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ARTICLE *in* KIDNEY AND BLOOD PRESSURE RESEARCH · FEBRUARY 2014

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Kidney Blood Press Res 2013;38:83-91
DOI: 10.1159/000355754
Published online: February 22, 2014
Accepted: January 09, 2014

1423-0143/14/0381-0083$39.50/0

Original Paper

Analysis of Relative Expression Level of VEGF (*Vascular Endothelial Growth Factor*), HIF-1α *(Hypoxia Inducible Factor 1α*) and CTGF (*Connective Tissue Growth Factor*) Genes in Chronic Glomerulonephritis (CGN) Patients

Rafał Donderski[a] Joanna Szczepanek[b,d] Krzysztof Domagalski[c,d] Andrzej Tretyn[d] Jadwiga Korenkiewicz[e] Andrzej Marszałek[e] Andrzej Szymański[f] Zbigniew Wolski[f] Grażyna Odrowąż-Sypniewska[g] Jacek Manitius[a]

[a]Department of Nephrology, Hypertension and Internal Medicine; [b]Department of Pediatrics Hematology and Oncology; [c]Department of Infectious Diseases and Hepatology; [d]Department of Plant Physiology and Biotechnology, Faculty of Biology and Environment Protection, Center for Modern Interdisciplinary Technologies, Nicolaus Copernicus University Toruń; [e]Department of Clinical Pathology; [f]Department of Urology; [g]Department of Clinical Laboratory Medicine, University Hospital, Bydgoszcz, Nicolaus Copernicus University, Toruń, Poland

Key Words
Chronic kidney disease (CKD) • Chronic glomerulonephritis • Relative gene expression level • Tubulointerstitial compartment damage

Abstract
Background/Aims: Analysis of gene expression in renal tissue is considered to be a diagnostic tool predicting the clinical course of glomerulonephritis. The present study quantified the relative transcript levels of VEGF, CTGF and HIF-1α in renal tissue to establish their relationship with some clinical variables in patients suffering from chronic glomerulonephritis (CGN). ***Methods:*** 28 patients (6F and 22M, mean age 51.2±15.0) with CGN were enrolled. Type of CNG recognized by kidney biopsy (histopatological evaluation) was as follows: minimal change disease (MCD)-3pts, IgA nephropathy-5pts, FSGS-3pts, membranous nephropathy-4pts, mesangio-proliferative glomerulonephritis-3pts; MPGN-1pts, lupus nephritis-6pts, granulomatosis with polyangitis-2 pts; hypertensive nephropathy- 3pts. Renal tissue from 3 individuals with normal eGFR and histology was taken as control. Mean clinical follow-up of patients was 12 months after biopsy. eGFR and daily urinary protein excretion (DPE) was assessed

Rafał Donderski MD

Dept. of Nephrology, Hypertension and Internal Medicine
Nicolaus Copernicus University in Toruń, Skłodowskiej-Curie No 9, 85-094 Bydgoszcz (Poland), Tel. +48-052-5854030, Fax +48-052-585-40-30, E-Mail rafdon@o2.pl

Kidney & Blood Pressure Research

Kidney Blood Press Res 2013;38:83-91
DOI: 10.1159/000355754
Published online: February 22, 2014

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at the time of biopsy and then in 6 months intervals. Real-time PCR was used to determine relative gene expression. The housekeeping gene *GAPDH* was used as normalization control. ***Results:*** At the time of the biopsy relative expression of 3 analyzed genes was diminished in comparison to control. There were statistically significant differences in VEGF gene relative expression level in patients which varied according to eGFR and tendency in patients which varied according to DPE. HIF-alfa and CTGF gene showed only a tendency. ***Conclusions:*** Overexpression of the VEGF gene in subjects with DPE>3,5g may point to insufficient oxygen supply in renal tissue which may result in tubulointerstitial fibrosis with further functional renal impairment and decline of eGFR.

