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Association of cytokeratin 7 and 19 expression with genomic stability and favorable prognosis in clear cell renal cell cancer

Kirsten D. Mertz[1,2], Francesca Demichelis[1,2], Andrea Sboner[3], Michelle S. Hirsch[1,2], Paola Dal Cin[1,2], Kirsten Struckmann[4], Martina Storz[4], Simone Scherrer[4], Daniel M. Schmid[5], Räto T. Strebel[5], Nicole M. Probst-Hensch[6], Mark Gerstein[3,7,8], Holger Moch[4] and Mark A. Rubin[1,2,9,10]*

[1]*Department of Pathology, Brigham and Women's Hospital, Boston, MA*
[2]*Harvard Medical School, Boston, MA*
[3]*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT*
[4]*Department of Pathology, Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland*
[5]*Department of Urology, University Hospital Zurich, Zurich, Switzerland*
[6]*Molecular Epidemiology, Institute of Social and Preventive Medicine, University Hospital Zurich, Zurich, Switzerland*
[7]*Department of Computer Science, Yale University, New Haven, CT*
[8]*Program in Computational Biology and Bioinformatics, Yale University, New Haven, CT*
[9]*Dana Farber Cancer Institute, Boston, MA*
[10]*Broad Institute of MIT and Harvard Medical School, Cambridge, MA*

The purpose of our study was to demonstrate that distinct cytogenetic alterations in the most common subtype of renal cell cancer, clear cell renal cell carcinoma (ccRCC), are reflected in protein expression profiles. We performed conventional cytogenetics and immunohistochemical analysis for cytokeratins (CKs) on 126 ccRCCs. Protein expression was evaluated *in situ* using a semiautomated quantitative system. The results were validated using an independent cohort of 209 ccRCCs with long-term follow-up. Cytogenetic alterations were identified in 96 of 126 ccRCCs, most of them involving chromosome 3 through loss, deletion or translocation. Expression of CKs and E-cadherin in ccRCC was associated with lack of cytogenetic alterations and low nuclear grade. In the validation set, CK7 and CK19 protein expression was associated with better clinical outcome. At the multivariate level, the best model included metastatic status and CK19 expression. Expression microarray analysis on 21 primary ccRCCs and 14 ccRCC metastases identified genes significantly associated with CK7 and CK19 expressing ccRCCs. Two novel ccRCC biomarkers associated with the CK7 positive ccRCC phenotype, PMS2 and MT1-MMP (MMP14), were further validated. We conclude that the variability observed for CK expression in ccRCC can be explained by genetic heterogeneity. Distinct molecular subtypes of ccRCC with prognostic relevance were identified, and the CK7/CK19 expressing subtype is associated with better outcome.

Key words: clear cell renal cell cancer; cytogenetics; cytokeratin; gene expression profiling; prognosis

The 2004 World Health Organization classification of renal tumors,[1] which was built upon previous classification systems,[2,3] has refined the classification of adult renal epithelial neoplasms, taking into account morphological, clinicopathological and cytogenetic features. Several studies have shown that renal epithelial neoplasms have distinctive microscopic and molecular features.[4–6] Although tumor stage at presentation, histological tumor grade and histological subtype are the principal prognostic factors of renal cell carcinoma (RCC), the clinical behavior, proclivity for metastasis, and potential response to therapy cannot be predicted thus far by histopathologic markers.[4] Patients with clear cell RCC (ccRCC) have an increased risk of cancer specific death when compared to patients with papillary or chromophobe RCCs.[7] However, significant heterogeneity is observed amongst ccRCCs as evidenced by variability in protein expression and disease progression.

Cytogenetic analysis of benign and malignant epithelial tumors of the kidney has identified associations between morphological features and chromosomal aberrations.[8–10] Despite the frequent associations between morphological and karyotypic findings in epithelial kidney tumors,[11–13] the association between protein expression of cytokeratins (CKs) and karyotypic alterations in ccRCC has not been addressed. Clear cell RCC is an extremely heterogeneous tumor with inconsistent immunohistochemical expression patterns.[14,15] We hypothesized that the variability observed for CK expression and other markers of differentiation may be best explained by genetic heterogeneity.

Cytokeratins are the fundamental markers of epithelial differentiation,[16,17] and CK expression patterns are largely retained during neoplastic transformation.[18] The CKs found in simple epithelia (CK7, CK8, CK18, and CK19) are expressed in normal renal tubules and renal neoplasms. The majority of ccRCCs are positive for CK8 (about 40%) and CK18 (95–100%),[14,15] similar to the normal proximal renal tubule, suggesting that this is the site of origin for this subtype.[19] As almost all ccRCCs express CK18,[14,15] we did not expect it to be a useful distinctive marker and therefore excluded this CK in the present study. CK7 and CK19 are also simple epithelial CKs but show a more restricted expression in ccRCC (each about 10–15%), making these 2 CKs useful markers to define certain ccRCC subgroups.[14,15] In the normal kidney, CK7 and CK19 are expressed by distal tubules and collecting ducts but are absent in proximal tubules. The expression of CK7 and CK19 in ccRCC subsets suggests that these ccRCCs may represent specific subtypes with potential origin from the collecting duct system or with neo-expression of these CKs.[20]

Here we demonstrate significant associations between the expression of specific CKs, the cell adhesion protein E-cadherin and cytogenetic alterations. Two ccRCC subtypes defined by CK7 and CK19 expression were determined to be associated with genetic stability, a distinct molecular signature as determined by expression array profiling and a more indolent clinical course.

