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ORIGINAL ARTICLE

Immunohistochemical marker panel differentiates between the three most common subtypes of renal cell carcinoma independent from histomorphologic criteria

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Abstract To develop a reliable immunohistochemical marker panel differentiating between the three most common renal cell carcinoma (RCC) subtypes without inclusion of histomorphologic criteria we investigated protein expression of vimentin, glutathione S-transferase alpha (GST-α), CD10, CD117 (C-KIT), carbonic anhydrase 2 (CAII), parvalbumin, alpha-methyl-CoA-racemase (AMACR), and cytokeratin-19 (CK 19) in 65 age and stage matched trios of clear cell carcinoma, papillary renal carcinoma and chromophobe renal carcinoma using tissue microarrays. All markers displayed significant differential expression among the subtypes ($p<0.001$) except CAII ($p=0.192$). According to positive (LR+) and negative (LR−) likelihood ratio, six markers (CD10, GST-α, AMACR, CK19, C-KIT and arvalbumin) demonstrated acceptable or good values to detect certain subtypes of RCC, but failed in terms of ruling out the respective subtypes. Based on the individual performance of these six markers, we combined them and reviewed each single case: LR+ for detection of clear cell RCC considerably increased by application of the six marker panel, but did not exceed 10. LR− was still >0.1; in case of papillary RCC LR+ rose beyond 10, but LR− remained >0.1. LR+ for recognition of chromophobe RCC rose far beyond 10, but LR− remained >0.1. Thus, the panel can reliably recognize two main RCC subtypes without inclusion of histomorphologic features. Further improvement is needed for consistent detection of clear cell RCC and for dependably ruling out all three main subtypes as well as identification of rare variants and benign lesions like oncocytoma.

Keywords Renal cell carcinoma · Subtype differentiation · Renal mass biopsy · Immunohistochemistry

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Introduction

Renal neoplasms comprise several distinct clinicopathologic entities with potential prognostic and therapeutic differences. More than 95% of clinically significant lesions can be diagnosed as one of the common subtypes of renal epithelial tumors: clear cell renal cell carcinoma (cc-RCC), papillary

RCC (pp-RCC) and chromophobe (cp-RCC) [1]. These distinct types of renal tumors may have overlapping morphological features including variable cytoplasmic staining and architectural patterns, making diagnosis sometimes challenging [2]. Considering the increasing role for percutaneous biopsy procedures in the management of solid renal masses, differentiation between the three subtypes has become still more difficult and subjective in many cases [3, 4]. In these small biopsies, the entire range of cytoarchitectural features may not be fully represented. Each of these three carcinoma subtypes is accompanied by the expression of a certain spectrum of antigens that can be detected immunohistologically. The list of candidate genes has expanded considerably in the recent past and several molecular markers have been investigated to optimize subtype differentiation in RCC [2, 5]. We found GST-α and CD10 as commonly quoted markers for clear cell RCC, CK-19 and AMACR for papillary RCC and C-KIT Parvalbumin plus Vimentin for chromophobe RCC.

cc-RCC is frequently associated with high expression of GST-α [6, 7] and CD10 [8–12]. Both markers are already in use to differentiate the most common subtypes in RCC [5, 11, 13]. Furthermpre, lack of expression for C-KIT [14, 15] and Parvalbumin [5, 16] in connection with cc-RCC histology is discussed as a typical feature of this subtype. Subtype specific markers in terms of pp-RCC by contrast are not as pronounced. High AMACR expression is seen both in pp-RCC and cc-RCC [17, 18]. Although CK 19 was reported to display strong positivity in pp-RCC specimens [19], validation is still lacking. Lack of immunoreactivity for C-KIT and Parvalbumin is also a typical characteristic of pp-RCC [16, 20–22] while chromophobe (cp) RCC was repeatedly shown to be associated with highest expression of both markers: Sensitivity was consistently reported to be about 100% (C-KIT [15, 21–23], parvalbumin [16, 24–26]). Vice versa loss of expression for Vimentin is referred to as a distinguishing marker for cp-RCC and specificity is reported to be about 80–100% in the literature [24, 25, 27]. CA-2 expression in RCC was found to be highly sensitive in terms of chromophobe RCC in one of the earliest gene array profile studies [28]. However, further publications analyzing CA-2 immunostaining in RCC tissue specimens are lacking.

Our objective was to investigate the selected markers as a panel in an adequate case numbers of the three RCC main subtypes. Depending on the generated sensitivity and specificity for each examined marker we were searching for a marker panel allowing the differentiation of the three RCC subtypes with a maximum of likelihood and a minimum of markers. For this purpose, we constructed a tissue microarray containing 65 cc-RCC, pp-RCC and cp-RCC specimens, respectively. All cases were matched regarding age, grade and tumor stage leading to a comprehensive study cohort for all tumor subytpes.

