M2 Macrophage Marker Chitinase 3-Like 2 (CHI3L2) Associates With Progression of Conventional Renal Cell Carcinoma

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Abstract. *Background/Aim:* In spite of early detection, approximately 15% of the small renal cell carcinomas (RCC) will develop metastasis within 5 years follow-up. The aim of this study was to identify new biomarkers to estimate the postoperative relapse of the most common conventional RCC. *Patients and Methods:* Tissue multi arrays of conventional RCC without metastasis at the time of operation from a cohort of 634 patients were analysed by immunohistochemistry for expression of the chitinase 3-like protein 2 (CHI3L2). Cancer specific survival of patients was estimated with Kaplan–Meier analysis, univariate and multivariate Cox regression models. *Results:* Kaplan–Meier analysis estimated a shorter cancer-free survival for patients with CHI3L2 positive tumors. In multivariate analysis, the CHI3L2 positivity associated with a 3.5 times higher risk for tumor relapse ($p < 0.001$). *Conclusion:* Expression of CHI3L2 in tumor cells of conventional RCC define a group of patients at high risk for postoperative progression.

The widespread use of imaging techniques in routine diagnosis results in detection of a growing number of renal cell carcinomas (RCCs) confined to the kidney (1, 2). However, in spite of the early detection, approximately 10-15% of the small RCCs operated with curative intent will develop metastasis within 5 years follow-up. Therapies targeting VEGF and mTOR pathways or recently used biologic and immunomodulatory therapies can prolong the life of patients

having metastatic disease. Therefore, there is a need for biomarkers to estimate postoperative tumor relapse. The close follow-up of these patients enhances the chance to detect metastasis as early as possible and start targeted therapy.

By applying global gene expression analysis using the Affymetrix array we have identified increased expression of several genes including chitinase 3-like protein 2 (CHI3L2), in most commonconventional RCCs with rapid progression leading to death of patients within 3 years after operation. The CHI3L2 expression was first demonstrated in chondrocytes and synoviocytes and CHI3L2 is recognized as a specific biomarker of progression of osteoarthritis (3, 4). There is a well documented correlation between expression of another chitinase (CHI3L1) and tumor progression (5-9). However, no data are available on the possible correlation between CHI3L2 expression and biological behaviour of conventional RCC.

The aim of this study was to analyse the expression of CHI3L2 in a cohort of conventional RCCs confined to the kidney at the time of operation and estimate the predictive value of its expression for postoperative tumor relapse. To achieve our goal, 634 clinically localized conventional RCCs at the time of operation were subjected to CHI3L2 immunohistochemistry.

Patients and Methods

Affymetrix array analysis. For gene expression analysis we used RNA isolated from 12 conventional RCCs leading to death of patients within 3 years of follow up and from another 12 tumours without progression during 8 years follow up. Gene expression analysis was carried out using the Affymetrix Human Genome U133 Plus 2.0 array as described earlier (10). Differentially expressed genes were identified by using the Gene Set Enrichment Analysis (11). Data of the expression profile have been deposited in the NCBI Gene Expression Omnibus under the accession number GSE11151.

Clinical data and tissue samples. A total of 634 patients who underwent radical or partial nephrectomy for conventional RCC between 2000 and 2013 at the Department of Urology, University

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of Pecs, Hungary were enrolled in this study. None of the patients had detectable metastatic disease at the time of operation. Data on regular follow-up, tumor relapse and tumor specific death were obtained from the Renal Tumor Registry of the Department of Urology. Follow-up was defined as the time from operation until the last recorded examination or cancer specific death. Patients who died from disease other than RCC were excluded from this study. Preoperative clinical staging included abdominal and chest computed tomography scans (CT). Bone scans and brain CT scans were performed only when indicated by clinical signs. The presence of nodal involvement was confirmed by histology, whereas distant metastases by radiographic examination. Patients were followed up every 6 months during the postoperative period, by abdominal ultrasound and measurement of serum creatinine and eGFR, and yearly by CT.

The histological diagnosis and TNM classification were reevaluated by a genitourinary pathologist (GK) according to the Heidelberg and TNM classification systems by applying a 3 scaled tumor grading system (12, 13). 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)

Tissue microarray (TMA) and immunohistochemistry. Representative tumor areas were marked in H&E stained slides of conventional RCCs. Two to five biopsies were obtained from each tumor, corresponding to areas of different morphology and/or tumor grade. TMA was constructed by one of the authors (GK) using a Manual Tissue Arrayer (MTA1, Beecher Instruments, Inc., Sun Prairie, WI, USA) and 0.6 mm core biopsies.

