Role of new biomarkers in the progression of conventional renal cell cancer

Doctoral (PhD) theses

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ABBREVIATIONS

AWOD	alive without disease = tumormentes túlélő
CD14	cluster of differentiation 14
CI	confidence interval
CXCL1	Chemokine Ligand 1
CXCL8	Chemokine Ligand 8
DOD	dead of disease
ECM	Extracellular matrix
EMT	epithel-mesenchyma transformation
FPRL1	formyl peptide receptor like-1
HNF1A	hepatic nuclear factor 1 alfa
IL1A	Interleukin 1-alfa
IL1B	Interleukin 1-beta
IL6	Interleukin 6
LBP	Lipopolysaccharide binding protein
MMP12	matrix metallopeptidase12
MMP2	mátrix metallopeptidase 2
MMP9	mátrix metallopeptidase 9
MODY	maturity-onset diabetes of the young
MTA1	Manual Tissue Arrayer
MTSCC	mucinous tubular and spindle cell carcinoma
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
p38	Mitogen Activated Protein Kinase p38
RCC	renal cell cancer
RR	relative risk
SAA1	serum amyloid A1
SLC6A19	Solute carrier family 6 member 19
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
TLR4	toll-like receptor 4
TMA	tissue microarray
TMEM27	transmembrane protein 27

1. INTRODUCTION

Renal cell carcinoma (RCC) represents 2-3% of all cancers occurring with the highest incidence in Western countries. In 2012 approximately 84.000 patients were diagnosed with RCC in European countries and 35.000 died due to metastatic disease (Ferlay et al. 2013). The most common conventional RCC (also called clear cell RCC), which makes up 75% of renal malignancies has a high mortality, nearly every third patient have or will develop metastatic disease during the postoperative course. Surgery is the primary treatment for RCC and recent adjuvant therapy can only prolong the life of patients with metastatic disease. Due to the widespread use of modern imaging techniques the number of patients with incidentally detected small RCC at an early stage is increasing. However, in spite of early detection, approximately 15% of clinically localised tumours operated with curative intent will develop metastasis within 5 years follow-up. Until now, only few biomarkers exist to identify RCC with high risk of postoperative relapse. Some of the new biomarkers are included in clinical and pathological prognostic scoring systems.

2. AIMS

The main objective of our work was identifying new biomarkers suitable to predict the outcome of organ confined conventionel renal cell cancer. Progression of malignant tumors is in association with the changes of the relations between the cancer and its microenvironment and with the dedifferentiation of tumor cells. Three genes were choosen to asses these processes.

A, Inflammatory changes in tumor stroma have important role in progression. Therefore we have choosen for further investigation the acute phase protein coding *SAA1* gene.

B, Remodelling and degradation of the surrounding stroma is mandatory for invasive growth and spreading of cancer cells. Previous studies have revealed that the metalloproteinase coding *MMP12* gene is upregulated in highly malignant sarcomatous RCC. To asses the role of extracellular matrix degradation in tumor progression we have analysed the expression of MMP12 gene in conventional renal cell cancer.

C, During development and progression the vast majority of tumors retain their epithelial characteristics and turn into an unpolarized sarcomatoid form. This transformation is needed for tumor cell outspread. To study the role of this cell structure change in tumor progression we have identified the *TMEM27* gene, which codes the transmembrane protein TMEM27 localized exclusively to the brush border of proximal tubules

The selection of the investigated biomarkers was based on the results of global gene expression analysis (Affymetrix) followed by reverse transcription PCR of tissue samples from RCCs with and without progression and from RCCs with sarcomatoid features. These previous studies were carried out in the Molecular Oncology Laboratorium leaded by Prof. Gyula Kovács at the Ruprecht Karl University of Heidelberg and in the Genomics Core Facility of European Molecular Biology Laboratory in Heidelberg.

3. METHODS

3.1. Patients and tissue samples

Patients subjected to tumour nephrectomy between 2000 and 2010 at the Department of Urology, University of Pecs were enrolled in a retrospective study. Data on regular follow-up and tumour specific death was obtained from Registry of the Department of Urology (Betegregiszter, Intramed, eMedSol). Follow-up was defined as a time from the operation until the last recorded control or cancer specific death. Patients who died from causes other than RCC are not counted in this measurement. Preoperative clinical staging included chest X-ray and abdominal and computed tomography scans (CT). Bone scintigraphy and brain CT scans were obtained only when indicated by clinical signs. The presence of nodal metastasis was confirmed by histological, whereas distant metastases by radiographic examination. In postoperative period patients were observed in every 6 month by abdominal ultrasound and measurement of serum creatinine and eGFR and by chest X-ray. Abdominal and pelvic CT was carried out every year. The pathologic features of all tumours were re-evaluated by an experienced uropathologist (GK) according to the the Heidelberg classification system. The stage and grade was defined using the 2009 TNM classification and a three scale grading system. The collection and use of all tissue samples for this study was approved by the Ethics Committee of the University Pecs, Hungary (No. 5343/2014).

