

Aim of the study: Despite significant progress in the pathology of clear cell renal cell carcinoma (ccRCC), diagnostic and predictive factors of major importance have not been discovered. Some hopes are associated with insulin-like growth factors. The aim of the study was to compare the expression of genes for insulin-like growth factor family in tumours and in tissue of kidneys without cancer.

Material and methods: Fifty-two patients years with clear cell renal cell cancer were qualified to the study group; patients nephrectomised because of hydronephrosis were included in the control group. Expression of genes were evaluated by RT-PCR.

Results: Expression of *IGFR-1* gene in tumour accounts for about 60% of cases. The incidence is higher than in corresponding adjacent non-cancerous kidney tissues and higher (but with no statistical significance) than in kidney without cancer.

Expression of *IGFR-2* gene in tumours has not been established. The incidence of the expression in corresponding adjacent non-cancerous kidney tissues is small. Expression of this gene has been present in all specimens from kidneys without cancer.

Expression of *IGFBP-3* gene ascertained in all (except four) cases of ccRCC and in the majority of clippings from adjacent tissue. It was not found in kidneys from the control group.

IGF-1, IGF-2, and IGFR-1 mRNA copy numbers in ccRCC were higher than in the material from the control group

Key words: insulin-like growth factors, clear cell renal cell carcinoma, RT-PCR.

Contemp Oncol (Pozn) 2016; 20 (2): 130–136
DOI: 10.5114/wo.2016.58720

Expression of insulin-like growth factor family genes in clear cell renal cell carcinoma

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Introduction

The incidence of renal cancer is increasing annually by about 2% [1]. According to recent data it represents 2–3% of all human malignant neoplasms [2]. In Poland, as well as in the Czech Republic, it is burdened with a high mortality level, and therefore it is considered one of the most important urological cancers in Central and Eastern Europe [3]. Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. Clear cell renal cell carcinoma (ccRCC) is a distinct subtype of RCC, and the most frequent. Kidney cancer develops as a result of abnormalities in the genetic material, which occur as a result of inheritance as well as changes acquired as a result of environmental impact [4]. Tumour stage and grade are considered as the only widely accepted prognostic markers of RCC.

Insulin-like growth factors 1 and 2 (IGFs) are peptides with promitotic and antiapoptotic effects. The action of both IGFs goes through the activation of receptor IGF type 1 (IGFR-1), a transmembrane receptor with tyrosine kinase activity, and in a small part through the activation of insulin receptor [5]. Another type of receptor, IGF receptor type 2 (IGFR-2), has no tyrosine-kinase activity and does not exert mitogenic and antiapoptotic action. This receptor is identical to the mannose-6-phosphate receptor (IGF-II/M6P-R). Activity of IGFs is regulated by their binding proteins (IGFBPs), most important of which is IGFBP-3. Activation of IGFR-1 promotes growth of many cancers [6–8]. Elevated level of IGF-1 and reduced IGFBP-3 in blood can be associated with an increased risk of cancer [9]. Increased incidence of colorectal cancer has been seen in acromegaly, in which hypersecretion of growth hormone (GH) is followed by elevated IGF-I levels [10]. The GH/IGF system is implicated in growth regulation of the kidney during embryogenesis. Treatment with GH and IGF increases kidney size and glomerular filtration rate [11, 12]. IGFs are also involved in pathological process, such as nephritis and glomerulosclerosis [13]. Against this background we may suspect that IGFs can promote the development and growth of ccRCC.

The aim of the study was to demonstrate the expression of genes for IGFs, IGFBP-3, and IGF receptors in ccRCC.

