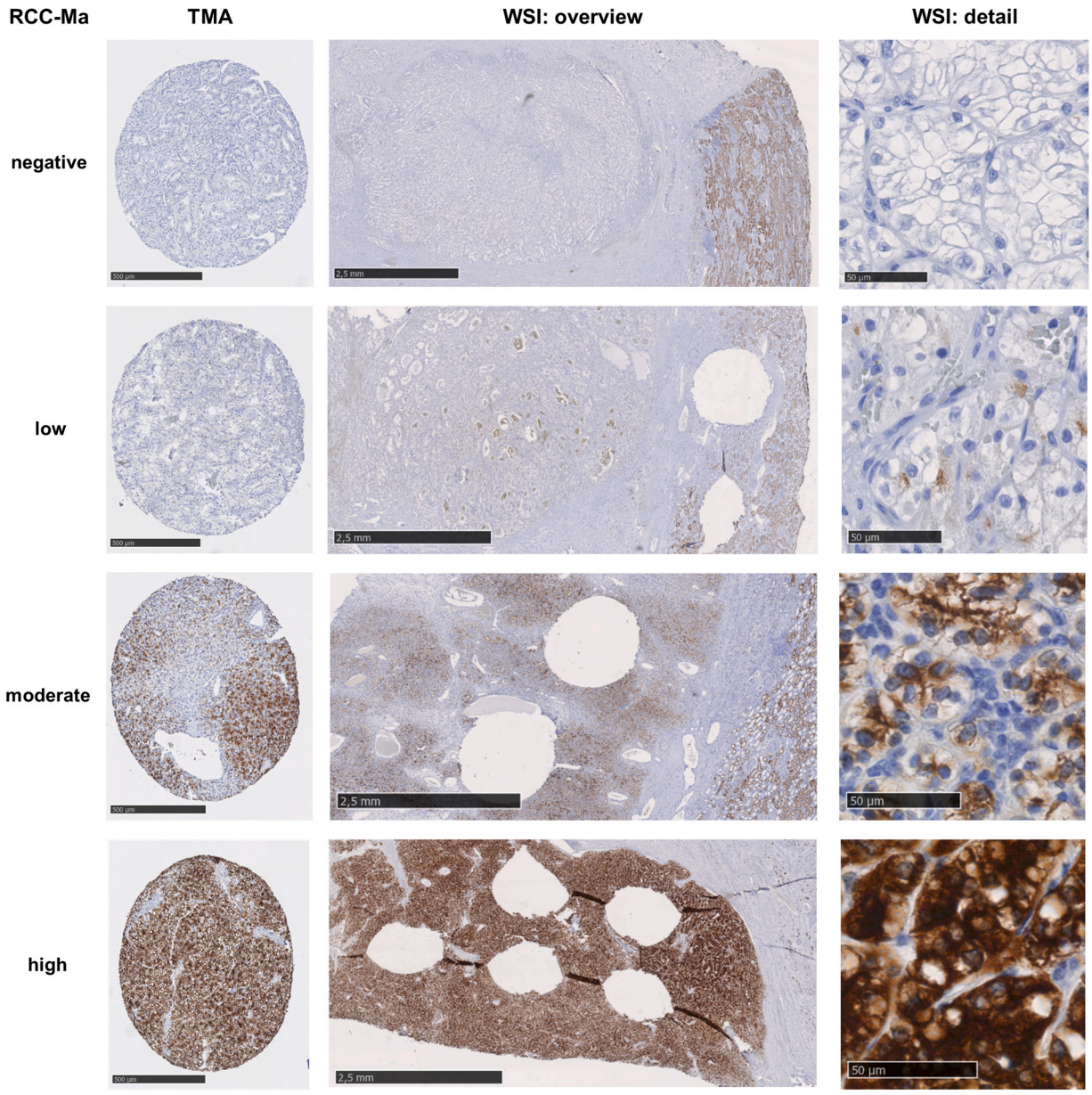

Fig. 4. Correlation of the immunohistochemical RCC-Ma stainings between the tissue microarray (TMA) and corresponding whole slide images (WSI). Representative cases of RCC-Ma negative clear cell renal carcinoma (ccRCC) and ccRCC with low, moderate and high RCC-Ma positivity were selected. The corresponding whole slides, which were used for the construction of the TMA, were stained for RCC-Ma with the same staining protocol.