Introduction

Percutaneous fine-needle kidney biopsy is considered to be a gold standard in the diagnostics of kidney diseases. Histological analysis of renal tissue may be helpful in detecting its structural changes, both in the glomerular and the tubulointerstitial compartment. The assessment of immunoglobulin and complement fractions is an integral part of this evaluation. Novel diagnostics approach in kidney diseases compromise: gene expression analysis of renal tissue samples obtained during kidney biopsy, and analysis of urinary proteins, mainly low molecular weight proteins (LMWP) the excretion of which may correlate with severity of tubulointerstitial lesions. [1].

The analysis of mRNA level for molecules known to be involved in pathogenesis of tubulointerstitial lesions may be helpful as prognostic tool in renal diseases and can correlate with the slope of renal function determined by eGFR measurements [2]. Gene expression data usually correlate with clinical course and biopsy histology, and they provide potential insight into the "cellular programme" of the underlying disease [3, 4]. Moreover, the upregulation of intrarenal genes expression may precede structural alterations. In our study we wanted to evaluate the relative expression of VEGF (*vascular endothelial growth factor*), CTGF (*connective tissue growth factor*) and HIF-1α *(hypoxia inducible factor 1α)*, genes, which play a role in regulating angiogenic, fibrogenic, inflammatory, and apoptotic renal processes [5-8].

Material and Methods

28 patients (pts) (6F and 22M, mean age 51.2±15.0) with mostly primary chronic glomerulonephritis (CGN) recognized in kidney biopsy were investigated. In each individual, 3 tissue specimens were collected. Histopathological and immunohistological evaluation of renal tissue samples was performed. Each specimen was considered to be representative as it contained more than 10 glomeruli. Molecular analysis was carried out in Biotechnology Department of Nicolaus Copernicus University in Toruń. Indications for renal biopsy were: nephrotic syndrome, proteinuria of less than 3,5g per day, hematuria (without hematuria of urological origin), rapid decline in renal function (RPGN) associated with changes in urinalysis. Histopatological characteristics of patients are presented in Table 1. Renal tissue from 3 individuals with normal renal function and histology was used as a control. The normal renal tissue samples were obtained during total nephrectomy performed after diagnosis of renal tumors. The control tissue was obtained from kidneys with no evidence of tumor obstruction or vessel invasion. The samples were obtained from areas remote from the malignancies. All studied subjects had no history of hypertension or renal disease.

Median clinical follow-up of patients was 12 months after biopsy procedure. eGFR (ml/min) using the MDRD equation was calculated. eGFR was followed for 12 months at 6 months intervals (T_0-T_6-T_{12}). Mean values were: $eGFR_0$ -55.8±31.5; $eGFR_6$- 55.1± 28.8; $eGFR_{12}$ – 54.7±30.5 respectively. Daily urinary protein excretion (DPE) was evaluated at the time of the biopsy and was expressed in g/24h. Mean values calculated at 6 months intervals were: DPE_0- 5.48±5.15; DPE_6 -3.37±3.43; DPE_{12} 1.58±1.88 respectively.

Hypertension was diagnosed in 23 patients (82,1%). They were treated with ACE-I – 23 pts; AT II antagonists – 21pts; calcium channel blockers- 20 pts; diuretics- 23 pts. Mean blood pressure in office

measurements was SBP-137,5±10,2mmHg and DBP-86,52±7,59mmHg. Renal anemia (HB concentration <13,0g/dl in males and <12,0g/dl in females) was diagnosed at the time of kidney biopsy in 6 individuals. These were cases of rapid progressive glomerulonephritis (RPGN) related to lupus nephritis and vasculitis. Immunosuppressive agents were administered to 19 patients in accordance with biopsy findings suggesting active renal lesions. The treatment was carried out accordingly to current standards.

The study protocol was approved by the local Bioethics Committee of the Nicolaus Copernicus University Collegium Medicum in Bydgoszcz.