Materials and methods

Clinical data

A total of 65 age-, stage-, grade- and gender-matched trios of each clear cell, papillary and chromophobe RCC were retrieved from archives of three pathology institutes (Table 1). Patients with familial RCC syndromes (e.g., von Hippel–Lindau disease) were excluded. All cases were reviewed by two experienced uropathologists (A.H. and H.M.) according to the 2004 WHO classification and 2002 UICC TNM system. A consensus of the histopathological classification was reached for all cases. Cases with questionable histology or other histopathological entities were excluded from the study.

Immunohistochemistry

A Tissue Micro Array (TMA) of representative areas from formalin-fixed, paraffin-embedded tumor material was established as described previously [29]. Briefly, suitable areas with representative tumor were marked on standard haematoxylin and eosin (H&E)-stained sections, punched out of the paraffin block and inserted into a recipient block. The punch diameter was 1.5 mm. The freshly cut 5-μm sections of the resulting blocks underwent strictly synchronous treatment throughout all staining procedures. After deparaffinization, rehydration and microwave treatment according to standard procedures, staining with primary monoclonal antibodies to AMACR (Dako M361,1:10), Vimentin (Dako M0725, 1:50; no microwave treatment), Cytokeratin 19 (Dako M0888, 1:100) and CD117 (Dako A4502, 1:200) were performed on a Ventana Nexes immunohistochemistry device. The antibodies to GST-α (Acris Antibodies BP144, 1:50), CAII (Santa Cruz Biotechnology

Table 1 Patient's tumor characteristics

RCC subtype	Clear cell	Papillary	Chromophobe
Total number (*n*)	65	65	65
pT1a	24	24	24
pT1b	18	18	18
pT2	10	10	10
pT3a	8	8	8
pT3b	5	5	5
pN2	2	2	2
G1 (Fuhrman)	44	44	44
G2 (Fuhrman)	14	14	14
G3 (Fuhrman)	7	7	7
Male Pat.	39	48	30
Female pat.	26	17	35
Median pat. age	66 (25–88)	66 (26–85)	66 (36–84)

sc25596, 1:50), Parvalbumin (Abcam, ab11427, 2 μg/ml) and CD10 (Novocastra NCL-L-CD10-270, dilution: 1.25) were used together with the Envison + Dual Link System HRP detection (Dako). Hale's colloidal iron staining was performed using Muller–Mowry staining method [30].

Evaluation of immunoreactivity was performed in accordance to previously published studies using an approach incorporating staining intensity as well as amount of stained cells or nuclei.

AMACR staining was negative if intensity was not exceeding weak staining in at least 5% of tumor cells. When at least 5%, but not more than 50%, of cells were stained weakly, moderately or strongly, this was considered focal positive. When more than 50% of cells were stained weakly, moderately or strongly, the case was rated diffusely positive. Vimentin and Parvalbumin staining were negative if less than 30% of cells demonstrated cytoplasmic reactivity or staining was not more than moderate. Only strong staining in at least 30% of cells was considered positive. CK-19 staining was negative if no peroxidase reaction was observed. Weak reactivity or moderate to strong staining in less than 30% of cells was rated weakly positive, while moderate to strong staining in at least 30% was considered as strong positivity. CD117 was rated positive if weak staining was exceeding 50% of cells or reactivity was at least moderate. GST-α staining was negative if no reactivity was observed. If less than 30% of cells stained or staining was weak, the case was rated weakly positive. When these criteria were exceeded, staining was considered as strongly positive. CD10 staining was considered positive if a minimum of 2% of cells demonstrated at least weak membranous staining. CA-II staining was considered positive when moderate and strong immunoreaction was present in at least 10% cells and considered negative when no or weak staining was present.

Scoring of the immunohistochemical data was done by two Uro-Pathologists (A.H. and H.M.) who had no knowledge of the clinical data.

Statistical analysis

Statistical analyses were completed using SPSS version 15.0 (SPSS, Chicago, IL). Values of $p<0.05$ were considered significant. Exact χ^2 tests were used to study the statistical association between the three groups.

Results

Of the 195 tumor specimens on the array, 159 to 176 could be successfully analysed for the respective stainings. Non-informative spots in the majority of cases resulted from missing tissue or tumor cells.

GST-α staining resulted in strong positivity in 93.8% (60/64) cc-RCC tumors. This was in contrast to papillary and chromophobe tumors, which expressed GST-α in 44.1% (26/59) or 19.6% (9/46) of cases. This differential expression was significant ($p<0.001$).

In total, 77.8% (42/54) of the cc-RCC specimens were marked by strong CD10 reactivity, while pp-RCC tumors stained positive in 51.8% (27/56) and cp-RCC tumors displayed reactivity in 29.5% (13/44). These subtype specific differences again reached statistical significance ($p<0.001$). Sensitivity and specificity resulted in positive likelihood ratio (LR+) for diagnosis of cc-RCC using GST-α that was below 10 (1.9); for CD10, it was 2.8. LR− for exclusion of cc-RCC was 0.4 for GST-α and 0.1 for CD10 (Fig. 1).