Material and methods

Patients qualified to radical nephrectomy were included to the study. The exclusion criteria were as follows: diagnosed diabetes mellitus, previously

treated cancer, any form of hormonal therapy. Sixty-two patients suffering from kidney cancer were included into the examined group and 30 patients (age 32–63) nephrectomised because of hydronephrosis were included into the control group (the number of patients is smaller than in the tested group, but it results in a small number of nephrectomies made for reasons other than cancer). Permission for the study was obtained from the Ethics Committee of Silesian Medical University (NN 6501-2004/06/07), and each patient gave written informed consent. Patients were operated and tissues were collected for histopathology. Cancer tissues and tissues from the pole of the kidney opposite to the tumour were collected for RNA extraction and immediately stored in the tank with liquid nitrogen. Only materials obtained from patients with tumours qualified as ccRCC were included to further study. Finally, 52 patients aged 35–65 years were qualified into the examined group. The clinical-pathological characteristics of this group are presented in Table 1. For all patients, nuclear grading was determined by the criteria proposed by Fuhrman *et al.* [14].

RNA extraction from tissue specimens

Total RNA was extracted from cells using an RNeasy mini purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Quantitative RT-PCR assay

Expressions of genes were evaluated using real-time RT-PCR and SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit; QIAGEN, Valencia, CA). The analysis was performed using an Opticon™ DNA Engine Continuous Fluorescence Detector (MJ Research, Watertown, MA). GAPDH gene was included to monitor the RT-PCR efficiency as an endogenous positive control of amplification and integrity of extracts.

Oligonucleotide primers (Table 2) were designed in the Department of Molecular Biology of Silesian Medical University on the basis of reference sequences (<http://www.ncbi.nlm.nih.gov>). Detection of *GAPDH* gene was performed according Ercolani *et al.* [15], and β -*actin* was detected according to Nakajima-Iijima *et al.* [16].

Table 1. Characteristics of the study group

Characteristics	Number	Age (years)
All group	52	41–65
Male	25	41–65
Female	27	41–65
Tumour size (cm) \leq 7	34	
Tumour size (cm) $>$ 7	18	
TNM		
T1a	13	
T1b	21	
T2a	6	
T2b	1	
T3a	7	
T3b	2	
T4	2	
Regional lymph nodes		
N0	41	
N1	11	
N2	0	
Distant metastases		
M0	52	
M1	0	

To quantify the results obtained a standard curve method was employed. Commercially available standards of β -*actin* (*ACTB*) cDNA (TaqMan® DNA Template Reagent Kit; PE Applied Biosystems, Inc., Foster, CA) were used at five different concentrations to simultaneously detect the expression profile of each gene. The obtained results of mRNA copy number were recalculated per μ g of total RNA.

Statistical methods

Statistical analyses were performed using Statistica 9.0 software (StatSoft, Tulsa, OK). Values were expressed as mean and standard error, as well as median and dispersion for tested parameters. Non-parametric Mann-Whitney U test was used for analyses. The prevalence of expression of tested parameters was calculated, and the results were compared using χ^2 test, and χ^2 with Yates' correction, depending on the calculation of the expected value. Cra-

Table 2. The sequence of primers used for RT-PCR

Gene	Sequence of primers	Oligonucleotide sequence	Localization mRNA	Amplimer
Homo sapiens insulin-like growth factor-1 (IGF-1)	IGF-1 F IGF-1 R	5' TGCTTCCGGAGCTGTGATC 3' 5' GATCCTCGGGTGGCATGTCACCTTCACT 3'	452-470 645-673	221 bp
Homo sapiens insulin-like growth factor receptor-1 (IGFR-1)	IGFR-1 F IGFR-1 R	5' ACGCCAATAAGTTCGTCCACAGAGACCT 3' 5' GAAGACTCCATCCTTGAGGGACTCAG 3'	3425-3452 3587-3612	187 bp
Homo sapiens insulin-like growth factor-2 (IGF-2)	IGF-2 F IGF-2 R	5' CGTTGAGGAGTGCTGTTTCC 3' 5' GGGGTATCTGGGAAGTTGT 3'	750-769 860-879	129 bp
Homo sapiens insulin-like growth factor receptor-2 (IGFR-2)	IGFR-2 F IGFR-2 R	5' TGGCAGGTCTCTGACTCAGAAGCTAAC 3' 5' GTCACAGTAGAAGAAGATGGCTGTGGAGC 3'	4028-4055 4121-4149	121 bp
Insulin-like growth factor binding protein-3 (IGFBP -3), transcript variant 2	IGFBP-3 F IGFBP-3 R	5' GCTACAGCATGCAGAGCAAGT 3' 5' CAGCTGCTGGTCATGTCCTT 3'	986-1006 1069-1088	102 bp