Table 1. Histological characteristics of patients

Histopathology evaluation	Number of cases
Minimal change disease	3
IgA nephropathy	5
FSGS	3
Membranous nephropathy	4
Mesangioproliferative glomerulonephritis	3
MPGN	1
Lupus nephritis	4
Granulomatosis with polyangiitis	2
Hypertensive Nephropathy	3
Control	3

Abbreviations: FSGS: focal segmental glomerulosclerosis, MPGN: membranoproliferative glomerulonephritis

Histopathological evaluation

Two renal tissue samples obtained from the left kidney were sent to the Department of Clinical Pathology of the Nicolaus Copernicus University Collegium Medicum in Bydgoszcz. After fixing in 10% formalin overnight paraffin block was prepared (one formalin fixed specimen). Hematoxylin-eosin, trichrome Masson, Congo red staining and PAS reaction were performed on deparaffinized and dehydratated paraffin tissue sectioned at 4-μm thickness. The other specimen were left unfixed for immunofluorescence examination. The tissue sample was frozen at -30°C. In 4 μm tissue sections the IgG, IgM, IgA, C3 and fibrinogen were determined by means of fluorescein-labeled anti-human serum.

Molecular analysis

Renal tissue samples were taken from each patient during routine biopsy procedure for real-time polymerase chain reaction (RT-PCR) assessment of the mRNA levels for investigated genes. The samples were transported in liquid nitrogen or RNalater solution, then stored at the temperature of -80˚C for subsequent RNA extraction performed in the Department of Biotechnology, Nicolaus Copernicus University.

Sample preparation

Buffer RLT (Qiagen) was used for the lysis of homogenized tissues before RNA isolation. Total cellular RNA was extracted from samples with the use of TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. RNA was additionally purified with DNase I (Fermentas) and phenol–chloroform extraction. RNA quality and quantity were confirmed with agarose gel electrophoresis and spectrophotometry (NanoDrop). 4 μg of the purified RNA was reverse transcribed by using the Transcriptor High Fidelity cDNA Synthesis Kit (containing reverse transcriptase and random primer hexamers), following the manufacturer's recommendations (Roche). In order to assess the quality of the obtained cDNA and the specificity of the primers, and to develop appropriate reaction conditions, especially the annealing temperature of primers for each gene, a conventional PCR was performed.

Quantitative RT-PCR

The following primers have been used to measure RNA abundance (5` to 3`):
VEGF forward primer: AAGGCTGAGCTGGAGGAAG, *VEGF* reverse primer: GGAGCATGATTGAGACTCGC
CTGF forward primer: TGACCGCCGCCAGTAT, *CTGF* reverse primer: GAGGAGGACCACGAAGGC
HIF1α forward primer: TGCTCATCAGTTGCCACTTC, *HIF1α* reverse primer: CCAAGCAGGTCATAGGTGGT
GAPDH forward primer: GAGTCAACGGATTTGGTCGT,
GAPDH reverse primer: TTGATTTTGGAGGGATCTCG.

We used the housekeeping gene *GAPDH (glyceraldehyde 3-phosphate dehydrogenase)* as normalization control. The comparative cycle time (Ct) value of the target PCR was normalized by subtracting the Ct value of *GAPDH*. Each reaction contained 1 μl standard/cDNA template, 0.1 μl each primer for the gene (final concentration 0.3 μM), 8.8 μl water, and 10 μl FastStart SYBR Green Master following the standard PCR program suggested by the manufacturer (Roche). The reactions were carried out on a Quantica instrument (Techne), using the following temperature program: 95°C for 10 minutes (activation of FastStart Taq DNA Polymerase), then 40

cycles of 95°C for 15 seconds, 61°C for 15 seconds and 72°C for 30 seconds. The reactions were performed in duplicate.