3.2. Tissue microarray

Paraffin blocks of foetal and adult kidney and tissue microarrays (TMA) containing conventional RCCs as well as other types of renal tumours were used for this study. From tumours with areas of different morphology or grade 2-4 biopsies were taken. For internal control of antibody staining foetal and adult kidney biopsies were included in the arrays. TMAs was constructed from paraffin embedded material after marking the areas of interest on H&E stained slides using a Manual Tissue Arrayer (MTA1, Beecher Instruments, Inc., Sun Prairie, USA) and 0.6 mm core biopsies.

3.3. Immunohistochemistry

The 4μm sections of TMA were dewaxed in xylene and rehydrated in graded ethanol. sections After deparaffinisation and rehydration of, antigen demasking was performed by boiling the slides in 10 mM sodium citrate buffer, pH 6,0 (TMEM27, SAA1, MMP12) and TE buffer, pH 9,0 (HNF1A) in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase activity and nonspecific staining were blocked by incubation with 0.3% hydrogen peroxide containing 1% normal horse serum for 10 minutes at room temperature. Slides were then incubated overnight at 4°C in moist chamber temperature with rabit anti-SAA1 antibody (ab655, Abcam, Cambridge, UK) at 1:100 dilution and with rabbit anti-TMEM27 antibody (ab200664,Abcam, Cambridge, UK) at the dilution of 1:1000 and with rabbit anti-HNF1A antibody (PA5-22310, Thermo Fisher Scientific, Budapest, Hungary) at the dilution of 1:500 and with rabbit anti-MMP12 antibody (NBP1-31225, Novus Biologicals, Littleton, CO, USA) at the dilution of 1:250. HRP conjugated anti-rabbit secondary antibody (MACH4 Universal HRP-Polymer, Biocare Medical, Concord, USA) was applied for 30 minutes and colour was developed using the AEC substrate (DAKO, Glostrup, Denmark). Tissue sections were counterstained with Mayer's haematoxylin (DAKO). The slides were evaluated twice blinded to the clinical data.

3.4. Statistical analysis

Data analysis was performed with the SPSS Statistics software package version 23.0 (IBM,35 Armonk, NY, USA). Correlation between gene expression and clinicopathological parameters was calculated with the Chi-square test. The impact of the different variables (age, sex, size of tumour, TNM classification, grade, stage, metastasis and gene expression) on the survival time of the patients was estimated with Kaplan-Meier analysis, the comparison of survival curves was made with the Log rank test. Univariate and multivariate survival analysis was performed with the Cox regression model. Patients alive and disease free were censored. Differences were considered significant at $P<0.05$.

4. RESULTS

4.1. SAA1

4.1.1. SAA1 expression by immunohistochemistry

We enrolled 429 patients without metastatic disease at the time of operation. The average follow-up time of patients was 73.53±4.6 months. Follow-up was defined as a time from the operation until the last recorded control or cancer specific death. During the follow-up 78 (18%) patients developed metastasis and died due to cancer. Patients who died from causes other than RCC were excluded from this study. The male female ratio was 266 to 163 (62% and 38%), the mean age of patients 61.4±11.2 and the average size of tumours 50.1±25.5 mm.

Immunohistochemical analysis did not detected SAA1 expression in foetal or adult kidney. The vast majority of RCCs were negative for SAA1 staining. A weak to strong staining reaction for SAA1 was detected in 20 of the 429 tumours. The SAA1 protein is accumulated at the cell membrane forming sometimes small protein globlets but in cases with more intensive reaction also cytoplasmic staining occurred. Small cysts lined with tumour cells within the solid tumour tissue contained SAA1 positive fluid. In some cases large SAA1 positive globlets were seen by weak cytoplasmic staining.

4.1.2. Comparison of SAA1 expression with clinicopathologic characteristics

The relationship between SAA1 protein expression and clinicopathologic characteristics is summarized in Table 1. The SAA1 expression showed a significant correlation with the cancer specific survival, tumour size, grade and T classification ($p <0.001$).

4.1.3. Correlation between SAA1 expression and survival

The Kaplan-Meier survival analysis using log-rank test estimated a short disease specific survival for patients with SAA1 positive tumours (Fig. 1). The positive staining indicated median survival time of 58 months (33 to 82, 95% CI). Univariate analysis as showed in Table 2 revealed that patients with SAA1 positive tumour have a significantly increased risk of cancer specific death (RR 6.61; 95% CI-3.70-11.83). Multivariate analysis was also performed to assess the independent prognostic value of SAA1 staining in relation to known clinicopathological prognostic variables (Table 2). SAA1 expression, in addition to T classification and tumour grade was statistically significant (RR-2.11; 95% CI-1.14-3.92; $P=0.017$).