Diffuse AMACR staining was observed in 27.0% (17/63) of the cc-RCC tumors and in 34.1% (15/44) of the cp-RCC specimens, while papillary carcinomas displayed diffuse staining pattern in 88.3% (53/60). Overall, 74.6% (47/63) pp-RCC tumors were strongly positive for CK-19. In contrast, only 15.4% (10/65) cc-RCC tumors and 23.9% (10/43) cp-RCC tumors were CK-19 positive. Subtype specific differences for CK-19 reached statistical significance ($p<0.001$). LR+ for diagnosis of pp-RCC by AMACR dropped below 10 (2.9), as it did for diagnosis pp-RCC tumors by CK19 (4.0). LR− for exclusion of pp-RCC was 0.3 for AMACR and 0.2 for CK-19 (Fig. 2).

Overall, 76.1% (35/46) of the cp-RCC tumors stained C-KIT positive. In contrast, only 4.8% (3/63) pp-RCC tumors expressed C-KIT, and all 60 (100%) cc-RCC specimens were C-KIT negative. These staining differences were highly significant ($p<0.001$). Parvalbumin expression was positive in 87.8% (35/45) of the cp-RCC tumors, while 78.2% (43/55) pp-RCC tumors and all 62 cc-RCC (100%) were Parvalbumin negative; these observations were also statistically significant ($p<0.0001$).

LR+ for diagnosis of cp-RCC by C-KIT reached a value high that exceeded 10 (34.7), but dropped below 10 for detection of cp-RCC by Parvalbumin (7.5). LR− for exclusion of cp-RCC by C-KIT was 0.2, as well as by Parvalbumin (0.2) (Fig. 3).

Vimentin negative tumors were found in 95.7% (44/46) of the cp-RCC cases, while cc-RCC tumors expressed Vimentin in 35.9% (23/64) and pp-RCC tumors in 38.7% (24/64). This differential expression was significant ($p<0.05$). Sensitivity and specificity resulted in LR+ of 8.8 and LR− of 0.8 (Fig. 4).

Carboanhydrase II staining was present in 79.7% (51/64) of cc-RCC cases and 81% (47/58) pp-RCC tumors. It was noted that 92.9% (39/42) of the cp-RCC tumors displayed positive CA-2 reaction. Significance for differential expression failed ($p=0.5$), and sensitivity and specificity resulted in LR+ far below 10 and LR− >0.1 (Fig. 4).