The amplification of the target sequence was detected using SYBR Green dye. Melting curve analyses were performed to verify the amplification specificity. At first, quantitative PCR reactions were carried out on a dilution series of a standard to obtain standard curves for both target and control genes. Concentrations of standards were determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer. The analysis was performed on serially diluted samples in triplicate. The specificity of the reaction was examined by analysis of the melting curve of the final amplified product. After 40 cycles, a melting curve was generated by increasing (by 0.5°C) the temperature from 70°C to 90°C, while the fluorescence was measured. The threshold cycle (Ct) was calculated based on the Second Derivative Maximum method using the Quansoft Software (Techne) by determining the cycle number at which the change in the fluorescence crossed the threshold. The qRT-PCR data were plotted as fluorescence signal versus cycle number. The relative level of expression was calculated using the Pfaffl method:

$$R=\frac{(E_{\text{target gene}})^{\Delta C_T \text{ target gene (control-sample)}}}{(E_{\text{GAPDH}})^{\Delta C_T \text{ GAPDH (control-sample)}}}$$

Statistical analysis

Results are expressed as mean ± SD. The relative gene expression levels are presented as n-fold difference relative to the control. Gene expression of the control group in the comparative C_T method always equals one. The data obtained were compared using Mann-Whitney non-parametrical tests. For all the tests p value of <0.05 was considered as statistically significant.

Results

Real-time PCR was used to determine the relative level of expression of selected genes. An ongoing experiment, we used non-specific detection of double-stranded DNA using SYBR Green dye.

For the purpose of analysis the following were used:
- standard curves (for the assessment of efficiency of reaction) (Figure 1A),
- amplification curves (allowing the determination of the threshold cycle Ct) (Figure 1B),
- melting curves of reaction products (for an analysis of the specificity of the amplicon synthesized) (Figure 1C).

The obtained gene activity expression data were normalized to the housekeeping gene - *GAPDH*.

The levels of *VEGF*, *CTGF*, *HIF1α* in the investigated group (black bars) and the control group (grey bars) were measured relative to *GAPDH*. The ratio of target genes to *GAPDH* is shown on the y-axis, the gene type analyzed is shown on the x-axis. Expression of *VEGF*, *CTGF*, and *HIF1α* was normalized to internal housekeeping gene control (*GAPDH*) using the Pffafl method (Figure 1). The relative expression level of 3 analyzed genes was evaluated in CGN patients (Table 2). In all analyzed tissue samples the relative expression level of investigated genes was lower in comparison to the control group. *VEGF* sample group was different from the control group. [P(H1)=0,003.] We found a trend toward lower expression level of *CTGF* (0,57) and *HIF-1α* (0,61) in the investigated group in comparison to control group (Figure 2). No statistical significance was discovered (Table 2). A higher relative expression of VEGF gene without statistical significance was found in pts with DPE>3,5g. (p<0,064) (Figure 3). We also found tendency toward higher relative gene expression for CTGF and HIF-alfa gene in pts with DPE>3,5g (without statistical significance). We found statistically significant differences in the relative expression of VEGF gene between groups with eGFR>60ml/min and eGFR<60ml/min (p<0,02), (Figure 4). VEGF overexpression was detected in patients with eGFR>60ml/min. Such statistically significant relationship was not

