ANTICANCER RESEARCH *39*: 1869-1874 (2019)
doi:10.21873/anticanres.13294

IL6 Shapes an Inflammatory Microenvironment and Triggers the Development of Unique Types of Cancer in End-stage Kidney

LEHEL PETERFI[1], MARIA V. YUSENKO[2] and GYULA KOVACS[1,3]

[1]*Department of Urology, Medical School, University of Pecs, Pecs, Hungary;*
[2]*Institute of Biochemistry, University of Muenster, Muenster, Germany;*
[3]*Medical Faculty, Ruprecht-Karls-University, Heidelberg, Germany*

Abstract. *Background/Aim: Chronic inflammation in end-stage kidney is associated with the development of pre-neoplastic lesions and renal cell tumors. The aim of this study was to clarify the role of the inflammatory microenvironment in this process. Materials and Methods: We used representative microscopic slides from 11 end stage-kidneys containing pre-neoplastic lesions and tumors and applied immunohistochemistry to detect IL-6, SAA1 and LBP expression. We also applied array-based comparative genomic hybridization (CGH) analysis to detect genomic changes in tumor cells. Results: We identified strong expression of IL6, LBP and SAA1 in activated stromal fibroblasts, in proliferating epithelial and tumor cells. Array CGH detected unusual genomic changes in tumor cells. Conclusion: Our data indicate that expression of IL6, acute phase protein SAA1 and LBP maintain a long-lasting inflammatory microenvironment that leads to remodeling of end-stage kidneys and the development of unique types of renal cell tumors.*

Chronic renal disease is characterized by gradually decreasing kidney function, which terminates in so-called end-stage renal disease (ESRD), and in acquired cystic renal disease (ACRD). ESRD kidneys display tubular atrophy, interstitial inflammation and fibrosis, as well as severe arterial, arteriolar and glomerular sclerosis, which results, either directly or indirectly, due to loss of functional and structural integrity (1). Despite of atrophic, scarred structures, ESRD/ACRD kidneys show a remarkable proliferative activity, especially in cells lining dilated tubules or small microscopic cysts. Remodelling of kidney structures is frequently accompanied by pre-neoplastic lesions and renal cell tumors (RCT) of unusual phenotype and genotype (2, 3).

The underlying molecular mechanism of structural changes and tumorigenesis in ESRD/ACRD kidney is not well known. The role of the microenvironment has been proposed to replace specific genomic alterations occurring in kidney cancers (4). Increased levels of hepatocyte growth factor (*HGF*) and its receptor c-met (*MET*), as well as ischemia, expression of hypoxia-inducible protein 2 (*HIG-2*) and hypoxia-inducible factor-1α (*HIF-1α*) have all been implicated in the remodeling processes of the kidney (5, 6). Recently, a global gene expression analysis revealed a unique expression signature containing a group of cytokines and chemokines, including IL6, and indicated the important role of the inflammatory microenvironment in ESRD/ACRD kidneys (7).

The aim of this study was to analyze the expression of the pro-inflammatory protein IL-6, as well as those of the acute phase proteins LBP and SAA1, using immunohistochemistry in ESRD/ACRD kidneys. We also characterized ESRD/ACRD associated tumors by array- based comparative genomic hybridization (CGH).

Materials and Methods

Tissue samples. Entire kidneys from 11 ESRD/ACRD cases were processed in several hundreds of paraffin blocks for histological analysis. The hematoxylin and eosin (H&E) stained slides were scored for: i) cysts, ii) small precursor lesions and iii) tumors. The diagnosis of tumors was established according to the Heidelberg Classification and Tickoo *et al*. (4, 8). Tissue multi array (TMA) was constructed from paraffin embedded ESRD/ACRD-associated tumors after marking the areas of interest on H&E stained slides. Core biopsies of 0.6 mm in diameter were placed in a recipient block by Manual Tissue Arrayer (MTA1, Beecher Instruments, Inc. USA).

Fresh tumor and kidney tissue samples from end stage kidneys were snap-frozen in liquid nitrogen immediately following

Correspondence to: Gyula Kovacs, Department of Urology, Medical School, University of Pecs, Munkacsy M. u. 2, H-7621 Pecs, Hungary. Tel: +3672507334, Fax: +3672242374, e-mail: g.kovacs@gmx.de, gyula.kovacs@urz.uni-heidelberg.de ORCID:0000-0003-2890-3781

Key Words: End stage renal disease, IL6, LBP, SAA1, micro-environment, tumor development.