Material and methods

Patients and tissue microarrays

Two hundred thirty-eight consecutive, unselected epithelial kidney tumors diagnosed between 1999 and 2004 were evaluated

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Kirsten D. Mertz's current address is: Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

***Correspondence to:** Department of Pathology and Laboratory Medicine, 1300 York Avenue, Room C 410-A (or Box #69), New York, NY 10021, USA. Fax: +212-746-8816. E-mail: rubinma@med.cornell.edu

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TABLE I – MONOCLONAL ANTIBODIES USED

Antigen	Clone	Dilution	Source	Staining pattern
CK7	OV-TL 12/30	1:100	DAKO	Cytoplasmic
CK8a[1]	CAM5.2	1:10	BD Biosciences	Cytoplasmic
CK8	35betaH11	1:50	DAKO	Cytoplasmic
CK19	RCK108	1:50	DAKO	Cytoplasmic
Pan-CKa[2]	Lu-5	1:50	Biocare Medical	Cytoplasmic
Pan-CKb[3]	AE1/AE3	1:50	DAKO	Cytoplasmic
AMACR	13H4	1:100	DAKO	Cytoplasmic
CD10	56C6	1:20	Vector Labs	Membrane
CD31	JC70A	1:40	DAKO	Membrane (vessel)
CD34	QBEnd10	1:400	DAKO	Membrane (vessel)
E-Cadherin	NCH-38	1:400	DAKO	Cytoplasmic, membrane
EMA	E29	1:200	DAKO	Cytoplasmic, membrane
EZH2	Ref. [24]	1:200	Ref. [24]	Nuclear
Ki-67	MIB-1	1:200	DAKO	Nuclear
Vimentin	Vim 3B4	1:400	DAKO	Cytoplasmic
WT-1	6F-H2	1:100	DAKO	Cytoplasmic
PMS2	A16-4	1:50	BD Biosciences	Nuclear and cytoplasmic
MMP14	113-5B7	1:1000	ACRIS	Membrane, cytoplasmic

CK, cytokeratin; EMA, epithelial membrane antigen; WT-1, wilms tumor 1.
[1]Reacts primarily with CK8 and weakly with CK7.–[2]Reacts with CK 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19.–[3]Reacts with CK 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 19.

both by histology and cytogenetics at the Brigham and Women's Hospital, Boston, MA. All morphologic diagnoses were made on the basis of hematoxylin and eosin stained slides by 2 pathologists (K.D.M, M.S.H), according to the 2004 World Health Organization classification of renal tumors.[1] Two RCC tissue microarrays (TMAs) were constructed of these tumors as previously described.[21] Four cores (0.6 mm in diameter) were taken from each representative tissue block. Among the 238 kidney tumors 129 were ccRCCs, and karyotype data were available for 126 ccRCCs. Karyotypic studies were performed on all cases according to standard protocols. Cytogenetic aberrations were reviewed by a cytogeneticist (P.D.C.) who determined if the karyotypes were normal, diagnostic for a particular epithelial tumor, or nondiagnostic. Histologic and cytogenetic diagnoses were recorded independently and compared for concordance. All data is stored in a database (http://rubinlab.med.cornell.edu/supplemental_data/cytogenetics_data/index.jsp).

An independent cohort of 209 ccRCC patients with long-term follow-up and cancer-specific death as endpoint was identified in the files of the Institute for Surgical Pathology, University Hospital Zurich, Switzerland. The clinical and pathological parameters of this cohort are described elsewhere (mean follow-up 52 months, range 1–179 months).[22]

All samples were collected from consented patients with prior institutional review board approval at each respective institution.

Immunohistochemistry

All TMA sections were freshly cut and immunostained as previously described.[23] Table I lists the monoclonal antibodies used in our study.

Semiautomated quantitative image analysis by chromavision

Evaluation of all immunohistochemical stainings was performed with the Chromavision (Chromavision Medical Systems, San Juan Capistrano, CA) Automated Cellular Imaging System (ACIS II), an upgraded version of the previously described ACIS System.[25] The system combines automated microscopy with computerized image analysis to generate continuous quantitative measurements of immunohistochemical stainings in terms of staining intensity (between 0 and 255) and percentage of stained tissue area (between 0 and 100). Each tissue microarray core was reviewed to ensure that protein expression measurements were taken from diagnostic regions and that staining scores obtained from nonrepresentative regions were excluded.

Statistical analysis

Unsupervised analysis of protein expression was performed by hierarchical clustering using average linkage method and correlation measure to evaluate case similarities. When analyzing protein expression data, single cluster enrichment for cytogenetic or histological characteristics was assessed by means. This analysis contains information from protein profiles of each tumor sample. We then focused our analysis on CK expression by correlating expression of each individual CK analyzed with either cytogenetic or histological characteristics of the tumors, by using unpaired student's *t* test (two-tailed *p*-value). For multiple hypothesis testing we applied Bonferroni correction, when appropriate.

For survival analysis, we used the Zurich cohort with 209 ccRCC cases. Kaplan–Meier analysis was used to present associations between cancer specific death and CK expression. Survival time was calculated from date of nephrectomy to date of death or to date of last clinical follow-up. Log-rank test was used to evaluate statistical significance of curve splitting. Cox proportional hazard regression analysis was used at univariate and multivariate level analyses. Backward Wald statistics was applied to identify the most parsimonious model. All statistics were performed using SPSS 16.0 for Windows (SPSS, Chicago, IL) with a significance level of 0.05 on two-tailed *p*-values.

