Molecular Profiling in IgA Nephropathy and Focal and Segmental Glomerulosclerosis

I. TYCOVÁ^{1*}, P. HRUBÁ^{1*}, D. MAIXNEROVÁ², E. GIRMANOVÁ¹, P. MRÁZOVÁ¹, L. STRAŇAVOVÁ¹, R. ZACHOVAL³, M. MERTA², J. SLATINSKÁ⁵, M. KOLLÁR⁴, E. HONSOVÁ⁴, V. TESAŘ², O. VIKLICKÝ^{1,5}

* These authors contributed equally to this work.

¹Transplant Laboratory, Institute for Clinical and Experimental Medicine, Prague, Czech Republic,

²Department of Nephrology Charles University and General University Hospital in Prague, First Faculty of Medicine, Czech Republic, ³Department of Urology Thomayer University Hospital, Prague, Czech Republic, ⁴Department of Clinical and Transplant Pathology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, ⁵Department of Nephrology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

Received May 25, 2017

Accepted August 1, 2017

On-line November 10, 2017

Summary

The aim of the study was to characterize by molecular profiling two glomerular diseases: IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS) and to identify potential molecular markers of IgAN and FSGS progression. The expressions of 90 immune-related genes were compared in biopsies of patients with IgAN (n=33), FSGS (n=17) and in controls (n=11) using RT-qPCR. To identify markers of disease progression, gene expression was compared between progressors and non-progressors in 1 year follow-up. The results were verified on validation cohort of patients with IgAN (n=8) and in controls (n=6) using laser-capture microdissection, that enables to analyze gene expression separately for glomeruli and interstitium. In comparison to controls, patients with both IgAN and FSGS, had lower expression of *BAX* (apoptotic molecule BCL2-associated protein) and *HMOX-1* (heme oxygenase 1) and higher expression of *SELP* (selectin P). Furthermore, in IgAN higher expression of *PTPRC* (protein-tyrosine phosphatase, receptor-type C) and in FSGS higher expression of *BCL2L1* (regulator of apoptosis BCL2-like 1) and *IL18* compared to control was observed. Validation of differentially expressed genes between IgAN and controls on another cohort using laser-capture microdissection confirmed higher expression of *PTPRC* in glomeruli of patients with IgAN. The risk of progression in IgAN was associated with higher expression *EDN1* (endothelin 1)

(AUC=0.77) and *FASLG* (Fas ligand) (AUC=0.82) and lower expression of *VEGF* (vascular endothelial growth factor) (AUC=0.8) and in FSGS with lower expression of *CCL19* (chemokine (C-C motif) ligand 19) (AUC=0.86). Higher expression of *EDN1* and *FASLG* along with lower expression of *VEGF* in IgAN and lower expression of *CCL19* in FSGS at the time of biopsy can help to identify patients at risk of future disease progression.

Key words

IgA nephropathy • Focal segmental glomerulosclerosis • Gene expression

Corresponding author

P. Hrubá, Transplant Laboratory, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 140 21 Prague, Czech Republic. Fax: +420 261 353 168. E-mail: petra.hrubá@ikem.cz

Introduction

IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS) are considered to be the most common glomerulopathies which in significant part of patients progress into end stage renal disease (ESRD) (Coppo *et al.* 2010, Moriyama *et al.* 2014). The

pathophysiology of the disease progression remains poorly understood.

Besides classical histopathological diagnostics (Chandramohan *et al.* 2009, Schmitt *et al.* 2014), molecular microscope may better evaluate dynamic molecular events which may be involved in the disease and progression pathogenesis.

Among well-known predictors of IgAN progression belong sustained hypertension, impaired renal function and persistent proteinuria (>1 g/d). The Oxford classification identified four pathological features (mesangial hypercellularity, endocapillary hypercellularity, segmental glomerulosclerosis, and tubular atrophy/interstitial fibrosis, resulting in a MEST score) that predicted renal outcome (Roberts *et al.* 2009). VALIGA study validated MEST score on 1,147 patients and showed that histological signs of chronic and irreversible damage (mesangial hypercellularity, segmental sclerosis, and tubular atrophy/interstitial fibrosis) had the strongest association with unfavorable outcome (Barbour *et al.* 2016, Coppo *et al.* 2014).

To date, three key molecules were found to be associated with progression of IgAN disease progression/recurrence after transplantation: galactose-deficient IgA1 (Gd-IgA1), IgG anti-Gd-IgA1 antibodies, and soluble CD89 (an Fc receptor for IgA) (Moldoveanu *et al.* 2007, Robert *et al.* 2015). Several studies identified numerous urinary biomarkers of progression of IgAN such as epidermal growth factor (Stangou *et al.* 2009), monocyte chemoattractant protein-1 (MCP-1), complement (C3a and C5a) or chemokine (C-X-C motif) ligand 1 (CXCL1) (Zhao *et al.* 2015).