Table 1. Relationship between SAA1 expression and clinicopathologic characteristics of conventional renal cell cancer

	No of cases (429)	SAA1 +	SAA1 -	P-value
Status				
AWOD	351	6	345	**P<0.001**
DOD	78	14	64	
Tumor size				
<4cm	192	2	190	**P<0.001**
4-7cm	153	5	148	
> 7cm	84	13	71	
Grade				
G1	278	2	276	**P<0.001**
G2	116	10	106	
G3	35	8	27	
T-stage				
1a-b	294	4	290	**P<0.001**
2	37	4	33	
3a-b-c	97	12	85	

AWOD: alive without disease, DOD: dead of disease

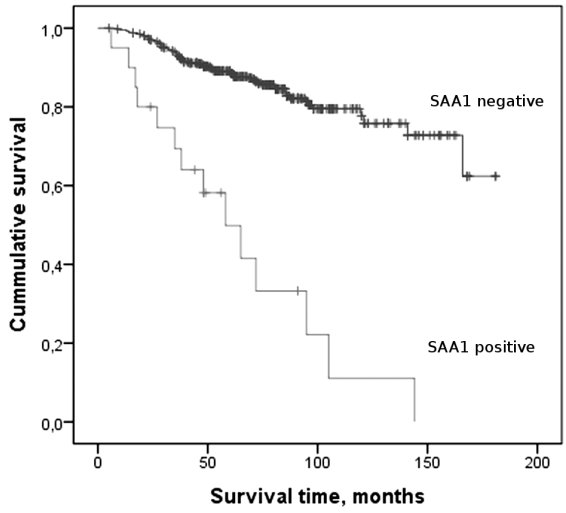

Figure 1. Kaplan-Meier survival analysis using log-rank test in SAA1 negative and SAA1 positive patients (n=429).

Table 2. Cox regression analysis of clinicopathologic parameters and SAA1 expression in relation to survival rates

	Univariate analysis		Multivariate analysis	
Parameters	**Relative risk (95%CI)**	**P value**	**Relative risk(95% CI)**	**P value**
Age (years)	1.02 (1.00-1.04)	0.088	1.01 (0.99-1.03)	0.234
Sex (male vs. female)	1.12 (0,73-1.71)	0.578	1.00 (0.63-1.59)	0.986
T-stage T1a-b		<0.001		<0.001
T2	4.95 (2.50-9.80)	<0.001	4.36 (2.09-9.07)	**<0.001**
T3a-b-c	8.74 (5.36-14.25)	<0.001	6.19 (3.50-10.93)	**<0.001**
Grade (G1 / G2-3)	3.84 (2.49-5.94)	<0.001	2.33 (1.39-3.91)	**0.001**
Tumor size (cm)	1.02 (1.02-1.03)	<0.001	1.00 (0.99-1.01)	0.314
SAA1 (+ / 0)	6.61 (3.70-11.83)	<0.001	2.11 (1.14-3.92)	**0.017**

CI: confidence interval

4.2. MMP12

4.2.1. MMP12 expression by immunohistochemistry

For MMP12 expression analysis 492 patients were enrolled (Cohort 1). We have separated 429 patients without progressive disease at the first observation into Cohort 2. Metastatic tumour was detected in 63 patients at the time of operation and further 85 patients experienced tumour relapse postoperatively. Altogether, 148 (30.1%) patients died due to metastatic disease. The male female ratio was 314 to 178 (63.8% and 36.2%) the average age of patients 61.52 years. Average size of tumours led to death of patients was 87 mm whereas the average size of tumours in the survival group was 51 mm.
As the percentage of positively stained cells made up at least 80 per cent of tumour cells in all positive biopsies, we did not evaluated the number of positive cells as a parameter. We have classified the staining intensity as follows: no staining, weak staining and moderate (in some cases strong staining)
First, we have analysed the expression of MMP12 in normal foetal and adult kidneys. Of interest, exclusively distal tubular cells displayed a strong positive staining with MMP12. Immunostaining with MMP12 antibody showed weak to moderate, sometimes strong reaction in 116 conventional RCCs whereas 376 tumours were negative for MMP12. Some examples are shown in Fig. 1B. Concerning the intensity of immunoreaction, the first statistical analysis failed to show substantial differences in cancer specific death between tumours with weak or moderate staining. Therefore, we have finally evaluated the result of immunohistochemistry as negative and positive MMP12 expression.

4.2.2. Comparison of MMP12 expression with clinicopathologic characteristics

The association between MMP12 expression and clinicopathological parameters such as RCC-related death, size, grade, T-stadium and stage of tumours, as well as coagulation

necrosis and metastasis at the time of operation is shown in Table 3. All parameters showed a significant correlation (<0.001) with MMP12 expression.

Table 3. Relationship between MMP12 expression and clinicopathologic characteristics of conventional renal cell cancer

	No of cases 492	**MMP12** +	-	**P-value**
Status				
AWOD	344	46	298	**<0.001**
DOD	148	70	78	
Tumor size				
< 4 cm	190	19	171	**<0.001**
4-7 cm	171	43	128	
> 7 cm	131	54	77	
Grade				
G1	272	22	250	**<0.001**
G2	140	44	96	
G3	80	50	50	
T-stage				
T1a-b	296	41	255	**<0.001**
T2	40	11	29	
T3a-b-c	148	59	89	
T4	8	5	3	
Stage				
I/II	320	448	272	**<0.001**
III/IV	172	68	104	
Necrosis				
yes	431	84	347	**<0.001**
no	61	32	29	
Metastasis *				
with	63	38	25	**<0.001**
without	429	78	351	

*at the time of surgery, AWOD: alive without disease, DOD: dead of disease.