RNA isolation

For cDNA microarray analysis, frozen material of 21 primary ccRCCs and 14 ccRCC metastases were randomly selected from the archive of the Institute for Surgical Pathology, University Hospital Zurich, Switzerland. All tumors were histologically evaluated by one pathologist (H.M.) and selected for the study on the basis of hematoxylin and eosin stained tissue sections. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA). RNA quantity was determined based on A_{260}, and the integrity of RNA was confirmed by agarose gel electrophoresis.

cDNA array analysis

Total RNA was amplified, labeled, and hybridized to HT_HG-U133A High-Throughput Arrays (Affymetrix, Santa Clara, CA) which were scanned using the HT Scanner, allowing 96 samples to be processed in parallel with minimal operator intervention. The original CEL files were processed with dChip (http://biosun1.harvard.edu/complab/dchip/) to obtain single data files comprising 22277 probe-sets. For each array, probe level intensities were normalized against the median array. Low expression values were filtered out with a high-pass filter and the data was

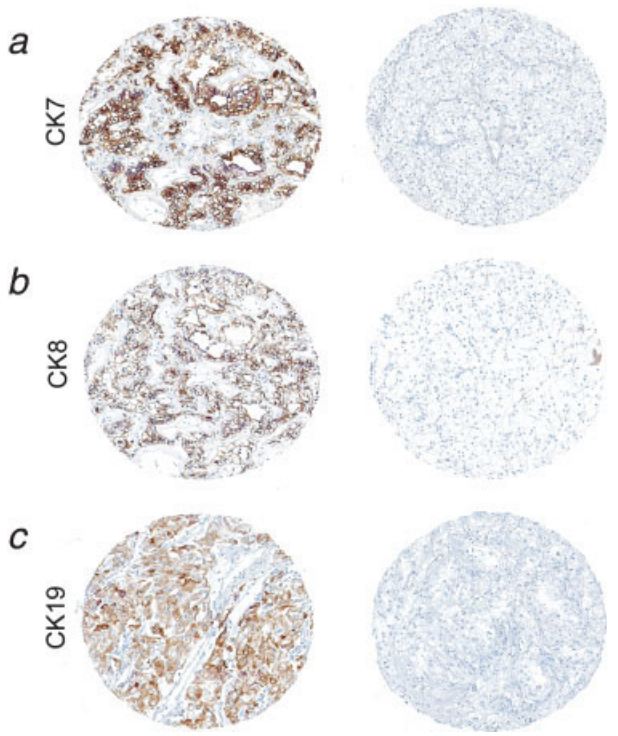

FIGURE 1 – Analysis of protein expression by immunohistochemistry on a tissue microarray, positivity or negativity for CK7 (*a*), CK8 (*b*), and CK19 (*c*).

log-transformed. The gene expression was standardized such that the distribution in each sample had median zero and standard deviation equal to 1.

The array probe sets for CK7 and CK19 were identified and analyzed. The array design includes 2 probe sets for CK7: 209016_s_at (chr12:50913304-50928976 (+) // 96.24 // q13.13) and 214031_s_at (chr12:50913483-50923188 (-) // 96.79 // q13.13). We computed the mean of the 2 probe sets to obtain a representative CK7 expression for further analysis. CK19 is represented on the array by one probe set (chr17:36933395-36938054 (-) // 99.41 // q21.2).

Pearson correlation for gene selection

Pearson correlation was used to select genes according to their relation to CK7 or CK19 across samples. As we had 2 probe sets for CK7, we computed the mean of the probe sets to obtain a representative CK7 expression. For CK19 with 1 probe set only, we computed the Pearson correlation of CK19 against every probe set. Genes with a two-tailed p-value < 0.001 were selected. Clustering analysis on gene expression data was performed as described for protein expression data.

Results

Associations between biomarker expression and cytogenetic profiles of ccRCC

The karyotypes of 126 ccRCCs from the Brigham and Women's Hospital Boston, obtained by conventional cytogenetic analysis, were compared with protein expression patterns. Sixteen immunohistochemical markers were selected (Table I), and the protein expression was evaluated by a semiquantitative image analysis system (Chromavision) (Fig. 1).

Within the group of 126 ccRCCs, we determined the number of alterations for each case and subdivided them in those without any cytogenetic alterations (30 cases, 23.8%), tumors with 1 or 2 alterations (37 cases, 29.4%), 3 or 4 alterations (31 cases, 24.6%), and more than 4 alterations (28 cases, 22.2%) (Supplemental Fig. 1). The most common cytogenetic alterations affected chromosome 3 in approximately one fourth of the cases (27%), most of them through translocation (42%), deletion (13.5%) or loss of the entire chromosome (26%) (Table II).

TABLE II – THE MOST COMMON CHROMOSOMAL ALTERATIONS IN A GROUP OF 126 ccRCCS AS DETERMINED BY CONVENTIONAL CYTOGENETICS

Alteration (on chromosome no.)	Number of cases (Frequency in %)
Gain (5)	11 (3.0)
Gain (7)	16 (4.4)
Loss (3)	25 (6.8)
Loss (8)	17 (4.7)
Loss (14)	16 (4.4)
Loss (Y)	27 (7.4)
Add (3p)	13 (3.6)
Del (3p)	13 (3.6)
t (3;a)	40 (11.0)

Add, addition; Del, deletion; t, translocation; t(3;a), translocation involving chromosome 3 and any other chromosome (a).