Table I. *Pertinent clinical-pathological data of tumors analyzed by array-CGH.*

Case	Age/ Gender	Renal disease	Size of kidney	Size of tumor	Tumor diagnosis	Genetic changes
1.	71/F	ACRD	12×6.5×5 cm	4.0 cm	RO	None
2.	61/M	ESRD	6.5×3×3 cm	3.0 cm	cRCC	-1,-3p,-4p,+5q,-6,-8p,-9,-10,-13q,-14,-15,-17,-18,+20,-21
3.	43/M	ACRD	9×5×4 cm	3.0 cm	cRCC	-3p,-14
4.	71/M	ESRD	6.5×3.5×2 cm	2.8 cm	cRCC	-3p
5.	61/F	ACRD	10×6×4 cm	3.8 cm	cRCC	-3p,+5,+7,-8p,-10q,-X
6.	33/M	ACRD	10×5×4 cm	3.5 cm	pRCT	+3,+X

+: Gain of chromosome; –: loss of chromosome; RO: renal oncocytoma; cRCC: conventional RCC; pRCT: papillary RCT.

nephrectomy and were stored at –80˚C. The remaining tissue was fixed in 4% buffered formaldehyde for histological report. The collection and use of all tissue samples for this study was approved by the Ethics Committee of the University of Heidelberg and University of Pecs, Hungary.

Immunohistochemistry. Paraffin blocks of normal and ESRD/ARCD kidneys as well as TMA containing tumors were used for immunohistochemistry. Following dewaxing and rehydration of the slides, antigen de-masking was performed in 10 mM sodium citrate buffer, pH 6.0 or TE buffer, pH 9.0 in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, Netherlands). Endogenous peroxidase activity and unspecific binding sites were blocked using 0.3% hydrogen peroxide containing 1% normal horse serum for 10 minutes at room temperature. Slides were incubated overnight with an anti-IL-6 polyclonal rabbit antibody (PA1-26811, Thermo Fisher, Budapest, Hungary) at 1:400 dilution, with an anti-LBP polyclonal rabbit antibody (HPA 001508, Atlas Antibodies, Stockholm, Sweden) at 1:250 dilution and with an anti-SAA1 monoclonal mouse antibody (ab655, Abcam, Cambridge, UK) at 1:100 dilution. HRP conjugated ready to use rabbit anti-mouse secondary antibody (HISTOLSMR, Histopathology Ltd, Pecs, Hungary) was applied for 30 min at room temperature. The signal was visualized using AEC (Amino-ethyl-carbazol) (DAKO, Glostrup, Denmark) and DAB (3,3'-Diaminobenzidin) substrate (DAKO). Tissue sections were counterstained with Mayer's haematoxylin (Lillie's modification, DAKO) for 10 sec, and they were finally mounted using Glycergel Mounting Medium (DAKO) or PERTEX (medite Ltd. Burgdorf, Germany). In the negative controls the primary antibody was omitted.

Array CGH. The high molecular genomic DNA was hybridized to a 44k CGH microarray platform (Agilent Technologies), according to the procedure by the manufacturer (version 5.0). Scanning and image analysis were carried out on a DNA Microarray Scanner (Agilent) according to the user's guide (version 2.0 or 5.0). The Agilent Scanner Control software (version 7.0) was used for the 5 μm scan resolution with 100% PMT for both channels. Feature Extraction Software (version 9.5) was used for data extraction from the raw microarray image files using grid template AMADID 014950. To visualise, detect, and analyse chromosomal patterns within the microarray profiles, CGH Analytics Software (Agilent Technologies) was used. Global ADM 2 algorithm with a threshold 6.0 and an aberration filter defaulted for a minimum of 3 probes per region was applied in the analysis. A copy number gain was defined as a log2 ratio >0.3 and a copy number loss was defined as a log2 ratio <–0. 3.