4.2.3. Correlation between MMP12 expression and survival

We have evaluated the cancer specific survival for all patients included in Cohort 1 and separately for those of Cohort 2. As it is shown in Fig. 2, the median survival time for MMP12 positive group in Cohort 1 was 58 months whereas it was 105 months in Cohort 2. Kaplan-Meier estimate analysis revealed that patients of Cohort 1 and Cohort 2 with MMP12 positive tumour have significantly shorter survival ($p<0.001$). Univariate analysis showed a significant correlation with all parameters listed in Table 1. Multivariate analysis of Cohort 1 revealed that T1-T2, grade, stage, necrosis, metastasis and size of tumour but not MMP12 expression were significant negative parameters predicting cancer specific survival (Table 4.). However, in Cohort 2 of patients without metastatic disease at the time of operation, MMP12

expression was an independent prognostic factor indicating a postoperative tumour relapse (p=0.048).

Table 4. Cox regression analysis of clinicopathological parameters and MMP12 expression in relation to cancer specific survival

Multivariate analysis		**All patients (492)**		**Patients without metastasis* (429)**	
Parameters		**Relative risk (95%CI)**	**P value**	**Relative risk (95% CI)**	**P value**
Age (years)		1.008 (0.991-1.024)	0.367	1.007 (0.986-1.028)	0.522
Sex (male/female)		0.958 (0.667-1.377)	0.819	0.927 (0.591-1.454)	0.743
T stage	**T1a-b**		**0.01**		**<0.001**
	T2	2.815 (1.477-5.367)	**0.02**	4.040 (1.974-8.268)	**<0.001**
	T3a-b-c	1.636 (0.819-3.270)	0.164	6.040 (3.540-10.304)	**<0.001**
	T4	2.570 (0.977-6.763)	0.056		
Grade (G1/G2-3)		2.006 (1.257-3.200)	**0.003**	2.180 (1.307-3.638)	**0.003**
Stage (I-II/III-IV)		3.492 (1.638-7.444)	**0.001**	2.635 (0.345-20.131)	0.350
Necrosis (yes/no)		1.833 (1.239-2.711)	**0.002**	1.550 (0.859-2.799)	0.146
Metastasis* (with/without)		5.805 (3.814-8.837)	**<0.001**		
Tumor size (cm)		1.006 (1.001-1.012)	**0.028**	1.002 (0.993-1.012)	0.631
MMP12 (0 / +)		1.260 (0.874-1.816)	0.216	1.612 (1.004-2.589)	**0.048**

CI: confidence interval, *at the time of surgery

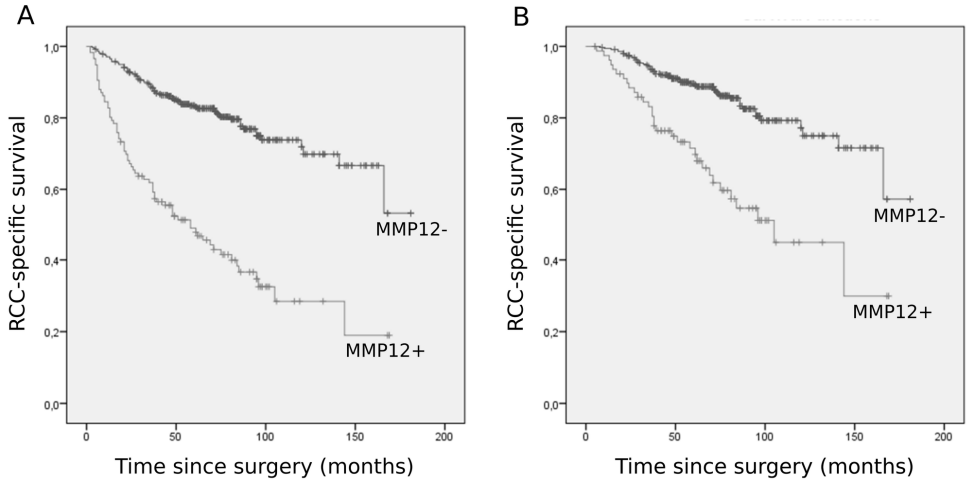

Figure 2. A.: Kaplan-Meier survival analysis using log-rank test in MMP12 negative and MMP12 positive patients in Cohort 1 (all patients). **B.:** Kaplan-Meier survival analysis using log-rank test in MMP12 negative and MMP12 positive patients in Cohort 2 (patients without metastasis at the time of surgery