The frequencies are evaluated against the total number of alterations (365) within the set of ccRCCs.

One aim of this study was to classify ccRCCs on the basis of their differential expression of epithelial markers. Cytokeratins (Pan-CKa, Pan-CKb, CK7, CK8a, CK8 and CK19), other epithelial markers (EMA, E-cadherin), and the mesenchymal marker vimentin, all of which are known to be positive in subsets of ccRCC, were evaluated (Table I). Representative positive and negative cores for CK7, CK8 and CK19 immunostaining are depicted in Figure 1, illustrating the heterogeneity for these immunohistochemical markers in ccRCC. The mean immunohistochemical staining intensity of each protein, based on absence or presence of each common lesion, and the two-sided p-value of the t test corrected for multiple hypotheses were determined. To investigate if there were associations between protein expression patterns and genomic lesions, we ran hierarchical clustering and evaluated the clusters for enrichment for any particular lesion. The clustering analysis output is visualized by a dendrogram organizing the samples based on similarity of their protein expression profiles. The expression values of the entire cohort of 126 ccRCCs are shown in the heatmap (Fig. 2). Tumors are annotated according to main chromosomal aberrations as listed in Table II, to their total number of cytogenetic alterations and to Fuhrman nuclear grade (FNG).

A small group of cases with high expression of CKs (Pan-CKa, Pan-CKb, CK7 and CK8a) was enriched for absence of cytogenetic alterations ($p = 0.02$) and for FNG 2 tumors ($p = 0.01$). A second subset of cases consisting of E-cadherin expressing ccRCCs with FNG 2 was also lacking any cytogenetic abnormalities ($p < 0.01$). In contrast, tumors with a high number of cytogenetic changes tended to express lower levels of CKs (CK7, Pan-CKb) and E-cadherin compared to tumors with a low number of cytogenetic changes.

We identified another ccRCC group which does not express CKs but vimentin. This group showed higher frequency of cytogenetic alterations and higher FNGs. Vimentin-positive tumors, which are frequently negative for markers of epithelial differentiation, most likely represent a subset of advanced tumors with aggressive clinical behavior.[26]

Survival analysis

A second independent cohort of 209 ccRCCs with clinical follow-up data was evaluated to test if CK expression in ccRCCs was associated with differences in prognosis. Sections of the TMA were stained for Pan-CKb, CK7, CK8a, CK8, CK19 and E-cadherin. The expression levels of each protein were determined by Chromavision and transformed into nominal variables, above and

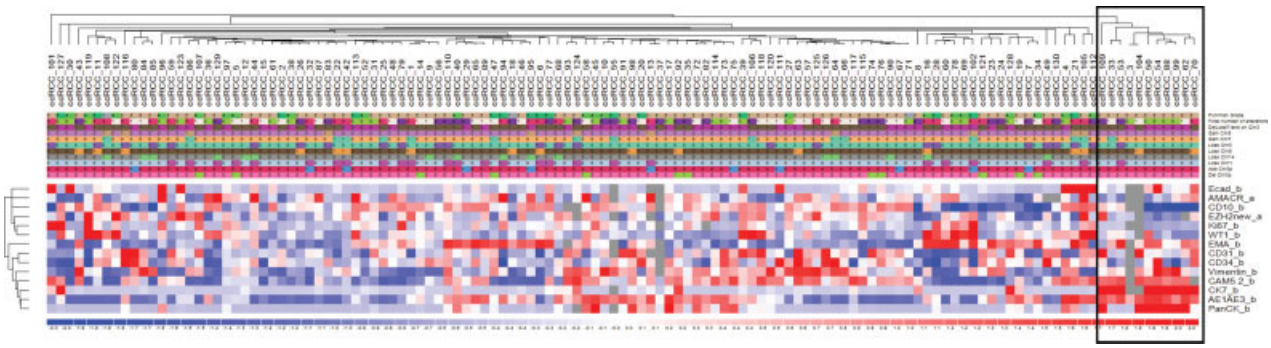

FIGURE 2 – Visual qualitative overview of ccRCC protein expression data. Hierarchical clustering revealed natural groups of tumors sharing similar protein expression patterns, and identified clusters enriched for particular lesions. This analysis included 125 ccRCCs and 14 immunohistochemical markers. The heatmap represents the protein expression percentages (in rows; red = high and blue = low protein expression) for each tumor sample (in columns). The clustering analysis output is shown by the dendrogram on the top of the figure. Tumors are annotated according to their Fuhrman nuclear grade (first row below sample ccRCC labels; FNG 1 = gray; FNG 2 = brown; FNG 3 = dark green; FNG 4 = light green), according to the total number of alterations (0 = 0 alterations, green; 1 = 1 or 2 alterations, white; 2 = three or four alterations, purple; 3 = more than 4 alterations, pink), according to presence of alterations on chromosome 3 resulting in a loss of genomic material (0 = no alteration on chromosome 3, pink; 1 = deletion, loss, translocation on chromosome 3, brown), and according to the main chromosomal alterations as listed in Table III. The CK cluster on the right side of the figure (black framed; $n = 11$) is enriched for absence of cytogenetic alterations ($p = 0.02$) and for FNG2 tumors ($p = 0.01$). E-cadherin positive cases ($n = 4$) also tended to be low grade tumors (FNG2) without genomic alterations, and these associations were statistically significant. In contrast, vimentin positive but CK negative tumors ($n = 19$) tended to have genomic alterations and higher FNGs.