Results

Histology of ESRD/ACRD kidney and tumor. Five kidneys were classified as ESRD whereas six cases with intensive cystic changes were classified as ACRD. Five tumors were diagnosed as papillary renal cell tumors (pRCT) and another 6 as conventional renal cell carcinomas (cRCC). We also found one oncocytoma, two ACRD-associated eosinophylic-vacuolated tumors, two chromophobe-like tumors, and one clear cell papillary RCC. Altogether, 65 small papillary, 42 chromophobe-like and 24 eosinophilic vacuolated pre-cancerous lesions were detected in the 11 kidneys collected from the nephrectomies (13).

Expression of IL6 in ESRD/ACRD kidneys and tumors. Diffuse *IL6* expression was seen in stromal fibroblasts and in some proliferating epithelial cells (Figure 1A). There was a positive immunostaining in dilated tubules showing papillary growth within the lumen. Scattered staining was detected in small atrophic as well as in dilated tubules lined with flat or cuboidal epithelial cells. IL6 positivity was seen in all conventional RCCs (Figure 1B) and in some of the chromophobe-like precursor lesions and tumors. ACRD-associated eosinophil vacuolated tumors displayed only scattered positivity with IL6, whereas the clear cell papillary tumor displayed IL6 positivity only in the stromal cells.

Expression of acute phase proteins LBP and SAA1. The LBP protein was highly expressed in the fibrotic stroma of ESRD/ARCD kidneys, whereas the SAA1 expression was less intensive in stromal areas (Figures 1C and D). Several tubules contained SAA1-positive fluid, which corresponds to elevated serum levels of IL6 due to acute phase response of the liver. Both LBP and SAA1 were also expressed in tumor cells (Figures 1E and F). Especially, chromophobe-like carcinomas and conventional RCCs displayed a moderate to strong LBP staining.