In FSGS, the increased serum creatinine, proteinuria, interstitial fibrosis and the presence of collapsing lesions were shown to be predictive for the progression into ESRD (Schwartz *et al.* 1999). The role of T-cell costimulating molecule B7-1 (CD80) in development of proteinuria in FSGS patients has been described (Kronbichler *et al.* 2016). Higher expression of some molecular markers of podocytes (podoplanin and podocin) were found in FSGS (Koop *et al.* 2003) and recently, it was shown that collapsing variant of FSGS with the worst prognosis had lower immunohistochemical expression of podocyte markers (CD10, α -actinin-4, and WT1) (Testagrossa *et al.* 2013).

In this study, we aimed to better characterize molecular profiles of IgAN and FSGS and to identify

potential molecular markers for the disease progression. Such markers might help to guide more specific and aggressive therapy for patients at risk.

Methods

Study cohort

Fifty patients in whom IgAN (n=33) or FSGS (n=17) was histologically diagnosed in 2 nephrology centers between January 2005 and May 2007 were prospectively enrolled in the study and followed for 12 months. The study protocol was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine in Prague (No. A 13-02-01) and General Teaching hospital in Prague (No. 19/06). Clinical and laboratory data were collected on the date the biopsy was performed and same data also one year after the biopsy. Patient demographic and clinical data are summarized in Table 1.

Control group consisted of eleven renal samples taken from “healthy” part of kidney in patients with renal cancer. All samples were taken during surgery, before beginning of renal cancer therapy to avoid the influence of anti-cancer drugs on the gene expression. Five males and 6 females were included to control group with the median age at the time of sampling 71.0 (44.0-81.0) years, the median body mass index (BMI) 27.5 (19.6-35.3) kg/m² and the median level of serum creatinine 90.0 (62.0-126.0) μ mol/l.

Based on the disease progression during the 12 month follow-up after kidney biopsy, two patient subgroups were identified. Patients in whom serum creatinine at 12-months increased for more than 20 % of baseline values or in whom the disease proceeded to ESRD were classified as “progressors. Patients in whom serum creatinine decreased during the 12-month follow-up or remained stable within normal range were classified as “non-progressors” (Table 2).

Validation cohort

As a validation cohort, samples from patients with IgAN (n=8) and controls (n=6) were enrolled between October 2013 and June 2015. Control samples were taken from “healthy” part of kidney in patients with renal cancer. Patients in the control group had median age at the time of sampling 71.8 (40.7-80.4) and from IgAN group 39.3 (25-63.6) years.

Table 1. Patients demographic and clinical data.

	IgAN Group	FSGS Group	* p value
Biopsy time point			
<i>N</i>	33	17	
<i>Male (%)</i>	61	71	0.548
<i>Age (years)</i>	38.6 (19.3; 65.6)	48.5 (25.0; 77.0)	0.103
<i>BMI (kg/m²)</i>	24.2 (19.9; 43.3)	26.8 (20.2; 39.5)	0.559
<i>Creatinine (μmol/l)</i>	124.0 (64.0; 528.0)	81.0 (60.0; 400.0)	0.062
<i>GFR (ml/s)</i>	1.05 (0.18; 3.01)	1.37 (0.23; 2.59)	0.089
<i>Proteinuria (g/day)</i>	1.48 (0.08; 15.15)	4.51 (0.88; 11.26)	0.010*
<i>Erythrocyturia (%)</i>	75	65	0.511
<i>Serum IgA (g/l)</i>	2.50 (1.17; 6.40)	1.93 (0.93-2.87)	0.034*
<i>Systolic blood pressure (mm Hg)</i>	140 (120; 220)	140 (110; 180)	0.713
<i>Diastolic blood pressure (mm Hg)</i>	85 (70; 120)	90 (70; 122)	0.827
<i>Cholesterol (mmol/l)</i>	5.20 (2.73; 11.30)	5.90 (3.08; 15.7)	0.180
<i>Triglycerides (mmol/l)</i>	1.73 (0.44; 5.66)	2.36 (1.02; 6.11)	0.031*
Therapy before biopsy			
<i>Prednisone / CPA (% (n))</i>	3 (1) - prednisone	0	1.000
<i>ACEi and/or ARB (%)</i>	48	63	0.372
Therapy after biopsy			
<i>Prednisone/CPA (%)</i>	67	47	0.229
<i>Prednisone alone (%)</i>	59	100	
<i>Prednisone + cyclophosphamide (%)</i>	41	0	0.067
<i>ACEi and/or ARB</i>	85	100	0.152
12-month follow-up			
<i>N</i>	32 (1 exitus)	16 (1 exitus)	
<i>Creatinine (μmol/l)</i>	116.7 (73.0; 600.0)	97.0 (77.0; 294.0)	0.186
<i>GFR (ml/s)</i>	1.12 (0.01; 2.40)	1.28 (0.70; 2.89)	0.144
<i>Proteinuria (g/day)</i>	0.54 (0.00; 7.68)	0.8 (0.05; 5.07)	0.709
<i>Prednisone/CPA (%)</i>	38	50	0.537
<i>Prednisone alone (%)</i>	92	100	
<i>Prednisone + cyclophosphamide (%)</i>	8	0	1.000
<i>ACEi and/or ARB (%)</i>	94	100	0.546