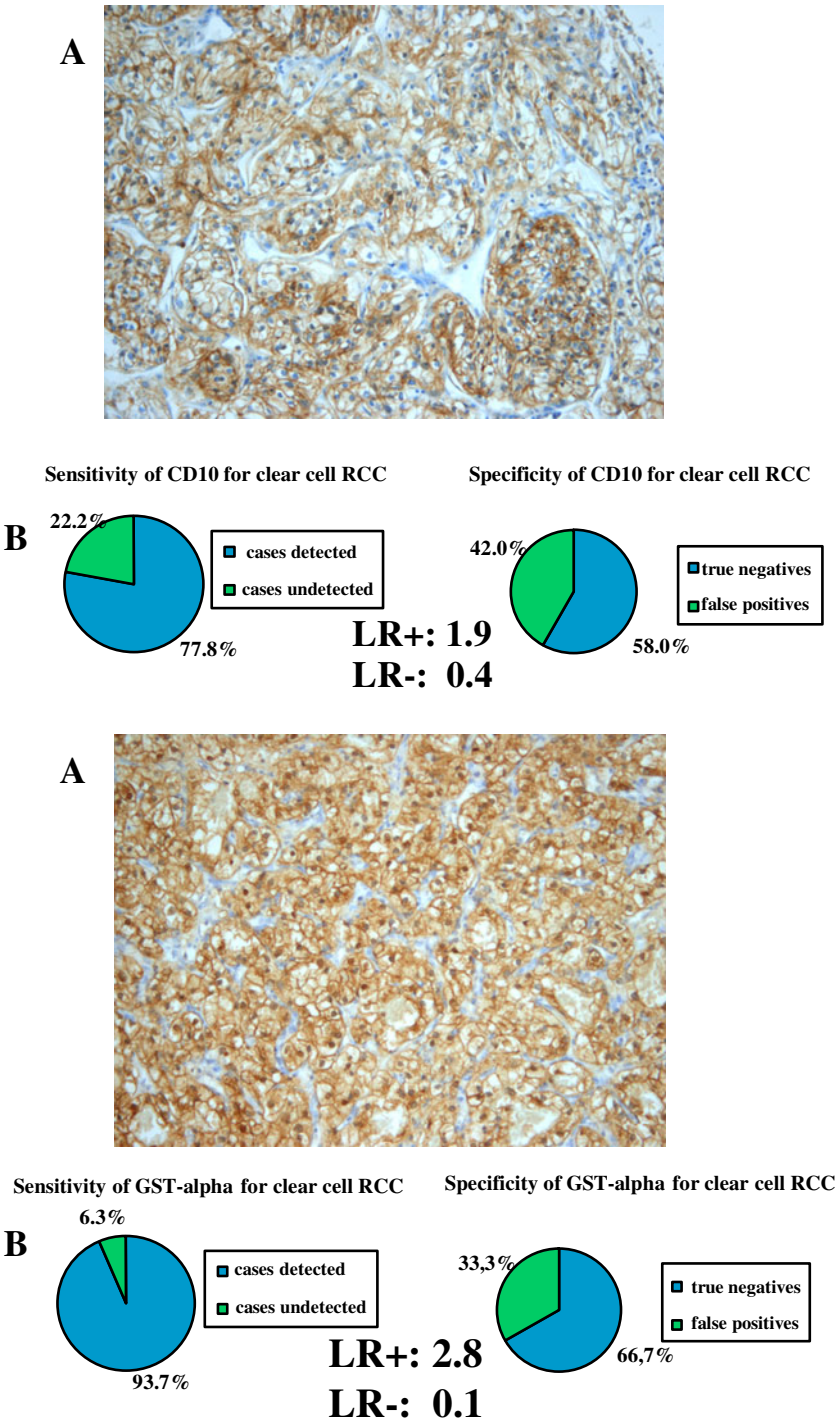

Fig. 1 GST-α and CD10 performance as potential markers to diagnose clear cell RCC. **a** CD10 and GST-alpha positive cc-RCC tumors. **b** Sensitivity and specificity resulted in LR+ <10 and LR- >0.1 for both markers

Hale's colloidal iron staining resulted in strong positivity in only 31.2% (20/64) cp-RCC tumors. This was in contrast to papillary and clear cell tumors, which were negative for Hale colloidal iron. This differential expression was significant ($p<$ 0.001). LR+ for detection of cp-RCC by Hale iron was 2.4. LR− for exclusion of cp-RCC by colloidal iron was 0.43.

Based on the performances of the markers for the detection of one subtype, we combined a marker set containing CD10, GST-α, AMACR, CK19, C-KIT and Parvalbumin and reviewed each case. Vimentin and CA-II were excluded due to their insufficient positive and negative likelihood ratios. To develop a six-marker panel algorithm, positive staining for each marker was scored +1 for the respective subtype. Accordingly, each RCC specimen was assigned the subtype with the highest score (Table 2). This way, 80.9% (148/183) of RCC cases could be assigned to one certain subtype.

As a result of this individual case analysis, LR+ for detection of clear cell RCC was increased by application