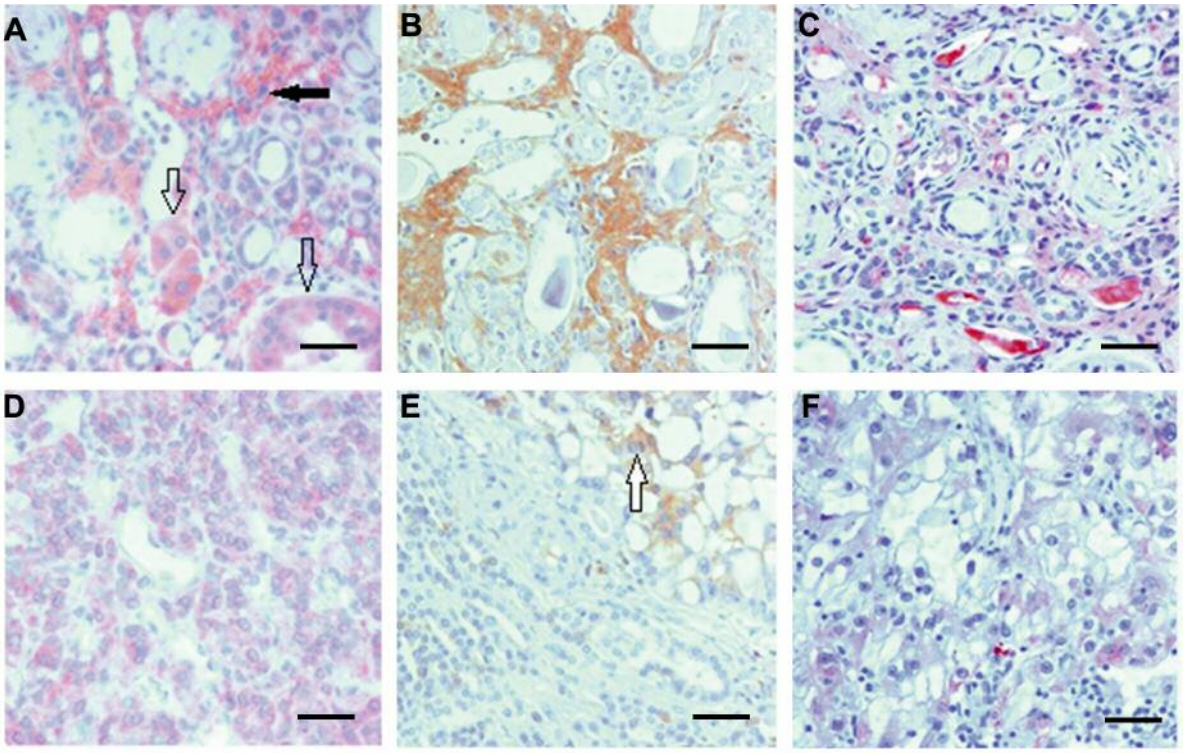

Figure 1. *Expression of IL6, LBP and SAA1 in ESRD/ACRD and tumors. (A) Strong IL6 expression in stromal fibroblasts (black arrow) and proliferation epithelial cells (open arrow), in eosinophilic vacuolated tumor (B) (open arrow). (C) Cytoplasmic immunoreaction of IL6 in a conventional RCC. (D) LBP expression in stromal fibroblasts and (D). (E) expression of SAA1 in stromal cells and (F) in some of the conventional RCC cells (Tumor 2). Scale bar: 25 μm.*

Genomic alterations in ESRD/ACRD tumors. High molecular weight DNA was available from six tumors (Table I). High resolution array CGH failed to detect any DNA changes in a renal oncocytoma (Tumor 1), which displayed an unusual histological pattern (Figure 2A and D). In four cRCCs a chromosome 3p loss was identified as expected. Tumor 2 showed an intensive inflammatory and sarcoid-like reaction (Figure 2B). However, considering the small size of Tumor 2, an unusual high number of additional genetic changes was detected (Figure 2E). Although Tumor 6 displayed a typical histology of a papillary RCT known in the general population (Figure 2C), CGH analysis revealed gain in chromosomes 3 and X (Figure 2F) instead of chromosome 7 and 17 trisomy, which is an unusual finding for papillary RCTs.

Discussion

We herein described the expression of *IL6, LBP* and *SAA1* in stromal fibroblasts, some proliferating epithelial cells in end stage kidneys, consistent with the presence of a pro-tumorigenic inflammatory microenvironment. Stronger expression correlated with the severity of structural remodelling and the increased amounts of fibroblasts in the scarred stroma. Moreover, the pre-neoplastic lesions and tumors appeared positive for the pro-inflammatory genes. Two of the six tumors displayed unusual histology, while one presented an unusual genetic abnormality, normally not found in such tumor types in the general population. Our observation strongly suggests an association between chronic, long-lasting inflammatory microenvironment in ESRD/ACRD and the development of morphologically and genetically distinct tumors.

ESRD/ACRD is the final stage of distinct types of kidney disease. The toxic effect as well as the reduced oxygen and nutrient delivery result in damaging the proximal tubules, which cannot easily convert from oxidative to glycolytic metabolism. Any type of damage to proximal tubular cells results in local production of pro-inflammatory chemokines and cytokines, including IL6, leading to an irreversible and progressive interstitial injury and loss of renal function (9).

The expression of various pro-inflammatory cytokines plays a crucial role in the remodeling of the extracellular matrix. Normal kidney fibroblasts can inhibit cancer development *via* direct cell-cell interaction by maintaining the normal tissue architecture (10). Active recruitment of inflammatory cells and progressive changes of stromal cells