Table 3. Presence of expression of genes for IGF-1, IGF-2, their receptors, and IGFBP-3 in ccRCC and their corresponding adjacent non-cancerous kidney tissues (NKT)

Group/gene		IGF-1	IGF-2	IGFR-1	IGFR-2	IGFBP-3
ccRCC (n = 52)	number with presence of expression	11	13	32	3	48
	%	21.2	25	61.1	5.7	92.3
NKT (n = 52)	number with presence of expression	5	15	11	11	42
	%	9.4	29.9	21.1	21.1	80.9
Differences between groups (χ^2)		$p = 0.1323$	$p = 0.6307$	$p < 0.05$	$p < 0.05$	$p = 0.1309$

Table 4. Number of mRNA copies per μg of total RNA of IGF-1, IGF-2, receptors, and IGFBP-3 in ccRCC and their corresponding adjacent non-cancerous kidney tissues (NKT). Full analysis for *IGFR-2* gene was not applied because of the small number of slices from ccRCC in which expression was observed

Group/gene		IGF-1	IGF-2	IGFR-1	IGFR-2	IGFBP-3
ccRCC (n = 52)	number with presence of expression	11	13	32	3	48
	average \pm SEM	24846 \pm 5986	146717 \pm 15928	33835 \pm 4562	5.7	6213 \pm 813
	median	30671	153206	25819	*8504; 14926;	4688
	min.–max.	6112–49640	64872–243151	4507–84890	20773	378–21022
NKT (n = 52)	number with presence of expression	5	15	11	11	42
	average \pm SEM	7351 \pm 3851	165416 \pm 23290	15721 \pm 2131	7377 \pm 1710	7651 \pm 1267
	median	5870	127212	113123	404848	5682
	min.–max.	3082–27298	73487–345742	10358–31404	147178–753475	1153–27198
Mann-Whitney U-test		$p = 0.05$	$p = 0.7943$	$p < 0.05$		$p = 0.4007$

min – minimum; max – maximum; * – number of copies in each of three samples demonstrated expression

Table 5. Presence of expression of genes for IGF-1, IGF-2, their receptors, and IGFBP-3 in ccRCC and kidneys with hydronephrosis (control)

Group/gene		IGF-1	IGF-2	IGFR-1	IGFR-2	IGFBP-3
ccRCC (n = 52)	number with presence of expression	11	13	32	3	48
	%	21.2	25	61.1	5.7	92.3
Control (n = 30)	number with presence of expression	23	24	14	30	0
	%	76.7	85.7	46.7	100	0
Differences between groups (χ^2)		$p < 0.05$	$p < 0.05$	$p = 0.2468$	$p < 0.05$	

mer's V was used to evaluate the degree of association between Fuhrman grade and IGFs family gene expression.

Results

Fuhrman grade

Distribution of Fuhrman nuclear grade was as follows: grade 1–13, grade 2–29, grade 3–8, grade 4–2.

Gene expression in ccRCC tissue and non-cancerous kidney pole tissue, and differences between groups (Table 3)

Only in small number of both tissues was expression *IGF-1* of genes observed. There was no statistically significant difference in the incidence of this expression. Expression of *IGF-2* genes was observed in a small number of cas-

es; the number of slices with expression was similar. The expression of *IGFR-1* gene was observed in 61.1% of slices of ccRCC. Incidence in cancer was higher than in tissue free of cancer. The *IGFR-2* gene expression was rarely observed in both tissues. In ccRCC it was observed only in three cases and in the free of cancer pole of kidney in 11 cases. The difference was significant. In two cases, expression occurred in both tissues (not presented). *IGFBP-3* gene expression was observed in the majority of cases in both groups.