4.3. TMEM27

4.3.1. TMEM27 expression by immunohistochemistry

For TMEM27 expression analysis 486 patients (Cohort 1) were enrolled in a retrospective study. From Cohort 1 we have selected 422 patients without metastatic disease at the time of nephrectomy (Cohort 2) to estimate the impact of TMEM27 expression in the prognostication of cancer specific death.
Thirteen per cent of patients in Cohort 1 were presented with metastatic conventional RCC at the time of surgery. In Cohort 2 further 17% developed relapse and metastatic tumour growth during the follow up. Altogether, 29% of the patients suffered from and died due to metastatic disease. The male female ratio was 313 to 173 (64.4% and 35.6%), the average age 61.3±11.3. The average tumour size of the 147 patients died until the end of follow-up was 86 mm whereas in the survival group only 52 mm.
We have selected the TMEM27, because it is expressed exclusively in proximal tubules, the suggested origin of conventional RCC. Immunohistochemistry revealed a faint membrane bound expression of the TMEM27 on the luminal surface of proximal tubules in foetal kidney. In adult kidney the TMEM27 expression was also localized to the luminal surface of proximal tubular cells but the staining was more intensive. One to four biopsies of 486 conventional RCCs embedded in TMA were stained with TMEM27 antibody. We found exclusively membrane positive staining with variable intensity in 356 tumours whereas 130 cases were negative for TMEM27. Tumour with multiple biopsies of distinct histological grades, when both positive and negative staining was noticed, was considered to be negative. To improve the specificity of TMEM27 staining in conventional RCC we have included other types of kidney tumours in our study. A weak positive membranous staining, sometimes only small areas of the specimen was seen in 9 out of 54 papillary RCT, most of them showing large eosinophil cells resembling those of the proximal tubules. None of the 21 chromophobe RCC, 33 renal oncocytoma, seven mucinous tubular and spindle cell carcinoma, six metanephric adenoma and nine Wilms' tumour displayed positive staining with TMEM27 antibody.
It was suggested that the transcriptional activator HNF1A regulates the TMEM27 expression. Therefore, we have analysed the HNF1A in our material. Although we confirmed the co-localisation of both genes to the proximal tubules, no correlation was seen between their expression in conventional RCC.

4.3.2. Comparison of TMEM27 expression with clinicopathologic characteristics

The relationship between TMEM27 protein expression and clinicopathologic characteristics is summarized in Table 5. The TMEM27 expression showed a significant correlation with the patient survival, tumour size, grade and T stadium, stage and metastasis at the time of operation.

4.3.3. Correlation between TMEM27 expression and survival

The average follow up time was 65.5±37.1 months. The Kaplan-Meier survival analysis using the log-rank tests indicated a poor disease specific survival rates for patients with TMEM27 negative tumour enrolled in Cohort 1 as well as in Cohort 2. The lack of TMEM27 indicated a median survival time of 39 months (23,6-54.4, 95% CI) for patients in Cohort 1 and 58 months (53 to 108, 95% CI) for patients enrolled in Cohort 2. In univariate analysis patients of cohort 1 and cohort 2 with TMEM27 negative tumour has a significantly increased risk of

cancer specific death when compared to TMEM27 positive tumours. We also performed a multivariate analysis to assess the independent prognostic value of TMEM27 staining in relation to known clinicopathological prognostic variables (Table 3). In cohort 1 tumour stage, size and metastasis at the time of presentation as well as the TMEM27 expression were statistically significant (RR-2.76; 95% CI-1.91-3.99; P<0.001). In cohort 2 without metastatic disease at the time of operation only T stadium and TMEM expression was significant (RR-2.95; 95% CI-1.81-4.80; P<0.001). Thus the lack of expression of TMEM27 is an independent negative biomarker indicating the poor postoperative cancer specific survival of patients with conventional RCC.

Table 5.. Relationship between TMEM27 expression and clinicopathologic characteristics of conventional renal cell cancer

	No of cases (486)	**TMEM27**		**P-value**
		+	**-**	
Age				0.567
mean		60.8	61.5	
Sex				0.877
male	313	230	83	
female	173	126	47	
Status				**<0.001**
AWOD	339	293	46	
DOD	147	63	84	
Tumor size				**<0.001**
< 4 cm	181	156	25	
4-7 cm	175	131	44	
> 7 cm	130	69	61	
Grade				**<0.001**
G1	265	239	26	
G2	140	95	45	
G3	81	22	59	
T-stage				**<0.001**
T1a-b	293	252	41	
T2	40	28	12	
T3a-b-c	145	75	70	
T4	8	1	7	
Stage				**<0.001**
I/II	316	270	46	
III/IV	170	86	84	
Metastasis *				**<0.001**
with	64	22	42	
without	422	334	88	

