The role of inflammatory signal transduction in the progression of conventional renal cell carcinoma

Doctoral (PhD) thesis

Author: Lehel Péterfi, MD

Department of Urology
Medical School, University of Pecs

Pécs, 2019

Head of the doctoral school: Prof. Lajos Bogár, MD, PhD, DSc
Program leader: Prof. László Pajor, MD, PhD, DSc
Mentor: Prof. Gyula Kovács, MD, PhD, DSc, FRCPath (UK)

1. Introduction

The role of inflammatory microenvironment in the tumor progression

"Lymphoreticular infiltrate" e.g. chronic inflammation at the sites of cancer was noticed by Rudolf Virchow in 1863. Today, a range of different scientific findings links cancer and inflammation. It is well demonstrated that several signalling pathways are involved in inflammation operating downstream of oncogenic mutations. Altered gene expression in tumour cells leads to recruitment of inflammatory cells producing chemokines and cytokines present in the stromal microenvironment. These pathways lead to activation of transcription factors (NF-kB, STAT3, HIF-1alpha) in tumour cells resulting in secerning of chemokines and cytokines. This in turn leads to recruitment of various inflammatory cells which also produces, together with the tumour cells, transcription factors. These transcription factors again lead to production of chemokines, cytokines and prostaglandin, continuing the circle of inflammation. This cancer-related inflammation finally leads to change various tumour-associated characteristics, like cell proliferation, survival, angiogenesis, tumour cell migration, invasion, metastasis, inhibition of adaptive immunity and altered response to hormones and chemotherapeutic agents. Tumours are not only mass of cancer cells, but contain many non-malignant stromal cells such as macrophages, lymphocytes, endothelial cells and fibroblasts. All these different cells and the cytokines which they secret, are involved in the neoplastic process, all of which together contributes to growth, progression and dissemination of tumour cells. One group of cells, which are a major component of the lymphoreticular infiltrate, are called tumour-associated macrophages (TAM). TAMs derive from the monocyte-macrophage lineage, and they are brought to the site of inflammation and tumour by chemokines. TAMs produce growth factors, angiogenic factors and protease-enzymes degrading extracellular matrix, all of which favours invasion and metastasis. Tumour-associated neutrophils

(TAN) play also a major role in cancer biology. The TANs migrate into tumour tissue under the influence of specific chemokines, cytokines and cell adhesion molecules, specifically the tumour microenvironment is responsible for their recruitment. Several cytokines (e.g CSF2, VEGF, IL-1ß and IL-6) secreted from tumour and stromal cells have been suggested to contribute to neutrophilia and to the induction of suppressive properties to these neutrophils. An inflammatory cytokine network may influence survival, growth, mutation, proliferation, differentiation and movement of tumour cells. Invasion of the tumour largely depends on its ability to degrade its local environment, allowing it to have space to expand. Matrix metalloproteases (MMP) produced by TAMs are activated by inflammatory cytokines, which release the enzymes into the extracellular matrix, digesting the environment, paving the way for invasion. Although several studies have been carried out to analyse the role of inflammatory microenvironment in tumour progression, only few have been concentrated on renal cancer.

In the last years by worldwide use of modern imaging techniques the number of incidentally detected pT1, pT2 or pT3 cRCC is steadily growing. In spite of early detection approximately 15-20% of these tumours progress and lead to metastasis. Therefore, it is necessary to find new biomarkers secreted by the tumour cells to estimate the clinical course of cRCC.

2. Objectives

The global gene expression analysis of cRCCs with and without progression by Affymetrix GeneChip platform detected the over expression of several genes including LBP and RARRES1 in cancers with progression. To confirm the RNS data, we have analysed a large number of well documented conventional RCCs with immunohistochemistry.

First, we have analysed the acute phase protein ***LBP*** gene to improve its role in the cRCC progression

Thereafter we have analysed on the same cohort of tumours the correlation between the carboxypeptidase inhibitor ***RARRES1*** and the carboxypeptidase ***AGBL2*** expression and tumour progression.

Because it was suggested that *RARRES1* gene use the tyrosine kinase ***AXL*** pathway and because the new tyrosine kinase inhibitors have been applied for target therapy, we have also analysed the AXL as well.

The final goal was to identify new biomarkers to estimate the prognosis of cRCC confined to the kidney at the time of operation.