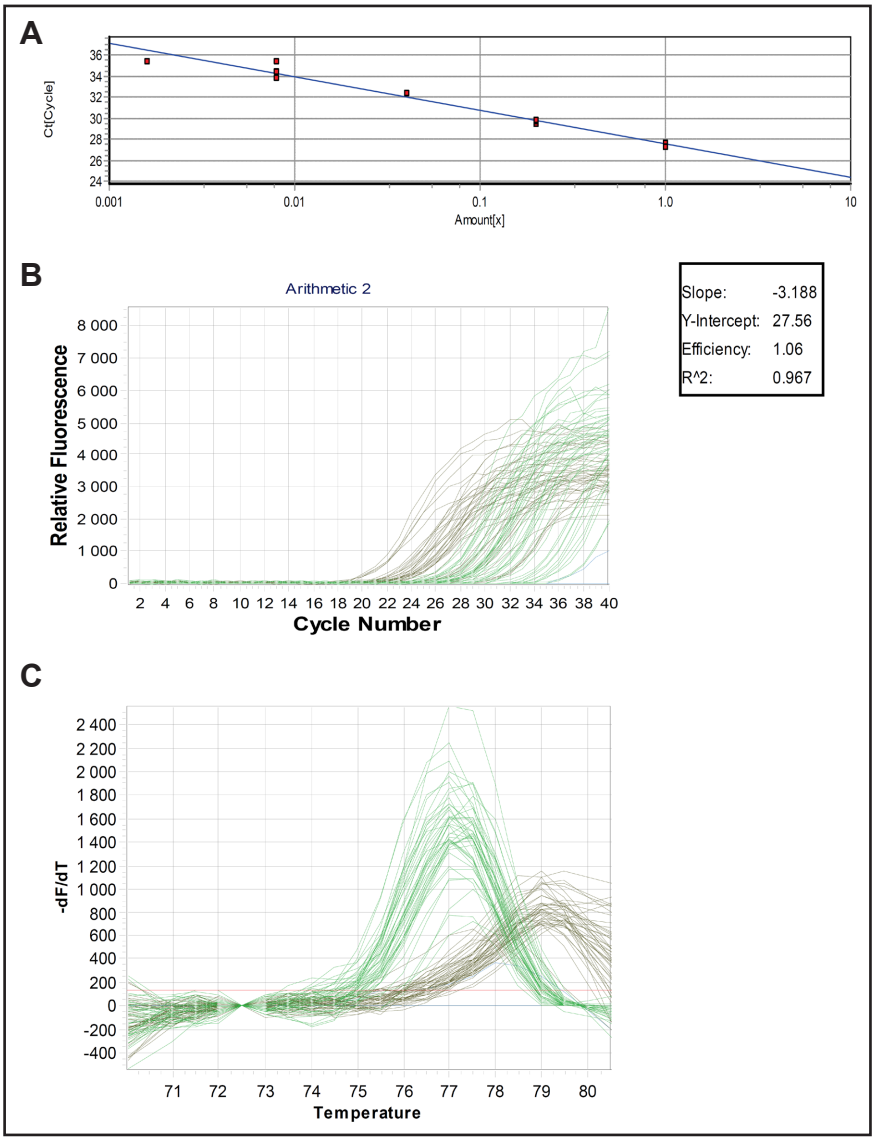

Fig. 1. Real-time RT-PCR of *VEGF* and reference gene. A – Standard curve for *VEGF* amplification. The curve is based on values obtained for decreasing serial dilutions of the sample cDNA; B – Amplification curves (the graph shows the amplification profiles of *VEGF* and *GAPDH* present in clinical samples. X axis, number of amplification cycles, Y axis, intensity of fluorescence in arbitrary units). Every sample was run twice. C – melting curves for *VEGF* and *GAPDH.*

Table 2. Results of relative expression level of investigated genes

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)
VEGF	TRG	1,06	0,300	0,163 - 0,485	0,107 - 1,445	0,003
GAPDH	REF	0,99	1,000			
CTGF	TRG	1,06	0,571	0,271 - 1,474	0,133 - 2,005	0,211
GAPDH	REF	0,99	1,000			
HIF1 alpha	TRG	0,95	0,614	0,106 - 1,549	0,048 - 22,535	0,542
GAPDH	REF	0,99	1,000			

Abbrevations: P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance, TRG - Target, REF – Reference

observed in CTGF and HIF-1 alfa. There was only a tendency. Patients' age, gender, or the presence of hypertension had no influence on the relative VEGF expression level except for DPE ($p<0,06$) and eGFR ($p<0,02$) (Mann-Whitney test). Patients' age, gender, DPE, eGFR, or presence of hypertension had no influence on the relative CTGF and HIF-1 alfa expression levels. No statistically significant differences in relative expression levels for VEGF, CTGF, HIF-1α were observed in a subset of glomerulonephritis with different histopathological pattern. Such evaluation was performed on 3 subgroups with the highest number of patients (IgA nephropathy, membranous nephropathy lupus nephritis).