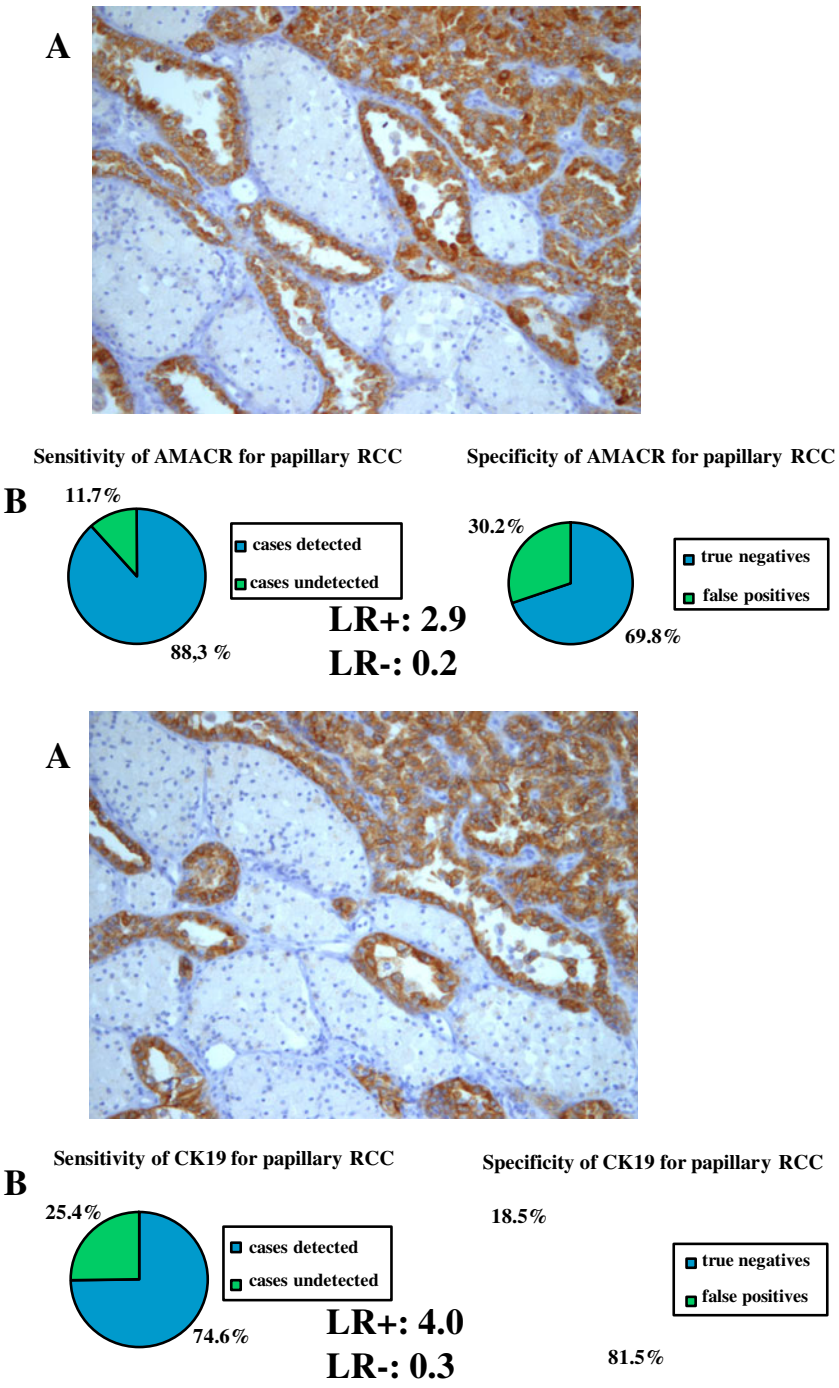

Fig. 2 AMACR and CK 19 performance as potential markers to diagnose papillary RCC. **a** AMACR and CK-19 positive pp-RCC tumors. **b** Sensitivity and specificity resulted in LR+ <10 and LR- >0.1 for both markers

of the six marker panel, but did not exceed 10 (7.4) and LR− remained >0.1 (0.3) (Fig. 5). The panel increased the LR+ for pp-RCC beyond 10 (17.7), thereby detecting papillary histology with high reliability; because of a LR− was still >0.1 (0.4), it cannot be used to definitely rule out this subtype. The panel enhanced LR+ for detection of cp-RCC far beyond 10 (30.2), but as LR− remains >0.1 (0.4), it cannot be used to rule out cp-RCC reliably. For this subtype, the panel performed slightly inferior to C-KIT alone.

Discussion

In the age of individual therapy, the approach to percutaneous image-guided RCC biopsy procedures plays an expanded role. However, difficulties in histopathological interpretation of the biopsy specimens—i.e., estimation of nuclear grading or correct classification of the certain RCC subtype—are reported in the literature [3, 31, 32]. As a result, various models have been proposed to simulate needle biopsy procedures for solid renal masses [33, 34]. We