Normal adult kidneys and TMAs containing conventional RCCs were used for immunohistochemistry. After dewaxing and rehydration, the 4- μ m thick sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6.0 in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). We used the EnVision FLEX System (DAKO, Glostrup, Denmark) for immunohistochemistry. Endogenous peroxidase was blocked with the Peroxydase-Blocking Reagent (DAKO) for 10 min at room temperature. The slides were incubated for 1 hour at room temperature with polyclonal rabbit anti-CHI3L2 antibody raised against the amino acids 27-374 of the CHI3L2 protein (Cat. Number PA5-21548, ThermoFisher Scientific, Budapest, Hungary) at 1:200 dilution. HRP conjugated secondary antibody (DAKO) was applied for 20 min and color was developed using the AEC substrate (DAKO). Tissue sections were counterstained with Mayer's haematoxylin (DAKO). Normal adult kidney included in the TMA was used as positive control. For the negative control, the primary antibody was omitted. The result was scored as membranous, cytoplasmic or negative staining. As more than 90% of tumor cells were positive at least in one of the core biopsies, no counting of positive cells was necessary. In cases with different staining in multiple biopsies from the same tumor, the positive staining was taken into account.

Statistical analysis. Correlations between categorical variables were estimated with Fisher's exact test. Estimates of the cumulative survival distributions were calculated by the Kaplan-Meier method, and the differences between the groups were compared using the log-rank test. The significance of clinical-pathological variables was evaluated using the univariate and multivariate Cox proportional hazard regression model. Analysis was performed using IBM SPSS

Table I. Association of CHI3L2 expression with clinical and pathological parameters (n=634).

	Nr. of cases (634)	CHI3L2 expression		p-Value
		Negative (502)	Positive (132)	
Gender				0.020
Male	371	282	89	
Female	263	220	43	
Status				<0.001
AWOD	534	465	69	
PTP	100	37	63	
Tumor size				<0.001
< 4 cm	251	222	29	
4-7 cm	251	193	58	
> 7 cm	132	87	44	
T-Stadium				<0.001
pT1	477	405	72	
pT2	87	64	23	
pT3	70	33	37	
Tumor grade				<0.001
G1	430	386	44	
G2	158	101	57	
G3	46	15	31	
Tumor stage				<0.001
I	473	403	70	
II	84	63	21	
III	77	36	41	

AWOD: Alive without disease; PTP: postoperative tumor progression.

Statistics v.25 for Windows (Inc. Chicago IL, USA). *p*-Value <0.05 was considered as the limit of statistical significance.

Results

Patients. A total of 634 patients diagnosed with conventional RCC confined to the kidney were included in the study. The mean age of patients was 61.4 $\pm$ 11.2 and 371 (58%) of the patients were male and 263 (42%) female. The mean follow-up time of patients was 73.53 $\pm$ 4.6 months. During the follow-up 100 (16%) patients developed metastasis or died due to metastatic cancer. The pertinent clinical and pathological parameters are shown in Table I.

Expression of CHI3L2 in conventional RCC. Global gene expression analysis revealed an upregulation of CHI3L2 RNA exclusively in conventional RCC with rapid progression (Figure 1A). Immunohistochemical analysis detected CHI3L2 expression exclusively in proximal tubular cells of normal adult kidney. A strong membrane attenuated expression of CHI3L2 was seen at the luminal surface of the tubular cells whereas cytoplasm displayed no or only weak expression

Figure 1. Expression of CHI3L2 protein in conventional RCC. **A)** Global gene expression analysis revealed a preferential expression of CHI3L2 in rapidly progressing conventional RCC. **B)** Expression of CHI3L2 at the luminal membrane of proximal tubular cells (PT-proximal tubules, DT-distal tubules). **C)** Negative reaction with CHI3L2 antibody. **D)** Strong membrane attenuated expression in “clear cell” conventional RCCs. **E)** Diffuse cytoplasmic expression of CHI3L2 protein. **F)** Strong CHI3L2 protein expression in tumor cells around a small necrotic area (arrows). **G)** CHI3L2 positive cells within a tumor stroma (arrows). Scale bar: 50 μ m.

(Figure 1B). The vast majority of conventional RCC (502 of 634) were negative for CHI3L2 immunoreaction in each core biopsy (Figure 1C). Among the negative tumors both “clear cell” as well as “granular cell” conventional RCC were observed. A strong membrane attenuated and submembranous expression or cytoplasmic expression of CHI3L2 protein was seen in at least one of the core biopsies of 132 tumors (Figure 1D and E). Of interest, we observed strong CHI3L2 immunoreaction in tumor cells surrounding small necrotic tumor areas (Figure 1F). Tumor associated macrophages displayed strong CHI3L2 staining in the inflammatory stroma of aggressive growing tumors (Figure 1G).