Number of mRNA copies in ccRCC and non-cancerous kidney pole tissue (Table 4)

IGF-1. Number of transcripts in ccRCC is higher than in non-cancerous kidney pole tissue. *IGF-2*. There is no significant difference in number mRNA copies.

Table 6. Number of mRNA copies per μg of total RNA of IGF-1, IGF-2, their receptors, and IGFBP-3 in ccRCC and kidney with hydronephrosis (control). Full analysis for *IGFR-2* gene was not applied, because only in 3 slices from ccRCC Full analysis for *IGFBP-3* gene was not applied because no expression of this gene was ascertained in the control group

Group/gene		<i>IGF-1</i>	<i>IGF-2</i>	<i>IGFR-1</i>	<i>IGFR-2</i>	<i>IGFBP-3</i>
ccRCC (n = 52)	number with presence of expression	11	13	32	3	48
	average \pm SEM	24846 \pm 5986	146717 \pm 15928	33835 \pm 4562	5.7	6213 \pm 813
	median	30671	153206	25819	*14926; 8504;	4688
	min.–max.	6112–49640	64872–243151	4507–84890	20773	378–21022
Control (n = 52)	number with presence of expression	23	24	13	28	0
	average \pm SEM	288 \pm 48	36560 \pm 3162	1845 \pm 318	7377 \pm 1710	
	median	240	39964	1560	2499	
	min.–max.	93–780	14317–56917	696–3734	917–24380	
Mann-Whitney U-test		$p < 0.05$	$p < 0.05$	$p < 0.05$		

min – minimum; max – maximum

IGFR-1. Number of transcripts is higher in cancer.

IGFR-2. Due to the small amount of cases in ccRCC no statistical analysis was performed.

IGFBP-3. There was no difference between groups.

Gene expression in ccRCC tissue and kidney with hydronephrosis (Table 5)

IGF-1 and *IGF-2.* Expression in the material from kidneys with hydronephrosis was ascertained as being more likely in specimens of ccRCC.

IGFR-1. Expression was more likely ascertained in ccRCC, but without statistical significance.

IGFR-2. As presented above in ccRCC, expression was been established (except in three cases). Expression was present in all specimens from kidney without cancer.

IGFBP-3 gene. Expression ascertained in all (except four) cases of ccRCC. Expression was found in kidneys with hydronephrosis.

Number of mRNA copies and differences between ccRCC tissue and kidney with hydronephrosis (control) (presented in Table 6)

IGF-1 and *IGF-2.* The number in tumours is higher than in kidneys from the control group.

IGFR-1. mRNA copy number in slices from ccRCC is higher than in kidneys of the control group.

IGFR-2. As shown above, gene expression was found only in three cases of ccRCC, no further analysis was carried out.

IGFBP-3. As shown above, the expression was not found in any slice from kidney without ccRCC. No further comparison between groups was performed.

Fuhrman grade and expression of tested genes (because of the small number of cases, grade 3 and 4 were grouped together)

IGF-1 gene. There was no expression in grade 3 and 4. Expression in grade 2 was more likely ascertained (31%) than in grade 1. V Cramer (VC) was 0.34, force of dependence was low.

IGF-2 gene. Expression was most frequently ascertained in grade 3 and 4 (40%) than in grade 2 (17.2%) and in grade 1 (15.4%). VC – 0.23. The force of dependence was low.

IGFR-1 gene. Expression in grade 3 and 4 was observed in 70%, in grade 2 – in 62%, in grade 1 – 38.5%. VC 0.40. The force of dependence was moderate.

IGFR-2 gene. No expression was observed in grade 1. In slices with higher Fuhrman grade it was observed in three cases.

IGFBP-3 gene. Expression was observed in almost all cases; there was no difference between groups.