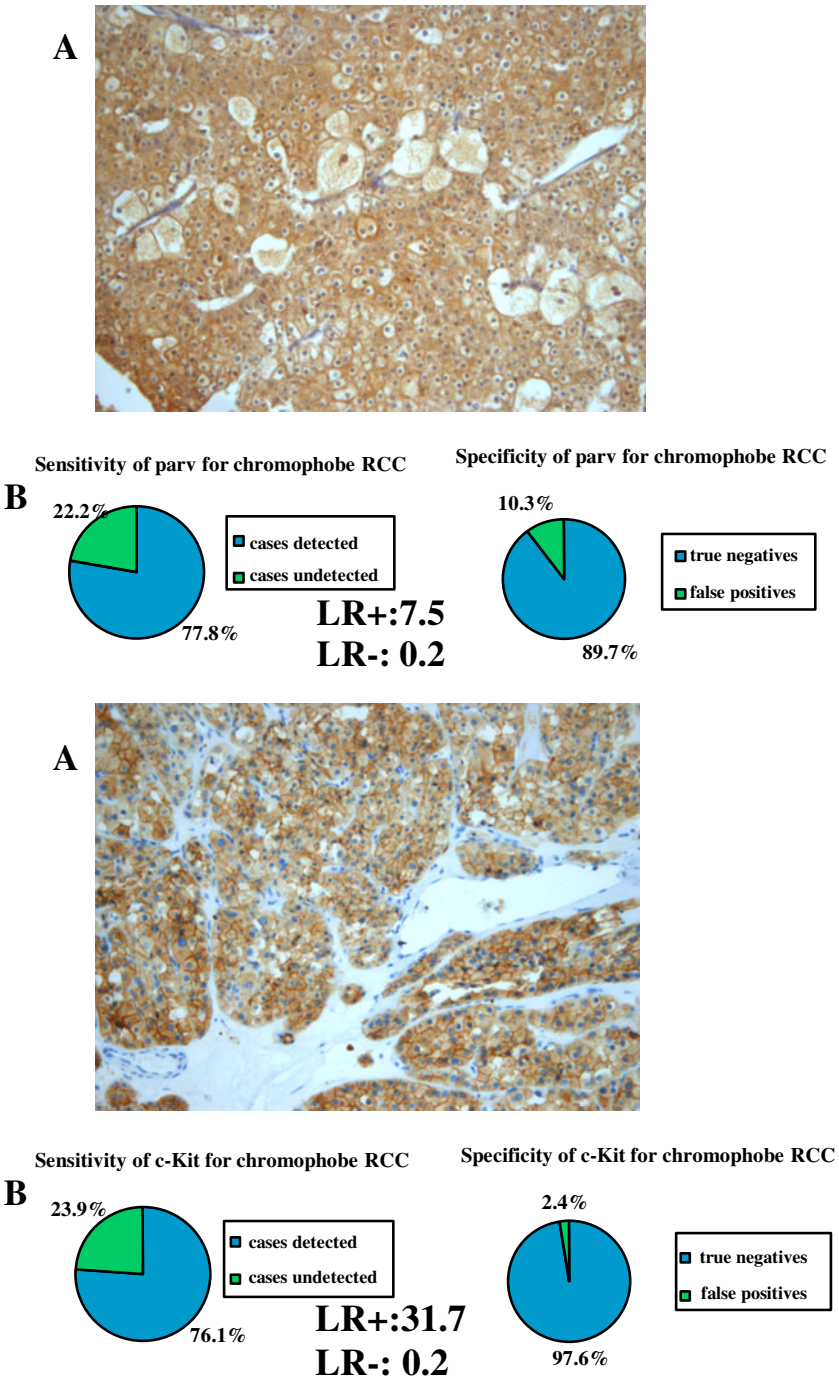

Fig. 3 C-KIT and parvalbumin performance as potential markers to diagnose chromophobe RCC. **a** Parvalbumin and C-KIT positive cp-RCC specimens. **b** Sensitivity for C-KIT resulted in LR+ >10; Specificity resulted in LR- >0.1 for both markers

applied a 1.5-mm punch needle for constructing a TMA, thereby mimicking the percutaneous biopsy technique. In this way, each core unit reflects a core conformation comparable to a Trucat needle biopsy core. Thus, the aim was to be able to differentiate between the three RCC main subtypes with a maximum of confidence and using a minimum of markers.

Several studies were dedicated to the development of a suitable immunohistochemistry panel to facilitate RCC subtype diagnosis. Yin-Goen et al. [35] describe 13 different immunomarkers, each of them highly specifically expressed in a particular RCC subtype. Takahashi et al. [36] proposed a classification model for kidney tumors using eight biomarkers based on previous gene expression profiling studies. Zhou et al. [11] used an immunohistochemistry set including 11 biomarkers differentiating not only clear cell and chromophobe RCC, but also papillary RCC type I from type II. Bazille et al. [37] reduced the marker panel from ten to only three biomarkers, which distinguished the different tumor subtypes in 79% of the cases. Similarly, Geramizadeh et al. [38] decreased