TABLE III – COX REGRESSION UNIVARIATE ANALYSIS OF THE ZURICH COHORT (209 ccRCCS)

	p-value	RR	95.0% CI for RR	
			Lower	Upper
Fuhrman Grade	<0.01	2.52	1.45	4.40
pT Stage	<0.01	2.69	1.71	4.23
Sarcomatoid Features	<0.01	2.07	1.36	3.16
Necrosis	<0.01	2.44	1.61	3.71
Nodal Status	<0.01	3.51	1.65	7.46
Metastatic Status	<0.01	4.79	2.63	8.73
Lymphocytic Infiltrate	0.01	2.48	1.24	4.94
Size (cc)	<0.01	2.43	1.349	4.39
Gender	n.s.	.81	.53	1.24
Age (years)	0.03	1.62	1.06	2.48
CK7	<0.01	0.52	.32	.85
CK8a	n.s.	0.76	.48	1.20
CK8	n.s.	0.79	.50	1.25
CK19	n.s.	0.80	.51	1.25

n.s., not significant.

For CK7, CK8a, CK8, and CK19, cases with expression above and below or equal to the median were compared.

below the median. Cox regression analysis at univariate and multivariate levels considered histopathological and clinical parameters as well as protein expression.

Cox regression results are summarized in Table III (univariate level). Figure 3 shows the survival curves for selected biomarkers. CK7 expression was associated with better outcome ($p < 0.01$, Fig. 3*a*), whereas CK8 (Fig. 3*b*) and CK19 (Fig. 3*d*) expression did not show long-term association. However, within the first 5 years after initial diagnosis, CK19 expression and CK8a expression were associated with a more favorable outcome ($p < 0.01$, Table IV, Fig. 3*d*; $p = 0.01$; Table IV, Fig. 3*c*).

Multivariate Cox proportional hazards regression analysis showed that metastatic status ($RR = 7.28$; 95%CI = 1.93–27.39; $p < 0.01$) and CK19 expression ($RR = 0.23$; 95%CI = 0.06–0.90; $p = 0.04$) emerged as independent prognostic factors for cancer-specific survival at a multivariate level.

When testing for association between expression of single proteins and histopathological characteristics of the 209 ccRCC tumors within this cohort, we detected a significant association between CK7 expression and low FNG ($p < 0.05$), low pathological stage ($p = 0.02$), absence of necrosis ($p < 0.05$), and absence of lymphocytic infiltration ($p < 0.01$) (Table V). All *p*-values are two-sided and corrected for multiple hypotheses.

Determination of CK7and CK19 specific signatures in ccRCC by microarray analysis

We further determined if CK7 and CK19 expression in ccRCCs is associated with specific gene expression signatures. Gene expression profiles were obtained from 21 primary ccRCCs and 14 ccRCC metastases by cDNA microarray analysis. Pearson correlation was used to estimate the relation between each single gene and CK7/CK19 expression, the 2 CKs identified as having prognostic relevance in ccRCC. Genes with a two-sided *p*-value <0.001 were selected. We identified 62 genes with a strong relation to CK7 (Fig. 4), and 182 genes related to CK19 (Supplemental Fig. 2).

Loss of CK7 expression in ccRCC was associated with significant dysregulation of genes involved in transcription, signal transduction, cell cycle regulation, DNA mismatch repair, cell adhesion and immune responses. To evaluate whether the transcripts identified could help distinguish between ccRCC subtypes defined by CK7 expression, we selected 2 candidates whose transcripts were up- (PMS2) or down- (MMP14) regulated in relation to CK7. For both PMS2 and MMP14, antibodies were commercially available.[27,28] PMS2 was strongly expressed in all ccRCCs positive for CK7. These cases had low or absent MMP14 expression. Representative TMA cores are depicted in Figure 5.

Thus, the data sets obtained by Pearson correlation for CK7 and CK19 represent a potential source of markers that may aid in distinguishing molecular ccRCC subclasses.

Discussion

Markers of epithelial differentiation, and among those CKs as constituents of the cytoskeleton in epithelial cells, show variable expression in ccRCC. In our study, we define a correlation between the heterogeneous expression of CK subtypes and the heterogeneous clinical behaviour of ccRCC. We demonstrate that the distinctive CK expression patterns of ccRCCs can be a surrogate for underlying genomic alterations, have predictive value for patient outcome and reflect different gene expression patterns in ccRCC subgroups.