3. Materials and methods

3.1. Patients and tissue samples

For evaluation of gene expression we have analysed tumour samples from 691 patients without metastasis at the time of presentation who undervent radical or partial nephrectomy for cRCC between 2000 and 2014 at the Department of Urology, University of Pecs, Hungary. Data on regular follow-up and tumour specific death was obtained from Registry of the Department of Urology. Follow-up was defined as a time from the operation until the last recorded control in 2018 or cancer specific death. Patients who died from causes other than RCC are not counted in this measurement. Preoperative clinical staging included abdominal and chest computed tomography scans (CT). Bone scans 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

yearly by CT. The histological diagnosis was performed by a genitourinary pathologist according to the Heidelberg and TNM classification systems. 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).

3.3. Tissue microarray (TMA) and immunohistochemistry

Paraffin blocks of fetal and adult kidneys and TMAs containing conventional RCCs were used for immunohistochemistry. From tumors with areas of different morphology and/or grade two to four 0.6 mm core biopsies were taken and used for TMA construction by a Manual Tissue Arrayer (MTA1, Beecher Instruments, Inc., Sun Prairie, USA). After deparaffinisation and rehydration the 4 um thick sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6,0 in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase activity and unspecific binding sites were blocked with 3% hydrogen peroxide containing 1% normal horse serum for 15 minutes at room temperature. Slides were incubated overnight at 4C in moist chamber with the primary antibody. 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. The following antibodies were used:

anti-LBP antibody (HPA 001508, Atlas Antibodies, Stockholm, Sweden) at the dilution of 1:250;

anti- RARRES1 antibody (HPA 001508, Atlas Antibodies, Stockholm, Sweden) at the dilution of 1:250;

anti-CCP2 (AGBL2) antibody (PA5-22310, Thermo Fisher Scientific, Budapest, Hungary), at the dilution of 1:200;

anti-AXL antibody (PA5-39124, Thermo Fisher, Budapest, Hungary) at the dilution of 1:200.

3.4. Statistical analysis

Data analysis was performed with the SPSS statistics software package version 20.0 (IBM,35 Armonk, NY, USA). Correlation between gene expression, clinical and pathological parameters was calculated with the Chi-square test. The effects of the different variables (age, sex, size of tumour, TNM classification, grades, stages, metastases and gene expression) on the survival time of the patients were estimated with Kaplan-Meier regression 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. LBP

Immunohistochemistry revealed the expression of LBP protein exclusively in proximal tubular cells of foetal and adult kidney. No LBP expression was seen in 588 (85%) tumours whereas a weak to strong cytoplasmic staining was detected in 103 (15%) of the 691 tumours. As we did not find substantial differences between weak or strong LBP expression and tumour progression, we have evaluated all cases with weak to strong expression as positive. As LBP expression is correlated with tumour progression, in cases with multiple biopsies the highest score was

evaluated. LBP expression was significantly correlated with the size, grade, T-classification stage and coagulation necrosis of cRCC as well as with cancer specific survival (all $p<0.001$). Using univariate survival analysis the T classification, grade, stage, necrosis and size of tumours as well as the LBP positivity are significantly associated with tumour progression and cancer specific death in a cohort of patients without metastasis at the first presentation (all but necrosis with $p>0.001$). Using multivariate analysis we have identified the T-classification, tumour grade and stage and LBP positivity as risk factors for postoperative tumour progression. The LBP expression was an independent negative survival factor indicating a nearly 4 times higher risk of cancer relapse (RR=3.60; 95% CI=2.15-6.04; $p<0.001$). Kaplan-Meier curves confirmed the significant prognostic value of LBP expression in cRCCs of patients without metastatic tumour at the first presentation.

4.2. RARRES1

4.2.1. Validation of RARRES1 expression in conventional RCC by immunohistochemistry

To validate the finding from the Affymetrix gene expression analysis we applied RARRES1 immunohistochemistry. First we have determined the RARRES1 protein expression in noncancerous foetal and adult kidneys. RARRES1 protein was detected exclusively at the luminal membrane of proximal tubular cells of adult and foetal kidney. Similar membranous expression was seen in 454 cRCCs displaying tubular or trabecular growth pattern. No expression was detected in 106 cRCC, whereas 131 tumours showed exclusively cytoplasmic RARRES1 expression. The expression of RARRES1 in distinct cellular compartment or lack of expression was significantly correlated with the size, grade, T-classification and stage of conventional RCC as well as with cancer specific survival (all $p<0.001$). The 5-year overall survival rate for the RARRES1 membranous, negative and cytoplasmic group were 97.6%, 79.8% and 61.6%, respectively. The mean

survival for patients with membranous RARRES1 staining was 170 (162-177) ± 4, with negative staining 135 (112-157) ± 11, and with cytoplasmic staining 90 (76-104) ± 7 months by overall survival of 158 (148-168) ± 5 months.