*at the time of surgery, AWOD: alive without disease, DOD: dead of disease

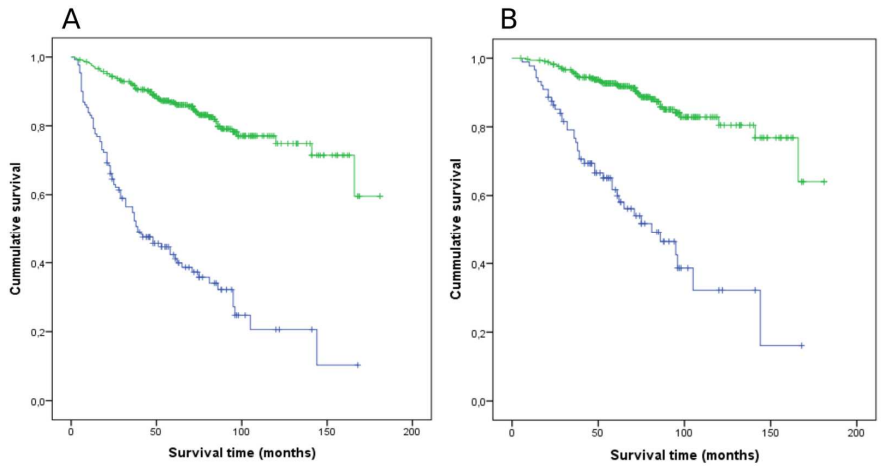

Figure 3. A.: Kaplan-Meier survival analysis using log-rank test in TMEM27 negative (line below) and TMEM27 positive (line above) patients in Cohort 1 (all patients). **B.:** Kaplan-Meier survival analysis using log-rank test in TMEM27 negative (line below) and TMEM27 positive (line above) patients in Cohort 2 (patients without metastasis at the time of surgery

Table 6. Cox regression analysis of clinicopathological parameters and TMEM27 expression in relation to cancer specific survival

Multivariate analysis	**All patients (486)**		**Patients without metastasis* (422)**	
Paramters	**Relative risk (95% CI)**	**P value**	**Relative risk (95% CI)**	**P value**
Age (years)	1.01 (0.99-1.03)	0.088	1.02 (0.99-1.04)	0.064
Sex (male/female)	0.94 (0.65-1.36)	0.768	0.83 (0.52-1.33)	0.451
T1a-b		0.025		**<0.001**
T2	2.08 (1.06-4.07)	0.032	4.16 (2.03-8.52)	**<0.001**
T3a-b-c	0.75 (0.36-1.57)	0.455	5.15 (2.95-8.97)	**<0.001**
T4	1.08 (0.39-2.96)	0.873		
Grade (G1/G2-3)	1.55 (0.94-2.54)	0.081	1.72 (0.99-2.99)	0.051
Stage (I-II/III-IV)	5.18 (2.48-10.82)	**<0.001**	2.35 (0.31-17.87)	0.407
Metastasis* (with/without)	5.97(3.92-9.09)	**<0.001**		
Tumor size (cm)	1.00 (1.00-1.01)	**0.003**	1.00 (0.99-1.01)	0.255
TMEM27 (+ / 0)	2.76 (1.91-3.99)	**<0.001**	2.95 (1.81-4.80)	**<0.001**

CI: confidence interval, *at the time of surgery

5. DISCUSSION

5.1. SAA1 and prognosis of conventional renal cell cancer

Kaplan-Meier analysis and Cox proportional regression model showed that SAA1 expression in tumour cells associates with poor patient survival. Multivariate analysis indicated that expression of SAA1 by tumour cells is a significant independent factor to predict cancer specific death. Thus, SAA1 is a valuable biomarker for estimating the risk for postoperative relapse of conventional RCC and can be used to stratify patients into low and high risk categories.

The acute phase protein SAA1 is secreted by the liver in response to inflammation. The transcription of SAA1 is induced by the inflammatory cytokines as IL1A, IL1B and IL6 [Kovacevic et al. 2008]. In turn, autocrine SAA1 amplifies the proinflammatory microenvironment by stimulating the expression of IL6, CXCL8 and CXCL1 and also the expression of metalloproteinases at tissue level [de Seny et al. 2013]. The SAA1 protein is one of the members of pro-inflammatory amplification loop, which plays a seminal role in the inflammatory microenvironment of cancer cells and in tumour progression. The SAA1 can also modulate cell adhesion and migration by inducing the expression of MMP-9 [Paret et al.2010]. The SAA1 has several receptors that mediate its various functions. The G protein-coupled formyl peptide receptor like-1 (FPRL1) has been described as the receptor involved in the cytokine induction and chemotactic activity of SAA1. The role of FPRL1 in the SAA1 mediated upregulation of MMP-9 and invasive growth of conventional RCCs was excluded. All probability, the signal transduction pathways in conventional RCC addressed by SAA1 is transmitted via TLR4 receptors and signals via p38 phosphorylation and NF-kB activation [Sandri et al. 2008]. Stimulation of TLR4 by LBP-CD14 complex, another acute phase protein promotes tumour invasion through NF-kB-dependent up regulation of matrix metalloproteinase-2 and beta-integrin [Harmey et al. 2002]. The dysfunctional immunity within tumour microenvironment promotes tumour progression by mediating proliferative and survival signaling and promoting angiogenesis, metastasis and drug resistance as well [Mantovani et al. 2008].