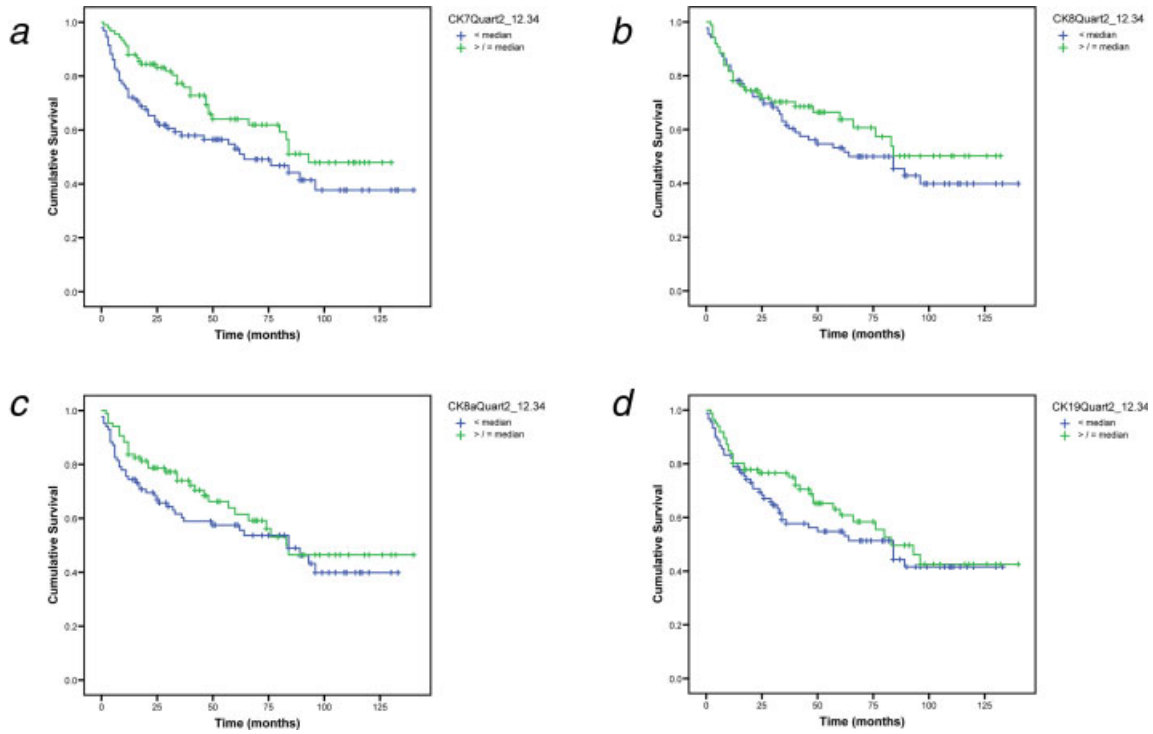

FIGURE 3 – CK7 and CK19 protein expression and survival in ccRCC. Kaplan-Meier curves for CK7 (*a*), CK8 (*b*), CK8a (*c*) and CK19 (*d*) protein percentages (above and below median). The green lines correspond to cases with protein expression equal to or above the median, the blue lines correspond to cases with CK expression below the median. (*a*) CK7 protein expression is associated with longer survival in ccRCC patients (log rank *p*-value = 0.03). (*b*) CK8 expression alone is not a statistically significant parameter for predicting outcome ($p = 0.31$). (*c*) Similarly, the predictive value of CK8a is limited, indicating a better prognosis within the first 5 years after initial diagnosis, but loosing statistical significance at later time points ($p = 0.24$). (*d*) CK19 protein expression shows significant association with longer survival within 5 years after initial diagnosis ($p < 0.01$), but is not significant on long term.

TABLE IV – COX REGRESSION UNIVARIATE ANALYSIS OF THE ZURICH COHORT (209 ccRCCS) WITHIN 5 YEARS FROM DIAGNOSIS

	p-value	RR	95.0% CI for RR	
			Lower	Upper
CK7	<0.01	0.52	0.32	0.85
CK8a	<0.01	0.47	0.28	0.78
CK8	0.01	0.54	0.33	0.88
CK19	<0.01	0.48	0.29	0.79

ccRCC cases with CK7, CK8a, CK8 or CK19 expression above and below or equal to the median were compared.

Our findings in ccRCC are consistent with CK expression studies in other malignancies. Schaller *et al*. have previously shown that loss of CK8 and CK18 in breast carcinomas was associated with a significantly worse prognosis, whereas the majority of patients with high CK18 remained recurrence-free.[29] In breast cancer cell lines, forced re-expression of CK18 was associated with reduced invasiveness *in vitro*.[30] A similar correlation between CK8/CK18 expression and reduced tumorigenicity was reported by Pankov *et al*. for murine pancreatic carcinoma cells.[31] Similar results were also obtained by Sommers and Thompson who showed that breast cancer cell lines became more aggressive as keratin filaments were replaced by vimentin, the intermediate filament-protein of mesenchymal cells.[32,33]

Our conventional cytogenetic analysis identified ccRCC subgroups with prognostically relevant CK expression patterns, indirectly suggesting that the number of cytogenetic alterations is linked to the biologic tumor behavior. It has been suggested that RCC progression is characterized by an accumulation of complex chromosomal alterations. Comparative genomic hybridization (CGH) analyses have shown that a high number of chromosomal copy number aberrations is associated with poor prognosis.[5] Interestingly, 24% of ccRCCs in our study did not reveal any conventional cytogenetic aberrations. Conventional cytogenetic analysis as applied for the Brigham and Women's Hospital cohort has its limitations, and therefore could affect our results. One of these limitations is the low resolution of the method. Furthermore, conventional cytogenetics can result in a bias towards tumor cells with a growth advantage *in vitro*. For these reasons, we validated our hypotheses on a large independent cohort of ccRCCs with long-term follow-up data.

Cytogenetic studies have shown that 3p deletions are linked to ccRCC, occurring in 40–70% of sporadic tumors with this histological subtype.[34–36] Previous molecular studies demonstrated that different genes on chromosome 3p are candidates for tumor suppressor genes (TSG), with *VHL* on 3p25.5 as the most frequently inactivated TSG. Although it is assumed that the inactivation of one or more genes on 3p plays a role in ccRCC initiation, it has been suggested that other cytogenetic alterations go along with ccRCC progression, but the nature and relevance of their interrelationship is poorly understood. Our data strongly indicate that there are ccRCC clusters defined by specific cytogenetic alterations, resulting in different CK expression patterns.