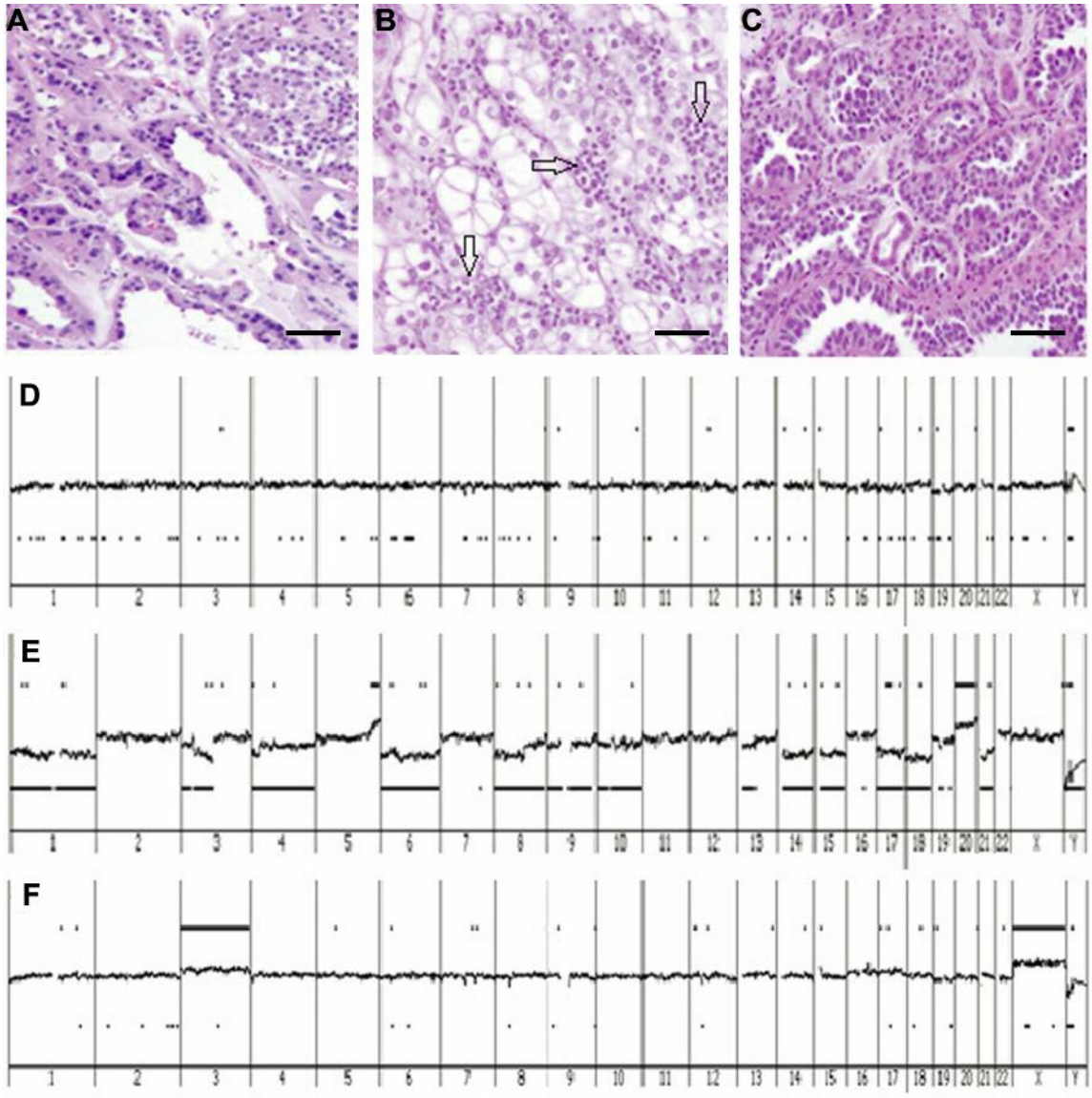

Figure 2. *Histology and genomic alterations of three tumors associated with ESRD/ACRD. (A) shows unusual histological pattern of an oncocytoma (Tumor 1). (D) no DNA changes has been detected by high resolution array CGH. (B) shows a conventional RCC (Tumor 2) with intensive immune cell infiltration (arrows). (E) in spite of the its small size this tumor displayed excessive genomic alterations. (C) shows a papillary RCT with tubulary and papillary growth patterns (Tumor 6,) and (F) trisomy in chromosomes 3 and X. Scale bar: 25 μm.*

can promote the development of cancer. The switch of normal stromal fibroblasts during long-lasting inflammation into activated fibroblasts, similar to the cancer-associated fibroblasts (CAFs), is one other important step supporting the development of cancer (11). The CAF-like fibroblasts producing IL6 in the stroma of end stage kidneys are largely responsible for the changes in the extracellular matrix, which supports tumorigenesis in that state. In addition to its other biological functions, IL6 can upregulate cytokeratin (12). It was shown recently that tubular cells in ESRD/ACRD kidneys shift their normal keratin profile from KRT8 and KRT18 to KRT7 and KRT19, allowing the cells to be more plastic (13).