Expression of CHI3L2 correlates with progression of conventional RCC. First, we evaluated the results of immunohistochemistry by Kaplan–Meier analysis according to negative, membranous and cytoplasmic positive cases and

found no significant difference between membranous and cytoplasmic expression. Therefore, we analysed both cytoplasmic and membrane attenuated expression together as positive. The association between CHI3L2 protein expression and clinical and pathological parameters is summarized in Table I. Expression of CHI3L2 showed a significant correlation with tumor relapse, size, grade and T classification. The Kaplan–Meier survival analysis using the log-rank test indicated a short disease-free survival for patients with CHI3L2 positive tumors (Figure 2). The 5-year survival rate for the CHI3L2 positive and negative group was 59.0% and 95.4%, respectively. Univariate analysis showed a significant association between size, T-classification, grade, and stage of tumors with CHI3L2 expression. Multivariate analysis was also performed to assess the independent prognostic value of CHI3L2 expression in relation to known clinicopathological prognostic variables. Positive CHI3L2

Figure 2. Kaplan–Meier estimates confirm the significant prognostic value of CHI3L2 expression in 634 patients with conventional RCC. The 5-year tumor-free survival rate for the CHI3L2 positive and negative group were 59.0% and 95.4%, respectively (Log rank (Mantel-Cox), $p < 0.001$).

expression showed a significant association with tumor grade (G2 and G3, $p = 0.004$, $p = 0.003$, respectively). The correlation between CHI3L2 positivity and postoperative relapse was statistically significant, marking a group of patients with more than 3 times higher risk for metastatic disease (RR=3.49; 95%CI=2.21-5.51; $p < 0.001$).

Discussion

In this study, detected cell membrane attenuated or cytoplasmic expression of CHI3L2 protein in 132 out of 634 conventional RCC confined to the kidney at the time of operation. Kaplan–Meier analysis and Cox proportional regression model revealed a significant correlation between CHI3L2 expression in tumor cells and postoperative tumor relapse. Multivariate analysis indicated that the expression of CHI3L2 by tumor cells was a significant independent factor of conventional RCC progression. Therefore, CHI3L2 is a valuable biomarker to stratify patients with conventional RCC into low- and high-risk categories.

The chitinase-like proteins belong to the glycoside hydrolase 18 family and exist in two isoforms, a major 50 kDa and a truncated 39 kDa isoform (14). The C-terminal domain of the truncated 39 kDa isoform has the capacity to bind to chitin, whereas the 50 kDa form is secreted by macrophages (15). The CHI3L2 binds to chitooligo-saccharides but it has lost the ability to hydrolyse them (16). CHI3L2 mRNA has been detected in lung, heart, and glioblastoma, but not in brain, spleen, or pancreas (3, 17). CHI3L2 is also expressed in macrophages stimulated by IL-4 and TGF β (18).

A recent study on human embryonic kidney cells (HEK293) and human glioblastoma (U87 MG) cells has shown that CHI3L2 signal transduction was regulated through the phosphorylation of ERK1/ERK2 kinases (19). CHI3L2 has been reported to enhance cell proliferation, colony formation and type II collagen expression at least in mouse chondrogenic ATDC5 cells (20). Recently, CHI3L2 has been identified as a pro-angiogenic and monocyte recruiting factor, and its elevated expression has been shown to be predictive for increased risk of distant metastases and poor response to neoadjuvant therapy of breast cancer (21). CHI3L2 has the ability to induce the migration of monocytes (22). The absence of clinical response to the therapy is associated with the presence of the M2 positive macrophage phenotype (22). Macrophages secrete chitinase like proteins including CHI3L2 (9). Recruitment of monocytes into tumor stroma is a critical process in all steps of tumor progression and may modulate the response to therapy (23, 24). In our study, expression of CHI3L2 was detected not only in the tumor cells but also in macrophages recruited to the tumor stroma.

An elevated level of expression and secretion of another chitinase 3-like protein 1, CHI3L1 (YKL-40) has already been correlated with poor outcome and short disease free survival of glioblastoma, breast, colorectal, lung, prostate, bladder, stomach and endometrial cancers (5-9). We showed, in this study, that positive immunostaining with CHI3L2 antibody is significantly associated with conventional RCC progression. We suggest that CHI3L2 expression can be used to identify a subset of conventional RCC with high-risk of disease progression and to optimize active surveillance and timely adjuvant therapy.

Conflicts of Interest

Authors have no conflicts of interest to declare.

Authors' Contributions

CP and GK designed the research study, GK constructed the TMA, CP and DB performed the immunohistochemistry, CP and MVY analysed the data. CP and GK wrote the manuscript, AS reviewed the manuscript.

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