Microarray analysis of malignancies from other organ sites has revealed molecular subtypes of tumors that are histologically indistinguishable, and gene expression profiles have been identified that correlate with clinical outcomes.[37–39] Global analysis of gene expression using cDNA microarray technology offers significant opportunities to identify novel markers that discriminate between classes of tumors and holds promise in identifying molecular subclasses of tumors with differing prognosis. Our data

TABLE V – FOR EACH IMMUNOHISTOCHEMICAL MARKER (PERCENTAGE OF TUMOR AREA EXPRESSING THE PROTEIN), WE TESTED FOR SIGNIFICANT DIFFERENCES WITH RESPECT TO CLINICOPATHOLOGICAL VARIABLES

	E-cadherin *p*-value (up-down) $N = 199$	Pan-CK, *p*-value (up-down), $N = 178$	CK7, *p*-value (up-down), $N = 184$	CK8, *p*-value (up-down), $N = 174$	CK19, *p*-value (up-down), $N = 176$
Event			0.02		
Fuhrman Grade			<0.005		(0.05)
pT Stage			0.002		
Sarcomatoid Features	0.01				
Necrosis	<0.01		<0.005		
Nodal Status					
Metastatic Status			0.03		
Lymphocytic Infiltrate			<0.01		
Size (cc)					
Gender					
Age (years)					

Mann–Whitney test was applied. All *p*-values are two-tailed. Uncorrected *p*-values are reported for all significant test results, and the reported results represent downregulations of the markers with respect to the clinicopathological features. Results in brackets are not significant, but revealed a tendency. If we consider multiple hypothesis correction, the bold printed *p*-values are still significant. Downregulation of CK7 expression appears to be strongly associated with aggressiveness.