4.2.2. Prognostic significance of cellular localisation of RARRES1 protein

Kaplan-Meier analysis confirmed the significant prognostic value of cytoplasmic expression or lack of expressions of RARRES1 in cRCCs of 691 patients (Log rank (Mantel-Cox), $p<0.001$). Univariate survival analysis revealed the significant association between T classification, grade, stage and size of tumours as well as the RARRES1 expression (all <0.001). Multivariate analysis showed a significant association between the cytoplasmic RARRES1 expression or lack of expression and tumour relapse. The lack of RARRES1 staining and also the cytoplasmic staining was an independent negative survival factor indicating a 7-8 times higher risk of cancer specific death of patients (RR=8.402; 95% CI=4.245 to 16.631, $p<0.001$ or RR=7.570; 95% CI=3.880 to 14.772; $p<0.001$).

4.3. Expression of the AGBL2 in conventional RCC

We have applied the carboxypeptidase AGBL2 immunohistochemistry in the same cohorts of 691 tumours. 180 tumours showed a cytoplasmic positivity, whereas 512 negative with the AGBL2 antibody. In the positive cases all cells of the tumours displayed a strong immunostaining with the AGBL2 antibody. Evaluation of the AGBL2 expression in relation to the clinical-pathological parameters showed significant correlation only with the stage ($p=0.033$) and grade ($p=0.007$) but not with size of tumours ($p=0.082$). In multivariate analysis only tumour grade ($p<0.001$) correlated with AGBL2 positive

immunohistochemistry. There was no significant association between tumour progression and AGBL2 positivity (RR=1.081 CI= 0.700 to 1.669, p=0.726).

4.4. Co-expression of RARRES1 and AGBL2

Out of 454 RCC with membranous RARRES1 expression 110 tumours showed AGBL2 positive staining as well. Only 7 of the 106 RARRES1 negative RCC displayed AGBL2 positivity, whereas 62 of the 131 cytoplasmic RARRES1 positive tumour showed AGBL2 positivity as well (Pearson Chi-Square test ($p<0.001$)

Table 1 Correlation between RARRES1 and AGBL2 expression

		Nr of cases (691)	RARRES1 expression			p-value
			mem (454)	neg (106)	cyt (131)	
AGBL2						<0.001
	neg	512	344	99	69	
	pos	179	**110**	7	62	

mem- membranous; neg - negative; cyt - cytoplasmic. Pearson Chi-Square test ($p<0.001$)

The Kaplan-Meier analysis of RARRES1 and AGBL2 co-expression revealed an excellent prognosis for 110 of the 691 patients with membranous RARRES1 and cytoplasmic AGBL2 expression. Only one of the 110 patients died due to disease during the follow-up of 60 months. The result of Kaplan-Meier regression analysison the co-expression of RARRES1 and AGBL2 suggests that RARRES1 expression is the driving force determining the outcome of disease.

4.5. AXL gene

4.5.1. Correlation of AXL expression with clinical-pathological parameters

Immunohistochemistry failed to detect AXL expression in normal foetal and adult kidney samples. No AXL expression was seen in 227 (33%) conventional RCCs. In 321 (46%) tumours a weak to strong membranous immunoreaction was detected, whereas a cytoplasmic staining was observed in 143 (21%) of the 691 tumours. The AXL staining patterns were significantly correlated with the tumour size, grade, T-stadium and stage of conventional RCC as well as with cancer specific survival (all $p<0.001$).

4.5.2. Cellular localisation of AXL is associated with tumour progression

Kaplan-Meier analysis revealed that patients having a cRCC with cytoplasmic expression or lack of the expression of AXL protein had a significantly shorter disease-free survival time compared with those showing membranous AXL expression. In the group of tumours with membranous AXL expression 97.5% of patients were alive, whereas with negative tumour 89.6% and with cytoplasmic positivity 65.9% at the time of follow-up of 60 months indicating a shorter cancer specific survival for cases with cytoplasmic AXL expression. Univariate Cox regression analysis showed that tumor size, T classification, grade, stage as well as cytoplasmic AXL positivity or lack of expression were significantly associated with tumour progression and cancer specific death (all $p<0.001$). In multivariate Cox regression analysis tumour grade and stage and AXL remained as independent predictor of cancer specific survival of patients. The lack of expression or cytoplasmic expression of AXL was an independent negative survival factor showing four or five times higher risk of cancer specific death (RR=4.110; 95% CI=1.999-8.449; $p<0.001$ and RR=5.048; 95% CI=2.391-10.657; $p<0.001$), respectively.