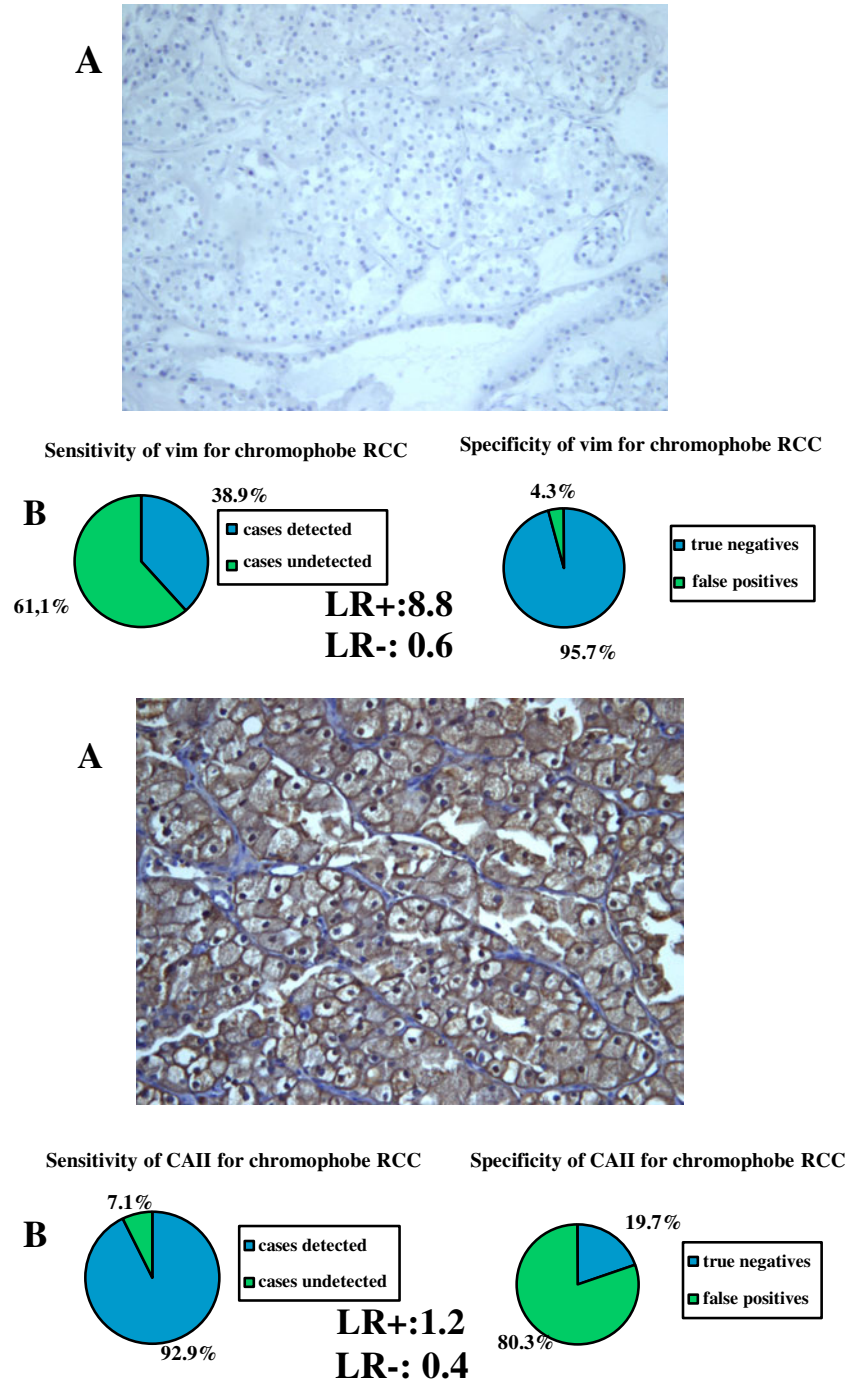

Fig. 4 Vimentin and CA-II performance as potential markers to differentiate chromophobe RCC. **a** CA-II and Vimentin positive cp-RCC specimens. **b** Sensitivity and specificity for both markers resulted in LR+ <10 and LR- >0.1

Table 2 Review of the six marker combination for each single specimen; positive staining for each marker scored +1 for the respective subtype

Positivity	CD10	GST-α	AMACR	ck19	c-Kit	Parvalbumin
Clear cell	+1	+1	0	0	0	0
Papillary	0	0	+1	+1	0	0
Chromophobe	0	0	0	0	+1	+1

the number of their marker set from ten primary candidates to five markers, which allowed them to determine the correct subtype in the majority of the analysed RCC specimens. Remarkably, the sensitivity and specificity rates of the examined markers vary widely from study to study. Moreover, some of the studies are underpowered in the case of papillary and—particularly—chromophobe subtype specimens.

Generally, GST-α, CD10, AMACR, CK-19 and Vimentin were found to be differentially expressed in the three

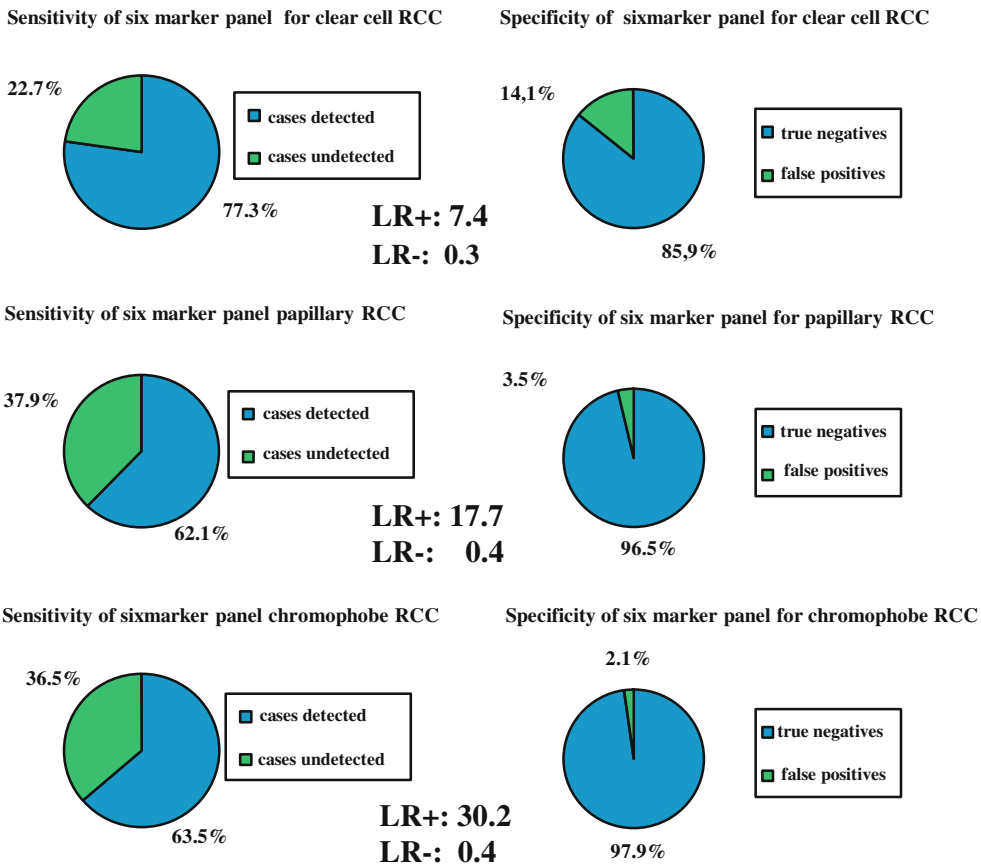

Fig. 5 Likelihood ratio positive (LR+) and likelihood ratio negative (LR−) for each of the three main subtypes utilizing the six marker panel

RCC main subtypes as described in the literature, although the differences between the subtypes were occasionally not as pronounced. For example, sensitivity for GST-α and CD-10 in terms of cc-RCC was comparable to the published data [6–10, 13]; however, a number of pp-RCC and cp-RCC tumors expressed GST-α and CD10, respectively. Consequently, specificity of these markers was affected and lower than that reported in earlier studies [7, 13, 15]. This might be attributed to the increased number of papillary and especially chromophobe RCC tumors in our study [8–10, 14, 25].