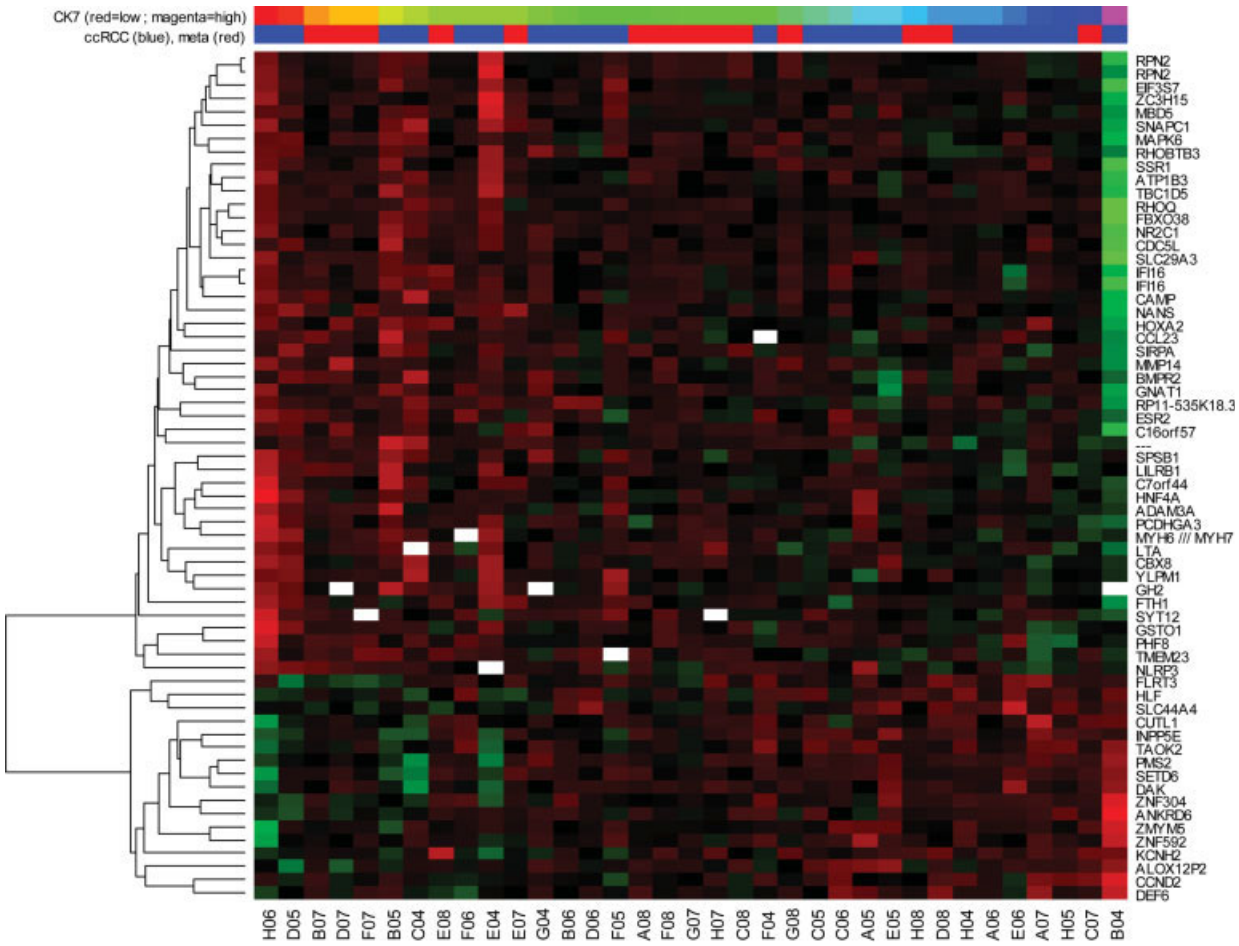

FIGURE 4 – Clear cell RCC gene expression data - 62 genes showed significant correlation with CK7 expression. Pearson correlation was used to estimate the relation between each single gene and CK7 expression in 21 primary ccRCCs and 14 ccRCC metastases. Genes with a *p*-value <0.001 were selected and color-coded: red = high expression, green = low expression, related to CK7. The hierarchical clustering is computed as average linkage with correlation distance. The 35 cases are further annotated according to their CK7 expression (first bar on the top; CK7 expression is color-coded as a gradient with red = low and magenta = high expression) and according to their status as primary (blue) or metastatic (red) ccRCCs (second bar on the top).

provide a starting point for identification of distinctive molecular signatures for ccRCC subtypes and may offer unique biological insights into these tumors. We demonstrate the potential benefits of 2 novel biomarkers in ccRCC with distinctive expression patterns that were identified in our analysis. The DNA mismatch repair protein PMS2 was found to be strongly expressed in CK7 expressing ccRCCs. Defects in DNA mismatch repair result in an accumulation of mutations that are associated with human cancers and diseases. While their contribution to gastrointestinal tumors is well studied, the role of mismatch repair defects in other tumor

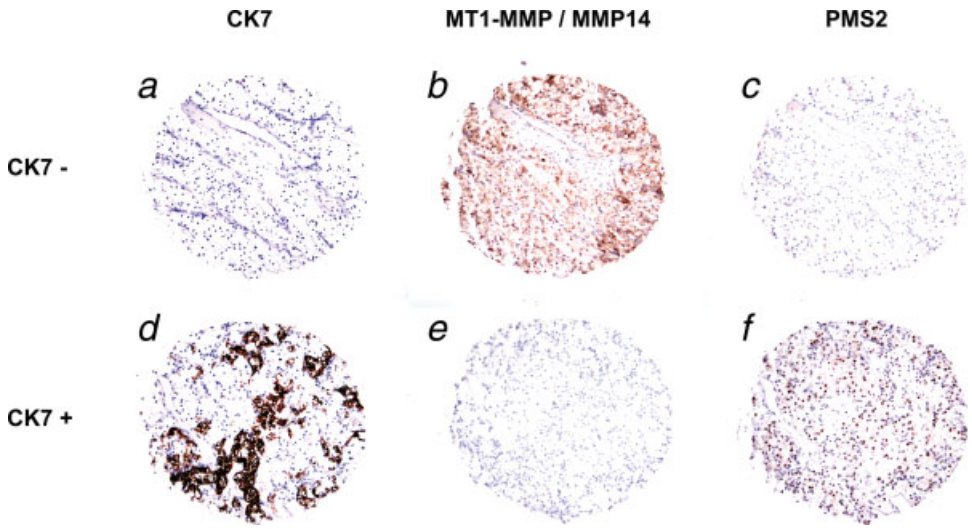

FIGURE 5 – Immunohistochemical analysis of PMS2 and MT1-MMP/MMP14 as 2 distinctive biomarkers in CK7 positive ccRCCs. (*a*) Example of a CK7 negative ccRCC with strong positivity for MT1-MMP/MMP14 (*b*), but without PMS2 expression (*c*). CK7 positive ccRCC (*d*) lacking MT1-MMP/MMP14 expression (*e*), but expressing PMS2 (*f*). The MT1-MMP/MMP14 immunohistochemical signal was located at the membrane and in the cytoplasm, whereas PMS2 showed mainly a nuclear staining pattern with weak cytoplasmic staining.

types is not well characterized. Norris *et al*. report a significant increase in the level of PMS2 in pre-cancerous and low grade prostate cancer as compared to higher grade tumors.[28] Our finding of increased PMS2 expression in CK7 positive ccRCCs is in line with this observation. However, further validation will be necessary to determine the prognostic significance of PMS2 expression in ccRCC.

In our study, we show the absence of matrix metalloproteinase (MMP) MT1 (also known as MMP14) in CK7 positive ccRCCs. A crucial step in tumor progression and invasion is the proteolytic degradation of the extracellular matrix (ECM) and basal membranes.[40] MMPs are zinc-dependent endopeptidases, which are largely involved in tissue remodeling, degradation of the ECM and tumor invasion.[41] Although most MMPs are secreted, the membrane type 1 matrix metalloproteinase (MT1-MMP) is localized in the cell membrane.[42] Its expression has been correlated with the invasive capacity of different tumors.[41] Our findings suggest that the good prognosis of CK7 expressing ccRCC can be partially explained by absence of MT1-MMP expression.

In summary, we have identified ccRCC subgroups, which are characterized by specific CK expression patterns and different prognosis. These findings may also have therapeutic relevance in the near future. Targeted delivery of liposome-encapsulated CK18 expression vectors by conjugation to anti-HER2/neu antibodies seems a promising approach in the treatment of breast cancer.[43] Similar treatment strategies could be envisioned for ccRCC. Biomarkers and pathways associated with CK7 or CK19 expression provide sources of novel therapeutic targets for ccRCC.

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