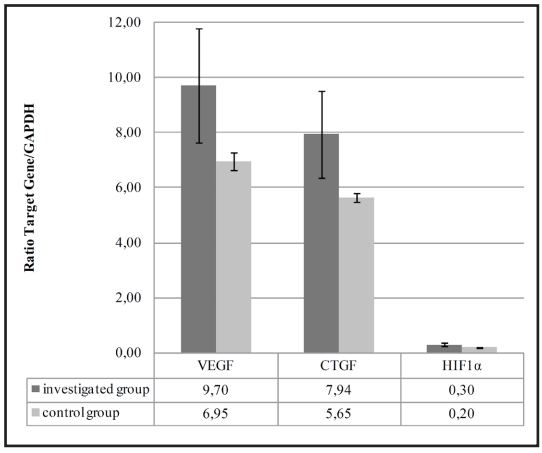

Fig. 2. Expression of analyzed genes in renal biopsies.

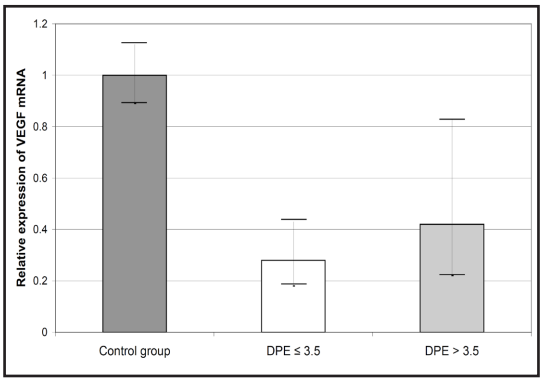

Fig. 3. Comparison of VEGF relative expression between control group and patients with different daily protein expression (DPE). Patients with DPE ≥3,5g showed higher VEGF relative expression in compare to patients with DPE ≤3,5g. (p<0,064).

Discussion

In patients with CKD, irrespective of its primary origin, many profibrogenic and proinflammatory cytokines and growth factors are involved in irreversible renal scarring, determining the future prognosis.

We found decreased relative expression of VEGF gene in comparison to control in all investigated patients regardless of histopathological type of glomerulonephritis. There are conflicting data concerning VEGF gene expression in kidney diseases. Boner et al. in a study of 8 patients with minimal change disease (MCD), using in situ hybridization in renal biopsy, found diminished VEGF gene expression in comparison to control. As a control they used normal renal tissue from areas without renal tumors. Simultaneously, in the investigated groups they found no differences in VEGFR-2 expression in comparison to control. VEGF plays a crucial role in the pathogenesis of proteinuria in MCD and other renal diseases associated with proteinuria. It is postulated that lower VEGF expression in MCD leads to the dysregulation of the renal repair process and it is thus impossible to maintain normal glomerular structure and function of capillary wall [9]. This may lead to persistent proteinuria. Shulman and co-workers demonstrated decreased VEGF expression in renal biopsies for different renal diseases (IgA nephropathy, SLE, MCD, amyloidosis, membranous glomerulonephritis, FSGS) in the sclerotic area, the area of marked amyloid deposition, and the area of crescent formation. In normal glomeruli in MCD there was a strong VEGF expression. Other investigators found increased VEGF expression not only in MCD but in other glomerular diseases as well [10]. In normal condition VEGF is an important microvascular permeability inducer release of which should be under strict control. In different glomerular diseases damage of visceral epithelial cells causes the enhancement of local VEGF release which in turn leads to increased glomerular permeability and alterations in glomerular endothelial cell function. Up-regulation of VEGF expression is involved in response to many renal pathologies such as diabetic nephropathy, FSGS, membranoproliferative and membranous glomerulonephritis [11]. It is difficult to