vimentin expression has been described previously and is associated with better long-term survival [28], whereas vimentin overexpression, defined as > 50 % positive tumor cells, is associated with unfavorable outcomes [29]. Thus, the results on vimentin as a prognostic factor remain inconclusive. Further studies on vimentin are warranted, because vimentin expression is associated with poor survival in gastric cancer [30], but favorable survival in endometrial cancer [31]. CAIX expression is considered a diagnostic hallmark of ccRCC, because it is a downstream target of the hypoxia-inducible factor 1a (HIF1a) pathway, which is in turn stabilized due to the mutational loss of the von-Hippel-Lindau protein. In the present study, 97 % of ccRCC were positive for CAIX, the majority of whom had moderate CAIX positivity. The negative cases were mostly partially dedifferentiated ccRCC, where the sarcomatoid component was part of the TMA. In this study, there was no significant association with survival (Table 3). In the literature, there were reports on CAIX as an independent prognostic factor for ccRCC [32,33], but also with weaker prognostic impact [34]. Investigations on the use as a predictive biomarker for cytokine therapy or targeted therapy for patients with metastasized RCC, such as sorafenib, found significant association of CAIX with response to therapy [35]. To date, CAIX does not contribute to choice of therapy. AMACR positivity is usually considered a diagnostic feature of pRCC. However, approximately 33 % of the ccRCC tumors in this study were AMACR-positive, in part with a surprisingly strong expression. Thus, AMACR positivity is not exclusive to pRCC, but is also seen in ccRCC. For the latter entity, there is no major prognostic value of AMACR positivity [36], which is in line with the results presented in this study. Low expression of AMACR in prostate cancer was associated with disease progress and death [37]. On the contrary, investigations on AMACR expression in ovarian or colorectal cancer showed equivocal impact on patient outcomes [38,39].

The major limitations of our work are the retrospective study design and the varying duration of FFPE tissue storage. The long time span of the selected patients may lead to differences in clinical and surgical

results due to changes in treatment modalities. For some patients in the study cohort, clinical outcomes were not available. In a preliminary data set of 32 ccRCC, immunohistochemical RCC-Ma stainings correlated significantly between WSI and TMA, but the cutoff value for RCC-Ma obtained from the TMA-based approach may be limited to larger ccRCC tumor specimens. Thus, further research is needed to validate this cutoff.

5. Conclusions

Taken together, the data indicate that the patient cohort included in this study is a well-characterized ccRCC cohort with comprehensive information on clinicopathological features. The clinical course of the disease is unbiased through modern adjuvant targeted therapies. RCC-Ma was an independent prognostic biomarker for DSS and is likely to have implications for monitoring of patients after surgery.

List of Abbreviations

AMACR: alpha-methylacyl-CoA racemase
CAIX: carboanhydrase 9
ccpRCT: clear cell papillary renal cell tumor
ccRCC: clear cell renal cell carcinoma
chRCC: chromophobe renal cell carcinoma
DSS: disease-specific survival
FFPE: formalin-fixed paraffin-embedded
ICI: immune checkpoint inhibitor
IHC: immunohistochemistry
IRS: immunoreactive score
LRS: Leibovich Risk Score
MLCNLMP: multilocular cystic renal neoplasm with low malignant potential
MITF-RCC: microphthalmia-associated transcription factor translocation renal cell carcinoma
OS: overall survival
pRCC: papillary renal cell carcinoma
RCC: renal cell carcinoma
RCCLS: renal cell carcinoma with leiomyomatous stroma
RCC NOS: renal cell carcinoma not otherwise specified
TMA: tissue microarray
WSI: whole slide images

Ethics approval and consent to participate

This study protocol was designed in accordance with the Declaration of Helsinki. The use of excess tissue samples older than four years was approved, and the need for written informed consent from the patients was waived by the Ethics Committee of the State Medical Association of Rhineland-Palatinate due to the retrospective design of the study (ethics approval number: 837.360.16 (10679)).

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CRediT authorship contribution statement

PJ Stenzel: Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Visualization, Project Administration; **KE Tagscherer:** Conceptualization, Writing - Review & Editing; **C Justenhoven:** Writing - Review & Editing; **PJ Wild:** Writing - Review & Editing; **A Haferkamp:** Writing - Review & Editing; **S Macher-Goeppinger:** Conceptualization, Writing - Review & Editing; **W Roth:** Conceptualization, Writing - Review & Editing; **S Frees:** Resources, Writing - Review & Editing, Supervision; **S Porubsky:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2025.155919.

Data Availability

The dataset analyzed during the current study is available from the corresponding author upon reasonable request.

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