During the acute phase reaction of the initial kidney disease IL-6 is released from macrophages and monocytes and may enhance LBP and SAA1 synthesis by stromal cells. As shown in this study, the stromal CAF-like fibroblasts release a large amount of IL-6 and induce LBP and SAA1 expression in the stroma. LBP catalyzes a signal *via* a CD14-enhanced mechanism to a receptor complex including TLR-

4, leading to the release of pro-inflammatory cytokines, such as IL-6, which in turn enhance LBP synthesis (14). The acute phase protein SAA1 also plays a role in the chemotactic recruitment of inflammatory cells to the site of inflammation and can activate the TLR4-dependent signalling. TLR4 can also recognize endogenous ligands, such as heat shock proteins, extracellular matrix components, including fibronectin and heparin sulphate, in response to tissue injury (15). Stimulation of TLR4 by the LBP-CD14 complex promotes *via* MyD88 invasion through NF-kB-dependent up regulation of matrix metalloproteinase-2 and beta-integrin (16).

One of the common mediators of carcinogenesis also includes an imbalance in the oxidative stress caused by inflammation (17). IL6 induces reactive oxygen and nitrogen species (RONS) causing mitochondrial and genomic DNA damage that may lead to tumor development (18). The higher frequency (83%) of *VHL* gene mutation in ESRD-associated conventional RCCs compared to those genetic alterations observed in RCCs in the general population (55%), also suggests a role for the hypoxemic-inflammatory cascade in tumorigenesis. RONS are also involved in the regulation of signaling pathways, including the activation of the hypoxia-inducible factor-1 (HIF1). Concerning the hypoxemic stage in ESRD/ACRD kidneys, high expression of hypoxia-inducible genes *HIG2, HIF1A* and *NFkB* triggering the expression of pro- and inflammatory proteins has been documented (6).

In agreement with previous findings, our work here recalls Virchow's observation that cancers occur preferentially at sites of chronic inflammation (19). The specific inflammatory microenvironment, the increased epithelial cell plasticity and high proliferation rate may be responsible for the structural changes and high frequency of tumor development. The increased production of free radicals causing genomic DNA alterations may additionally contribute to tumor development. There are several cancers whose development is linked to long-lasting inflammation, such as viral hepatitis and hepatocellular carcinoma, Helicobacter pylori infection and gastric cancer and Schistosomiasis and squamous cell bladder cancer (20). Our data also add ESRD/ARCD and associated tumors to this list, connecting them to chronic inflammation.

Conflicts of Interest

Authors have no conflicts of interest to declare.

Authors' Contributions

LP carried out the immunohistochemistry with IL6, SAA1 and LBP antibodies, MVY performed the array CGH and GK wrote the manuscript.

Acknowledgements

This work was supported by Grants of the Medical Faculty, University of Pecs, Hungary (PTE-AOK-KA-2018/16 and EFOP-3.6.3-VEKOP-16-2017-00009). The authors would like to thank Ms Barbara Kanyo for her excellent technical assistance, Drs. G. Staehler (Department of Urology, University of Heidelberg, Germany), B. Schulze-Brüggemann (Department of Urology, District Hospital Bad-Hersfeld, Germany), D. Ferluga (Insitute of Pathology, University of Ljubljana, Slovenia), and Mr. D. Cranston (Department of Urology, Radcliffe Hospital, Cambridge, UK) for providing us with specimens from end stage kidneys.

References

1 Hughson MD, Hennigar GR and McManus JFA: Atypical cysts, acquired renal cystic disease, and renal cell tumors in end-stage dialysis kidneys. Lab Invest *42*: 475-481, 1980. PMID: 7374111.

2 Chudek J, Herbers J, Wilhelm M, Kenck C, Bugert P, Ritz E, Waldman F and Kovacs G: The genetics and morphology of renal cell tumors in end-stage renal failure may differ from those occurring in the general population. J Am Soc Nephrol *9*: 1045-1051, 1998. PMID: 9621287.

3 Tickoo SK, de Peralta-Venturina MN, Harik LR, Worcester HD, Salama ME, Young AN, Moch H and Amin MB: Spectrum of epithelial neoplasms in end-stage renal disease: an experience from 66 tumor-bearing kidneys with emphasis on histologic patterns distinct from those in sporadic adult renal neoplasia. Am J Surg Pathol *30*: 141-153, 2006. PMID: 16434887.

4 Kovacs G: High frequency of papillary renal cell tumours in end stage kidneys – is there a molecular genetic explanation? Nephrol Dial Transplant *10*: 593-596, 1995. PMID: 7566566.

5 Konda R, Sato H, Hatafuku F, Nozawa T, Ioritani N and Fujioka T: Expression of hepatocyte growth factor and its receptor c-met in acquired renal cystic disease associated with renal cell carcinoma. J Urol *171*: 2166-2170, 2004. PMID: 15126778.