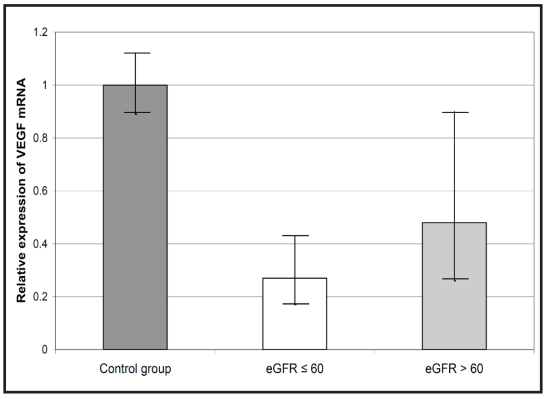

Fig. 4. Comparison of VEGF relative expression between control group and patients with GFR ≤60ml/min and GFR>60ml/min. Patients with GFR ≤60ml/min showed significantly lower VEGF relative expression in compare to patients with GFR >60ml/min (p<0,022).

say whether it is a primary or a secondary process related to the nature of nephropathy. Futhermore, it is postulated that some inflammatory cytokines may be involved in VEGF expression. Some of them such as IL-10 and IL-13 inhibit vascular permeability, and others such as IL-12 and IL-15 increase vascular permeability [12]. Besides, these inflammatory cytokines, other cytokines and growth factors such as TGF-β, PDGF, IL-6, IL-1β may increase VEGF expression [13]. In our study we found overexpression of VEGF gene in patients with substantial proteinuria (DPE>3,5g), (Figure 3). There are several well-known stimuli for VEGF release from the endothelial surface in renal diseases i.e. hypoxia, HIF-1 alfa, proteinuria. Increased VEGF expression may be beneficial as it may exert protective effect on renal tubules and stimulate peritubular microcirculation and peritubular blood flow via an increase in NO synthase expression. Moreover, we know that VEGF may halt peritubular capillary rarefaction. Therefore, VEGF administration may be a challenging method of treatment in renal patients. It is important to mention that high proteinuric patients (>3,5g) with the highest intraglomerular pressure may show higher degree of filtration barrier damage and thus higher VEGF expression level and VEGF release from the endothelium [14]. Another important issue is deficiency in oxygen delivery to renal tissue in high proteinuric patients and activation of HIF -1α pathway which stimulate VEGF expression. Therefore, overexpression of VEGF may indicate poor oxygen delivery which may in turn lead to tubulointerstitial fibrosis and further eGFR decline. On the other hand, there has been some evidence of decreased VEGF expression in overt diabetic nephropathy and it was associated with reduction in peritubular capillaries density. Significant inverse correlation between VEGF and proteinuria was reported [15]. In our study we reported statistically significant VEGF overexpression in pts with eGFR>60ml/min compared to pts with eGFR<60ml/min (Figure 4). That may be a sign of early lesions in the tubulointerstitial compartment. VEGF overexpression may precede renal tissue fibrosis and decline in eGFR. In the preliminary phase of kidney injury, as we mentioned before, increased VEGF expression may be observed and may exert a beneficial effect on renal microcirculation. Afterwards decreased VEGF expression may be observed and recognized, as a sign of angiogenesis impairment, capillary loss and development of glomerulosclerosis and interstitial fibrosis [16].