Discussion

Although renal cell carcinoma is one of the most common, so far prognostic and predictive factors of significant importance have not been found. Some hope could be associated with insulin-like growth factors. Their elevated levels in blood have been demonstrated in breast, colorectal, and prostate cancer [17, 18]. The value of this observation in renal cancer seems to be small. Moreover, Rasmuson *et al.* have shown that elevated levels of IGF-1 in sera may be a favourable prognostic factor [19]. Therefore, we assumed to track the expression of IGFs and their receptors genes in tumour tissue and diseased kidney was more appropriate than levels in serum.

Because it is impossible to take material from healthy kidney, we included material from kidneys removed because of hydronephrosis to the control group.

In tissue originating from ccRCC, the presence of IGF-1 mRNA occurs less frequently than in kidney with hydronephrosis. The presence of the expression tissue of kidney in free of cancer with ccRCC is similar to its presence in cancer. There is no expression in tumours with high (3 and 4) Fuhrman grade. So we can suppose that IGF-1 production in ccRCC can be blocked. This partly explains the cited above results of Rasmuson. Although the expression of *IGF-1* and *IGF-2* genes in tumours is observed less frequently than in tissue of kidneys free of cancer, the number of transcripts is several or even tens of thousands of times higher.

Promitotic and antiapoptotic activity of both IGFs were revealed after joining with IGFR-1. Expression of *IGFR-1*

gene in ccRCC tissue is admittedly not observed in all cases, but appears with a higher rate than in tissue from the pole of kidney free of cancer with ccRCC, and slightly more often but with no statistical significance than in kidneys with hydronephrosis. Again, when expression occurs, the number of mRNA transcripts in tumour is always dozens, hundreds, or even thousands of times higher than in kidney without cancer. IGF-1 expression has been demonstrated earlier in a few studies related to various cancers. These researches have been focused on the study of protein as well as genes. Tumours demonstrating the presence of receptor protein were often characterised by a particularly aggressive course [20, 21]. When studies were devoted to the gene, the relationship was not so clear. The possibility that increased expression of *IGFR-1* gene could be a negative prognostic factor in epithelial ovarian cancer was indicated by the results of Spentzos *et al.* [22], while Dziadziuszko *et al.* showed the opposite situation [23]. In his study a high *IGFR-1* gene copy number in tumour was associated with better survival of patients with operable non-small cell lung cancer. Increased expression of IGF-1 in ccRCC was demonstrated Parker *et al.* and Sichani *et al.* They highlighted that high expression of IGF-1 may be a negative prognostic factor [24, 25]. Increased expression of *IGFR-1* gene in ccRCC was shown by Yuen *et al.*, pointing out the possibility of blocking this receptor in patients with advanced cancer [26, 27]. Results of our study show that this expression is moderately rising with Fuhrman grade.

Expression of *IGFR-2* gene was detected only in three cases of ccRCC and was observed in all subjects from the control group. IGF-2 is a protein with a single transmembrane domain, short cytoplasmatic tail, and long extracellular domain consisting of three repeats [28]. One binding IGF-2 and two different repeats binding mannose-6-phosphate-tagged proteins such as renin, prolipherin, thyroglobulin, and the inactive form of TGF- β . Binding of IGF-2 leads to its internalisation/degradation. Loss of expression of the gene encoding this receptor was observed in breast cancer and hepatocellular carcinoma [29, 30]. Loss of heterozygosity in one copy of gene and missense mutation in the second were demonstrated in both. Lack of *IGFR-2* gene expression was also observed in Wilms' tumour, but in this case imprinting of the gene was the reason for this phenomenon [31]. In humans, in contrast to mice, the IGF-2 imprinting gene probably does not exist, except in Wilms' tumours [32]. A lack of IGF-2 increases IGF-2 availability to the cell. Expression of *IGF-2* gene in ccRCC tissue and diseased kidney was been observed in all cases, but autocrine/paracrine action was not necessary. The action of systemic IGF-2 was sufficient. We can assume that the lack of expression of the *IGFR-2* gene in tumour may be linked to the development of the disease. The expression in the part of the kidney free of cancer with ccRCC occurs rarely, but with higher frequency than in tumours. To explore the mechanisms of the development of ccRCC it is advisable to clarify whether the loss of expression concerns only the tumour or whether it is systemic. A very interesting group are the isolated cases (three persons) who developed ccRCC despite the fact that there was no decline of the expression of this gene. Currently they are in

a follow-up to investigate whether there are diversities in the course of disease in this group.