6 Konda R, Sugimura J, Sohma F, Katagiri T, Nakamura Y and Fujioka T: Over expression of hypoxia-inducible protein 2, hypoxia-inducible factor-1a and nuclear factor kB is putatively involved in acquired renal cyst formation and subsequent tumor transformation in patients with end stage renal failure. J Urol *180*: 481-485, 2008. PMID: 18550099. DOI: 10.1016/j.juro.2008. 04.006

7 Nagy A, Walter E, Zubakov D and Kovacs G: High risk of development of renal cell tumor in end stage kidney disease: the role of microenvironment. Tumor Biol *37*: 9511-9519, 2016. PMID: 26790439. DOI: 10.1007/s13277-016-4855-y

8 Kovacs G, Akhtar M, Beckwith BJ, Bugert P, Cooper CS, Delahunt B, Eble JN, Fleming S, Ljungberg B, Medeiros LJ, Moch H, Reuter VE, Ritz E, Roos G, Schmidt D, Srigley JR, Störkel S, van den Berg E and Zbar B: The Heidelberg classification of renal cell tumours. J Pathol *183*: 131-133, 1997. PMID: 9390023. DOI: 10.1002/(SICI)1096-9896(199710)183:2<131::AID-PATH931>3.0.CO;2-G

9 Daha MR and van Kooten C: Is the proximal tubular cell a proinflammatory cell? Nephrol Dial Transplant Suppl *6*: 41-43, 2000. PMID: 11143986.

10 Alkasalias T, Moyano-Galceran L, Arsenian-Henriksson M and Lehti K: Fibroblasts in the tumor microenvironment: shield or

spear? Int J Mol Sci *19*: 1532, 2018. PMID: 29883428. DOI: 10.3390/ijms19051532

11 Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K, Li X, Xiong W, Li G, Zeng Z and Guo C: Role of tumor microenvironment in tumorigenesis. J Cancer *8*: 761-773, 2017. PMID: 28382138. DOI: 10.7150/jca.17648

12 Toivola DM, Strnad P, Habtezion A and Omary MB: Intermediate filaments take the heat as stress proteins. Trends Cell Biol *20*: 79-91, 2009. PMID: 20045331. DOI: 10.1016/j.tcb.2009.11.004

13 Sarlos DP, Peterfi L, Szanto A and Kovacs G: Shift of keratin expression profile in end stage kidney increases the risk of tumor development. Anticancer Res *38*: 5217-5222, 2018. PMID: 30194170. DOI: 10.21873/anticanres.12845

14 Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, Polentarutti N, Muzio M and Arditi M: Bacterial lipopolysacchride activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. J Biol Chem *275*: 11058-11063, 2000. PMID: 10753909.

15 Akira S and Takeda K: Toll-like receptor signalling. Nat Rev Immunol *4*: 499-511, 2004. PMID: 15229469. DOI: 10.1038/nri1391

16 Kesenbrock K, Plaks V and Werb Z: Matrix metalloproteinases: regulators of the tumor microenvironment. Cell *141*: 52-67, 2010. PMID: 20371345. DOI: 10.1016/j.cell.2010.03.015

17 Chang CL, Marra G, Chauhan DP, Ha HT, Chang DK, Ricciardiello L, Randolph A, Carethers JM, Boland CR: Oxydative stress inactivates the human DNA mismatch repair system. Am J Physiol Cell Physiol *283*: C148-154, 2002. PMID: 12055083. DOI: 10.1152/ajpcell.00422.2001

18 Nagy A, Wilhelm M, Kovacs G: Mutations of mtDNA in renal cell tumours arising in end-stage renal disease. J Pathol *199*: 237-242, 2003. PMID: 12533837. DOI: 10.1002/path.1273

19 Virchow R: Die krankhaften Geschwülste (Dreissig Vorlesungen, gehalten wahrend desWintersemesters 1862-1863) Band I. August Hirschwald, Berlin, Germany.

20 Balkwill F, Charles KA and Mantovani A: Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell *7*: 211-217, 2005. PMID: 15766659. DOI: 10.1016/j.ccr.2005.02.013

Received January 31, 2019
Revised March 10, 2019
Accepted March 12, 2019