We found a tendency to overexpression of 2 other genes –CTGF and HIF-1α in pts with DPE>3,5g. CTGF plays an important role in the development of glomerular and tubulointerstitial fibrosis and it is overexpressed, especially in diabetic nephropathy, chronic allograft nephropathy. In normal kidney the cellular source of CTGF are podocytes. In human kidney diseases CTGF is produced by mesangial cells and tubular epithelial cells which leads, respectively to glomerulosclerosis and interstitial fibrosis [17]. Ito et al. analyzing 65 renal biopsies pointed out overexpression of CTGF in the extracapillary space and severe mesangial proliferative lesions of crescentic glomerulonephritis, IgA nephropathy, FSGS and diabetic nephropathy. In correlation with histopathological findings, CTGF up-regulation was mainly observed in the sites of tubulointerstitial damage and it correlates with the degree of damage [18]. In our study, we found increased HIF-1α gene expression (without statistical significance) in pts with clinically important proteinuria. HIF system (composed of 2 units: HIF1 and HIF2) interacts with transcription process of more than 100 genes, which are involved in, for example, glycolysis, angiogenesis, erythropoiesis, stimulation of collagen synthesis and cell cycle regulation or cell survival [19-21]. Rudnicki and co-workers identified up-regulation of HIF-1α in renal tissue of progressive kidney disease patients. The expression of HIF-1α correlated positively with the level of proteinuria whereas the expression of VEGF-A was negatively correlated to proteinuria [22]. There was no correlation between HIF-1α and the degree of interstitial fibrosis. VEGF-A expression was decreased in progressive patients in this study [22]. In our study, HIF-1α gene expression was higher in renal anemia patients compared to the rest of patients without anemia at the time of kidney biopsy. Increased HIF-1α gene expression as an answer to low oxygen delivery in renal tissue may exert protective role and attenuate kidney damage. On the other hand the activation of HIF-1α pathway in renal epithelial cells may promote renal fibrosis and the development of CKD [23]. There is a strong connection between VEGF and HIF-1α pathways in renal tissue.

Conclusion

Recent publications suggest diminished, normal or increased expression level of analyzed genes in comparison to control. In our study we found a lower level of relative expression of all investigated genes in the whole population of 28 pts but there was a trend toward increased VEGF, CTGF, HIF-1α in the subpopulation with DPE>3,5g, and VEGF overexpression in pts with eGFR >60ml/min. It should be remembered that we assessed relative gene expression in relation to control - normal renal tissue outside the tumor (based on histopathological evaluation). It is postulated that such tissue as a control may result in spuriously high level of, for example, VEGF mRNA and may thus affect the results of our study. High level of VEGF is detected in the area of surrounding the tumor. In theory, it is hard to exclude the influence of tumor metabolism products on the rest of renal tissue. A possible explanation for reduced gene expression observed in our study may be suspected superiority of other cytokines, growth factors such as, for example, FIH 1 (not evaluated in conducted study). The limitation of the study was inability to implement certain novel methods, such as laser microdissection analysis to analyzed gene expression separately in the glomerular, and the interstitial compartment [24, 25]. Data from other studies indicate that such evaluation should be considered. In our opinion, it is worth mentioning that a trend to higher level of relative VEGF gene expression in subjects with DPE>3,5g (and a trend toward a higher expression of 2 others genes) was detected. This may indicate oxygen supply deficiency in renal tissue which may result in tubulointerstitial fibrosis with further renal function impairment and eGFR decline in the near future. Therefore, this kind of diagnostic procedure may be valuable for prognosis the natural course of renal diseases.

In spite of the fact that molecular analysis is not performed as a routine procedure and quantitative molecular analyses are made in research settings, because of its informational value it can prove a popular clinical tool. Such molecular approach may be valuable not only in CKD patients undergoing conservative treatment but also in transplanted subjects when determining early allograft fibrosis.

Conflict of Interests

There are no conflicts.

Acknowledgements

This work was supported by Grant from Nicolaus Copernicus University, Toruń. We gratefully acknowledge the support of NERKADAR Foundation in Department of Nephrology, Hypertension and Internal Medicine, of Nicolaus Copernicus University.

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