Secreted acute phase proteins detected in the serum have been linked to tumour progression. Elevated serum level of SAA1 has been shown to correlate with clinical and pathological parameters and patients survival [Wood et al. 2010]. However, the serum concentration of acute phase reactants is not specific for any type of cancer and it may reflect bacterial infection and inflammation. Therefore, their serum level should be carefully interpreted only in knowledge of full clinical data. The SAA1 expression by the tumour cells itself can be used to identify subset of conventional RCC with high risk of disease progression.

5.2. MMP12 and prognosis of conventional renal cell cancer

Global gene expression analysis identified MMP12 as an up regulated gene in highly malignant sarcomatous RCC. Immunohistochemistry revealed weak to moderate cytoplasmic expression of MMP12 protein in 31% of conventional RCCs. It is generally accepted that conventional RCC derives from proximal tubules of kidney. Surprisingly, we found MMP12 expression exclusively in distal tubules of normal foetal and adult kidneys. Therefore, MMP12 positivity in conventional RCCs can be interpreted as de novo tumour specific gene expression. The cytoplasmic expression of the MMP12 is associated with the high risk of tumour relapse in a cohort of patients without metastatic disease at the time of operation. Kaplan-Meyer estimate also showed a significant difference ($p<0.001$) in survival of MMP12

negative and positive cases. Our results indicate that MMP12 overexpression correlates with poor prognosis of conventional RCC.

Matrix metalloproteinases are zinc-dependent endopeptidases playing a fundamental role in extracellular matrix (ECM) degradation [Nagase et al. 1999]. They are involved not only in breakdown of ECM in normal physiological processes during embryonic development, wound healing and tissue remodelling and but also in invasive growth and spreading of cancer cells [Lyu et al. 2005; Shuman Moss et al. 2012; Werb et al. 1992]. The extracellular matrix (ECM) plays a seminal role in the tumour progression and metastasis. One of the first steps in metastasis is the degradation of the basement membrane, a process in which MMPs have been implicated. Remodelling of ECM proteins such as collagen, fibronectin and laminin by proteinases has a strong influence on the tumour progression [Kessenbrock et al. 2010]. MMPs can modify the ECM which in turn mediates tumour progression by different signalling interactions [Nguyen-Ngoc et al. 2012]. MMPs are capable of degrading a wide range of bioactive molecules triggering cell migration, proliferation, angiogenesis and inflammatory reactions, all necessary to tumour progression [Butler et al. 2009].

MMP12 is known as macrophage metalloproteinase, has an inactive form of 54 kDa that is processed by cleaving N- and C-terminal residues resulting in a 22 kDa mature active form [Shapiro et al. 1993]. The role of MMP12 in the tumour biology is somewhat controversial. Depending on types of tissue and tumor analysed, MMP12 may have a tumour promoting as well as an anti-tumour effect. MMP12 expression is positively associated with the progression of several cancer types including lung, head and neck, skin, oesophagus, prostate and pancreas cancer and as we showed here of conventional RCC [Lv et al. 2015; Yang et al. 2012; Ding et al. 2002; Nabha et al. 2008; Kerkelä et al. 2002; Balaz et al. 2002]. MMP12 promotes migration and invasion of nasopharyngeal carcinoma cells [Chung et al. 2014]. In contrary, MMP12 expression has an anti-tumour effect in hepatocellular, gastric and colorectal cancers [Cheng et al. 2010; Yang et al. 2001, Gorrin Rivas et al. 1998]. The better prognosis of MMP12 secreting tumours was explained by the observation that MMP12 generates angiostatin from plasminogen, which inhibits endothelial cell proliferation necessary to tumour growth and metastasis [Gorrin Rivas et al. 2000].

5.3. TMEM27 and prognosis of conventional renal cell cancer

We have demonstrated the first time that lack of TMEM27 expression in conventional RCC correlates with sarcomatoid change and also associates with fatal progression of disease. Patients of Cohort 2, e.g. without metastasis at the time of nephrectomy, having TMEM27 negative tumours are at least 3 times more likely experience RCC specific death during follow-up than patients with TMEM27 positive tumours. Kaplan-Meier estimate indicates that lack of TMEM27 expression is negative and independent prognostic factor for progression of RCC. Taking into account all patients included in Cohort 1, the median survival estimate was 39 months (95% CI), whereas in Cohort 2, e.g. patients without progression at the time of operation the median survival was estimated to be 81 months (95% CI). Therefore, TMEM27 expression is a valuable prognostic factor predicting the postoperative tumour free survival of patients presented without metastasis at the time of diagnosis. The multivariate Cox regression model indicated that lack TMEM27 expression in tumour cells significantly associated with fatal outcome of disease of patients in Cohort 2. There is a significant correlation between TMEM27 expression and T-stadium of tumours (all <0.001) in multivariate Cox regression analysis. Our results suggest that TMEM27 immunohistochemistry in combination with T-classification may be a good marker for progression of conventional RCCs.