Another aspect concerns the TMA technology itself: due to possible heterogeneity within tumors, the use of one core per unit within the TMA may not fully represent the respective tumor as a whole. Antigen expression as demonstrated by a single tumor core might depend on its location within the tumor and not fully represent expression within the dominating histologic subtype.

The two papillary RCC associated markers, AMACR and CK19, demonstrated comparable sensitivity as in published data, but specificity of AMACR [17, 18] was impaired by positivity in non-papillary RCC tumors. Screening the literature for CK-19 expression in RCC, we encountered two relevant publications [19, 36] in this context. Despite the low case numbers, the authors found CK19 expression in pp-RCC tumors as well as in non pp-RCC tumors comparable to our findings. A potential future value for CK 19 was reported by Mertz et al. [39], who identified two distinct molecular subtypes of cc-RCC with prognostic relevance. These were defined by different CK19 (and CK7) expression. Thus, the variability observed for CK expression in cc-RCC can be explained by genetic heterogeneity.

Lack of Vimentin expression in chromophobe RCC histology is quite specific and reported for almost 100% of the chromophobe RCC cases in literature [15, 27]. Our analysis —95% of the examined tumors were Vimentin negative— corresponded well to these findings. However, Vimentin is also one of our markers, which displayed expression deviant from literature: We detected Vimentin staining in both papillary (40.2%) and clear cell (37.5%) RCC only in a subset of tumors compared to earlier reports, in which Vimentin expression both for cc-RCC and pp-RCC is reported to be up to100% [15, 19, 24, 27].

As noted above, Parvalbumin- and C-KIT expression is characterized as specific attribute of chromophobe RCC. Although our findings verified this observation, both markers are reported to be expressed to a higher extent in literature, reaching values up to100% for C-KIT [20–23] and Parvalbumin [7, 16]. However, LR+ for detection of cp-RCC by C-KIT was still 34.7. In contrast, the LR+ of Hale's colloidal iron staining was considerably worse. Thus, the

value of colloidal iron as a reliable marker for detection of chromophobe RCC cases is still on discussion [40].

Our analyses further confirmed the results of former investigations showing total lack of C-KIT [21–23] and Parvalbumin [16] in cc-RCC tumors as well as in pp-RCC tumors [16, 20–22].

In one of the earliest gene signature reports, CA-2 was identified as a potential marker for the differential diagnosis of chromophobe RCC [28]. However, the number of cases for the different subtypes was low. Our findings demonstrated high expression of CA II in all three RCC subtypes and consequently poor specificity, arguing against a utility of this marker for differential diagnosis of RCC.

After exclusion of Vimentin and CA-2, six immunohistochemical markers remained for the revision of all 183 RCC cases to simulate a diagnostic needle biopsy algorithm differentiating between the three RCC main subtypes without considering histopathology. As a result of this individual case review, the panel reliably detected two main RCC subtypes without inclusion of cytoarchitectural features, but failed to carry out a reliable detection of clear cell histology and to reliably rule out all three main subtypes.

Further limitations of our study are the lack of clear cell RCC cases comprised entirely or predominately of cells with eosinophilic cytoplasm as well as oncoctomas extending the differential diagnosis in tumors with eosinophilic cytoplasm beyond carcinoma.

Although there are several antigens that can be employed to separate oncocytoma from clear-cell RCC with eosinophilic cells and the eosinophilic variant of chromophobe RCC, immunohistochemical stains in these two tumors show substantial overlap. Thus, parvalbumin and C-kit, for example, are usually positive in both tumor entities. A recent update addresses to this issue proposing a three marker panel including C-KIT, CK 7 and PAX2, which has been reported to discriminate between oncocytoma and ch-RCC, to separate the four most common RCCs with eosinophilic cytoplasm reliably [41].

However, the present report highlights several important key points: With the investigation of 65 age-, grade- and stage-matched trios of clear cell, papillary and chromophobe renal carcinoma, this series comprises—to our knowledge—the largest and most homogeneous patient cohort studied so far. Moreover, scoring all punched biopsy cores without knowledge of the clinical data was the foundation for the design of a diagnostic algorithm imitating the potential difficulties in interpretation of a percutaneous needle biopsy.

All six markers are currently used as part of the daily routine in our immunohistochemistry laboratory unit; therefore, the panel enables cost-efficient and fast RCC subtype differentiation.

Our marker panel is a clear advancement in terms of immunohistochemistry application for RCC subtype differentiation. However, it needs further revisions, as shown by the relatively low specificity of values. Currently, further markers are evaluated for refinement of the panel.

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