5. Discussion

5.1. LBP

We found a strong correlation between cytoplasmic expression of LBP and postoperative progression of a subset of conventional RCC. Kaplan-Meier analysis and Cox proportional regression model showed that LBP expression in tumour cells associates with poor patient survival. Multivariate analysis indicates that expression of LBP by tumour cells is a significant independent factor to predict cancer specific survival in group of patients without detectable metastasis at the operation. LBP is an acute-phase protein that strongly modulates the response of immune system to endotoxins. LBP is constitutively produced by hepatocytes at a physiological level under normal conditions, but during an acute phase reaction IL-1 and IL-6 released from macrophages and monocytes strongly enhance LBP synthesis. At constitutive concentrations, LBP binds bacterial components such as lipopolysaccharide (LPS) and peptidoglycans and catalyzes their transfer via a CD14-enhanced mechanism to a receptor complex including TLR4 leading to the release of pro-inflammatory cytokines such as IL-6 which in turn enhance LBP synthesis. TLR4 can also recognize endogenous ligands such as heat shock proteins, extra cellular matrix components including fibronectin and heparin sulphate in response of tissue injury.

Conventional RCC is suggested to develop from proximal tubular cells. Expression of LBP in foetal and adult kidney is in accord with the involvement of proximal tubular epithelial cells in inflammatory processes in renal injury. The LPS-LBP-CD14-TLR4 signalling in microenvironment of cRCC can modulate the host immune system. Activation of TLRs initiates signalling cascades that mediate the release of cytokines from tumour cells, which recruit immune cells optimized for release of additional cytokines, pro angiogenic and growth factors that than continue to promote tumour survival and progression. Stimulation of TLR4 by the LBP-CD14 complex promotes tumour invasion through NFkB-dependent up regulation of matrix metalloproteinase-2 and beta-integrin. In summary, dysfunctional immunity within tumour microenvironment promotes its progression by mediating proliferative and survival signalling and promoting angiogenesis, metastasis and drug resistance.

In conclusion, we have identified the cytoplasmic expression of LBP in conventional RCC as an excellent marker to define a set of tumours with high risk of postoperative progression in patients without metastasis at the surgery. LBP staining may help to optimize active surveillance and direct adjuvant therapy with the ultimate goal of decreasing cancer progression. LBP immunhistochemistry is a cheap and reliable method to estimate the outcome of disease.

5.2. RARRES1 and AGBL2

Although the vast majority of cRCC (511 of 691) included in this study was classified as small (pT1a and pT1b) tumour at low risk of progression, 106 tumours developed metastasis during the follow-up or led to death of patients. Retaining the membranous RARRES1 expression at the tumor cell membrane marked a large group of patients (454 of 691) with excellent disease outcome. We found a positive correlation between the lack of expression of RARRES1 and postoperative tumour relapse in a group of patients with cRCC. These data might corresponds to the putative tumour suppressor or tumour progression suppressor function of RARRES1. RARRES1 expression was detected in different normal tissues such as prostate, lung, liver, heart and colon and in our study in the proximal tubules of embryonal and adult kidney. Decrease or lack of expression of RARRES1, frequently caused by promotor hypermethylation has been described in tumor cell lines and tumour tissues including prostate, endometrial, head and neck, nasopharyngeal, colorectal and gastric cancer and therefore it was suggested that RARRES1 is a tumour suppressor gene. However, not only the lack but also the strong cytoplasmic expression of RARRES1 had a significant association with postoperative tumour progression, the result which is not correspond to a “tumour suppressor” gene. Both the lack of expression or cytoplasmic expression of RARRES1 is a significant independent factor of predicting a 7 or 8 times higher risk for postoperative tumour

relapse in patients without detectable metastasis at the operation suggesting that the lack of membraneous expression is the precondition to aggressiv tumour behaviour.

RARRES1 (retinoic acid receptor responder 1) was first identified as a target gene induced by Tazarotene, hence its synonym TIG1 (Tazarotene-induced gene 1). RARRES1 has 6 exons and two alternatively spliced transcripts differing at the 3 end region. Based on the amino acid sequences RARRES1 is predicted to be transmembran protein with large extracellular region, a single pass hydrophic transmembran region and a small N-terminal intracellular region. Comparing the crystal structure of mouse latexin and the human latexin homologue TIG1 revealed that the basic surface of TIG1 may interact with membranes. A cell fractionation study revealed a full length isoform of 38 kDa RARRES1 as cell membrane bound. RARRES1 have also predicted to localise to other membranes including Golgi apparatus and endoplasmic reticulum. Another cellular fractionation study detected an approximately 50 kDa RARRES1 in the cytoplasmic but not in the membran fraction. Wether the cytoplasmic form of RARRES1 in cRCC corresponds to the 50 kDa protein found in RARRES1 transfected PC3 cell line is not yet known. What we know is that expression of RARRES1 at distinct cellular compartment is a strong new biomarker for estimating the risk of postoperative relapse and can be used to stratify patients with cRCC into high and low risk categories. Moreover, the membranous expression of RARRES1 with the cytoplasmic expression of the carboxypeptidase AGBL2 mark a group of tumours without any sign of progression during the average 60 month of follow-up.