TMEM27 was originally localized to the collecting duct [Zhang et al. 200]), and therefore it was called collectrin, but its correct localisation and function has now been cleared. The TMEM27 gene encodes a transmembrane protein that play an important role in trafficking amino acid transporters to the apical brush border of proximal tubules of the kidney [Verrey et al. 2009]. The expression of amino acid transporter SLC6A19 depends on its association with the TMEM27 at the cell membrane [Danilczyk et al. 2006]. The TMEM27 gene is localized to chromosome Xp22 outside of pseudo autosomal region and therefore, has no homologue on the Y chromosome. Of interest, fourteen of 18 Turner syndrome patients with X0 genotype enrolled in a genetic and phenotypic study revealed kidney malformation, most frequently horseshoe kidney which occurs in only one of 400 people in the general population [Pasquali et al. 2009; O Brian et al. 2008].

The TMEM27 is also involved in the control of insulin exocytosis by regulating the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) complex in beta cells of the pancreas Langerhans islet. The maturity-onset diabetes of the young (MODY) is characterized by impairment of glucose-stimulated insulin secretion from the beta cells [Fajans et al. 2001]. Mutation of the HNF1A gene causes one form of MODY called MODY3 [Yamagata et al, 1996]. Based on the co-localisation of TMEM27 and HNF1A expression in kidney and pancreas it was suggested that TMEM27 is a downstream target of HNF1A [Fukui et al. 2005; Yamagata et al. 2007]. We confirmed the localisation of TMEM27 to brush border and HNF1A to the nucleus of proximal tubules in normal kidneys but did not find any correlation between the positive staining of two genes in conventional RCCs.

There is no evidence from the spare data in the literature that TMEM27 is involved in any molecular pathway in tumour development or progression. Although TMEM27 expression in mouse was connected to proliferation of beta cells [Akpinar et al. 2005], others definitively disclosed this possibility (Altirriba et al. 2010). However, experimental work using siRNA silencing suggested that TMEM27 play a role in maintenance of primary cilia and polarity of renal epithelium [Zhang et al. 2007]. Our study revealed the loss of TMEM27 protein from membrane of highly malignant conventional RCC cells losing the cell polarity. These tumours undergone epithelium to mesenchyme transition, show higher biological malignancy marked by increased capacity to progression and metastasis.

6. THESIS

During the last decades the widespread use of imaging procedures has lead to early detection of RCC. The proportion of incidental T1a, T1b and T2 tumors is increasing in the surgical statistics of most urological centers. Altough these low stage tumors have lower progression rate than higher stage (T3, T4) tumors, 15-20% of them will develope metastases during follow up. Only few biomarkers are known to be able to identify these high risk patient group. We have described three markers which are suitable to predict the progression of conventional renal cell cancer.

A. We have proved that the cytoplasmatic expression of acute phase protein SAA1 is significantly associated with the postoperative progression of localised conventional RCC. Our data support that the changes in microenvironment of conventional RCCs play an importan role in tumor progression

B. Furthermore, we have verified that the cytoplasmatic expression of MMP12 in conventional RCC is in correlation with postoperative progression and shorter cancer specific survival. It can be explained by the fact, that not only the enhance of inflammatory processes, but the remodelling and degradation of tumor stroma is mandatory for tumor cell outspread and for the development of metastatis. MMP12 play an important role in this process.

C. Our study revealed the loss of TMEM27 protein from membrane of highly malignant conventional RCC cells losing the cell polarity. These tumours undergone epithelium to mesenchyme transition, show higher biological malignancy marked by increased capacity to progression and metastasis. Our finding makes TMEM27 gene for an excellent biomarker for estimation of postoperative cancer specific survival of patients with clinically localised conventional RCC at the time of operation.

Summarized, the SAA1, MMP12 és TMEM27 genes are excellent biomarkers, which are suitable to predict the postoperative progression in localised convetional renal cell cancer. The use of immunohistochemistry is a reliable and inexpensive procedure. These markers, together with other markers to be identified in further studies, will be able to use to foretell progression alone or in combination with other biomarkers or clinical factors. The integrated prognostical systems incorporating these new markers should undergo internal and external validation. The more intensive follow up of the high risk cases identified with this new prognostical system could lead to earlier diagnose of relapse promoting secound line surgical treatment like metastatectomy and removal of local recurrence. Early detection of relapse could make possible to start target therapy in time, before the decrease of patient's performance status. The risk adapted follow up supposedly could increase the efficiency of secound line treatment; therefor it could give survival benefit to the affected patients.

7. PUBLICATION

7.1. Publicaions supporting the dissertation

Andras Javorhazy, Nelli Farkas, Tamas Beothe, Csaba Pusztai, Arpad Szanto, Gyula Kovacs. Lack of TMEM27 expression is associated with postoperative progression of clinically localized conventional renal cell carcinoma.
J Cancer Res Clin Oncol 2016;142:1947-1953. **IF: 3.14**

Gyula Kovacs, Nina Kaerger Billfeldt, Nelli Farkas, Timea Dergez, **Andras Javorhazy,** Daniel Banyai, Csaba Pusztai, Arpad Szanto. Cytoplasmic expression of ß-catenin is an independent predictor of progression of conventional renal cell carcinoma: a simple immunostaining score.
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Oncol Letters (in press) **IF: 1.55**

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