The expression of *IGFBP-3* gene is in opposition to the expression presented above. It was present in over 92% of ccRCCs and has never been found in specimens from kidney free of cancer. Previous reports suggest that elevated serum concentration of IGF-1 protects against the development of cancer [17, 18, 33]. Our result does not exclude such a possibility. First of all, it concerns the tumour, and not the concentration in the blood. A few reports have presented results similar to ours. The first one, presented by Hintz *et al.*, shows the elevated expression of *IGFBP-3* gene in ccRCC tissue [34], and two others show the elevated expression of protein [35, 36]. Several factors are known to have the ability to influence the expression of this gene. The first one is IGF-1 [37, 38]. The impact of IGF-1 on IGF-1 expression was investigated by Rosendahl *et al.* [39]. Conducting the research on mice with xerotranted Caki-2 tumours (cell lines of human ccRCC), they showed that administration of IGF-1 stimulates the production of IGF-1 by tumours. The effect was stronger in early stages of tumour growth. IGF-1 in our case could provide a counterbalance for the mitogenic IGF-1 action and slow down rapid tumour growth. In this manner the milder course of illness in patients with higher IGF-1 observed by Rasmuson *et al.* could be explained. But the expression of *IGF-1* gene has been shown only in some ccRCCs. Systemic IGF-1 could promote this expression as well, but it is also present in persons without cancer, with no *IGFBP3* gene expression in the kidney. We can suppose that it is not the main reason for this expression. The cause of the observed (by us) *IGFBP-3* gene expression should be, however, the impact of hypoxia inducible factor (HIF)-1 α [40]. HIF-1 is a regulator of cellular response to hypoxia and its overexpression is observed in many tumours [41–43]. In normal oxygen conditions it is ubiquitinated. The ubiquitination requires binding to the von Hippel-Lindau (VHL) tumour suppressor protein [44]. In hypoxia or *VHL* gene mutation this process does not occur. Inactivation of the *VHL* gene due to mutation or hypermethylation is the most frequently described genetic alteration in ccRCC. According to some reports the prevalence may reach as much as 60–80% [45–47]. Induction of IGF-1 through HIF-1 α -mediated mRNA transcription has been described by Natsuizaka *et al.* [40]. It can be assumed that expression of IGF-1 appears as a counteraction of the promitotic action of IGF-1. Nevertheless, it may also have the opposite effect and promote the development of cancer [48]. In light of this, the expression of IGF-1 could indicate tumour malignancy. Taking into account the relationship between IGF-1 and the mutation of *VHL* gene, we may suspect that the observed *IGFBP-3* gene expression is an indicator of *VHL* gene mutation. Confirmation of this requires further research. Based on fairly unequivocal results indicating the presence of *IGFBP-3* gene expression in ccRCC and the healthy part of the kidney with cancer, it could be identified as a potential diagnostic marker of ccRCC. Our study is too small to fully demonstrate the utility of *IGFBP-3* gene as a biomarker, and it encourages further research. The follow-up is ongoing, and potential differences in the further course of

the disease between patients with present or absent of *IGFBP-3* gene expression would be an indication for studies on its prognostic and predictive value in ccRCC.

In conclusion, it should be said that the presented results show that there are basics for research on the use of the gene for *IGFBP-3* as a diagnostic marker for ccRCC and the gene for *IGFR-1* as a prognostic marker. Further research is also needed for a full explanation of the role of loss of *IGFR-2* gene expression in the development of ccRCC.

The authors declare no conflict of interest.

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Submitted: 18.10.2015

Accepted: 9.03.2016