5.3. AXL

Based on the cellular localisation we have separated three groups of tumours, one with membranous another with cytoplasmic AXL positivity and also a group of AXL negative

tumours. cRCC displaying membranous staining of AXL protein have an excellent prognosis. Patients with tumours dysplaying cytoplasmic expression have five times more risk to develop a metastatic disease during the five years follow-up. Until now, a single immunohistochemical study of AXL expression in RCC separated membranous and cytoplasmic staining, but evaluated the two forms together as positive result and did not correlated to disease outcome. In glioma cells only membrane attenuated AXL staining was reported, whereas other studies on distinct types of tumours applied an intensity and proportion score, staining intensity and per cent of positive cells or noticed simple as AXL positivity.

AXL is involved in several biological processes. Activation of AXL in tumor is linked to cell proliferation, survival, migration and invasion by activating oncogenic signaling pathways. The extracellular immunoglobulin-like and fibronectin-type III-like domains of AXL, which occur in the adhesion molecules of cadherin and immunoglobulin superfamily, suggest that AXL might regulate cell adhesion as well. AXL is expressed in tumor infiltrating macrophages and dendritic cells and has a function in limiting the innate immune response. Ablation of AXL signaling is associated with the increased expression of proinflammatory cytokines. And finally, upregulation of AXL leads to drug resistance of tumor cells, a function which makes AXL attractive for targeted therapy.

Taking into account the important function of AXL in several types of tumors novel forms of inhibition of AXL signaling by anti-AXL antibodies, by soluble AXL ectodomain or by small-molecule inhibitors have been elaborated. Recently, small kinase inhibitors for AXL have entered clinical trials. Cabozantinib, an AXL, MET and VEGFR2 inhibitor has been tested in the phase III METEOR trial and the therapy resulted in delayed RCC progression, and improved an objective response compared with everolimus. This finding has lead to inclusion of Cabozantinib in treatment of patients with metastatic conventional

RCC. The authors the METEOR study tested the MET expression in the same cohort of patients and concluded that MET expression is not correlated with the objective response to Cabozantinib. With all probability, Cabozantinib targeted the receptor tyrosine kinase AXL in metastatic cRCC. We suggest that AXL immunohistochemistry may help to optimize active surveillance of patients with cytoplasmic positive tumours and direct an early anti-AXL adjuvant therapy with the ultimate goal to achive a delayed progression

6. Conclusion

To continue the project of searching for new biomarkers for cRCC in the Department of Urology, we have analysed the acute phase protein LBP, carboxypeptidase AGBL2 and transmembrane carboxypeptidase inhibitor RARRES1 and tyrosine kinase AXL in a large serie of cRCC confined to the kidney at the time of operation.

The cytoplasmic expression of LBP protein shows significant correlation with the postoperative relapse of cRCC and delineates a group of patients with high risk of tumour progression.

The membranous expression of RARRES1 marks a group of tumours with good prognosis, whereas the lack or cytoplasmic expression of RARRES1 is associated with postoperative tumour relapse. Both the lack of expression or cytoplasmic expression identified a group of patients with 7-8 times higher risk of postoperative tumour progression. Of interest, all but one of the 110 patients having a tumour with membranous RARRES1 and cytoplasmic AGBL2 expression were tumour free during 60 month of follow-up time.

The tyrosine kinase AXL displayed a similar expression pattern to the RARRES1. The cytoplasmic expression of AXL showed a significant correlation with the postoperative

tumour relapse and separated a group of cRCC with a 4 times higher risk of tumour progression.

In Summary, we have identified three new biomarkers which predict the postoperative tumour relapse of cRCC confined to the kidney at the time of operation. Moreover, the cytoplasmic AXL positivity may direct the AXL kinase inhibitor therapy.

7. Acknowledgement

I would like to thank my mentor Professor Kovacs for introducing me in the scientific thinking and laboratory work and for our discussion on scientific moral and responsibility for the correct publication practice. Especially, I thank for discussions on several aspects of life outside the research work. I would like to thank the member of Molecular Oncology, Heidelberg for their valuable work, which was the starting point for my thesis. I thank Dr. Árpád Szántó for supporting my work in the laboratory and also Dr. Ákos Pytel my former mentor.

I thank my wife and my children for their patience and support.