Data are shown as medians (minimum-maximum). *p – values for categorical data were calculated by the *Fisher's test* and for continuous variables by *Mann-Whitney test*.

Renal biopsy

All biopsies were performed using a 14- or 16-gauge Tru-Cut needle (Uni-Cut Nadeln, Angiomed, Germany) guided by ultrasound (Toshiba, Power Vision 6000, Japan). Small portions (~2 mm) of renal tissue from the cortical zone were immediately stored in preserve solution (RNA later, Qiagen) for expression analysis, while the majority of renal tissue taken by core biopsy was used for routine histology performed by the standard method. Samples were routinely stained with

haematoxylin and eosin, haematoxylin and eosin with elastic stain, periodic acid-Schiff (PAS), aldehyde-fuchsin orange G (AFOG), Sirius red with elastic stain and periodic acid silver-methenamine (PASM).

RNA isolation

Renal tissue was homogenized, total RNA was extracted using RNA Blue (Top-Bio s.r.o. Czech Republic) and reverse transcribed into complementary DNA (cDNA), as described elsewhere (Platzer *et al.* 1994).

Table 2. Demographic and clinical data of progressors and non-progressors.

	IgAN Group		* p value
	Non-progressors	Progressors	
Biopsy time point			
<i>N</i>	21	7	
<i>Age (years)</i>	36 (19; 58)	45 (28; 65)	0.326
<i>BMI (kg/m²)</i>	24 (21; 40)	33 (25; 36)	0.395
<i>Creatinine (μmol/l)</i>	124 (75; 528)	335 (64; 471)	0.799
<i>GFR (ml/s)</i>	1.13 (0.2; 3)	0.3 (0.18; 1.3)	0.610
<i>Proteinuria (g/day)</i>	1.3 (0.1; 15.1)	4.5 (0.4; 5.5)	0.628
<i>Serum IgA (g/l)</i>	2.2 (1.2; 4.7)	4 (2.1; 5.1)	0.250
12-month follow-up			
<i>Creatinine (μmol/l)</i>	113.3 (73; 541)	460 (83; 600)	0.899
<i>GFR (ml/s)</i>	1.2 (0.5; 1.9)	0.9 (0; 2.4)	0.898
<i>Proteinuria (g/day)</i>	0.5 (0; 7.7)	1.4 (0.2; 2.7)	0.793
24-month follow-up			
<i>Creatinine (μmol/l)</i>	116.5 (70; 225)	600 (80; 600)	0.399
<i>GFR (ml/s)</i>	1.4 (0.4; 3)	0.5 (0; 1.5)	0.954
<i>Proteinuria (g/day)</i>	0.4 (0; 1.8)	1 (0.1; 1.4)	0.433
<i>Transplantation/Dialysis by 2 years (n, %)</i>	1 (4.8 %)	4 (57.1 %)	0.075

	FSGS Group		* p value
	Non-progressors	Progressors	
Biopsy time point			
<i>N</i>	9	4	
<i>Age (years)</i>	49 (25; 77)	34 (31; 66)	0.537
<i>BMI (kg/m²)</i>	28 (21; 40)	23 (20; 29)	0.064
<i>Creatinine (μmol/l)</i>	81 (71; 384)	88 (60; 114)	0.279
<i>GFR (ml/s)</i>	1.4 (0.4; 2.6)	1.4 (0.7; 1.9)	0.877
<i>Proteinuria (g/day)</i>	4.6 (1.5; 2.1)	6.3 (0.9; 11.3)	0.758
12-month follow-up			
<i>Creatinine (μmol/l)</i>	82 (77; 274)	111 (83; 153)	0.537
<i>GFR (ml/s)</i>	1.4 (0.6; 2.9)	1.1 (0.7; 1.9)	0.440
<i>Proteinuria (g/day)</i>	0.8 (0.2; 5.1)	0.9 (0.1; 1.3)	0.610
24-month follow-up			
<i>Creatinine (μmol/l)</i>	86 (72; 239)	99 (64; 120)	0.877
<i>GFR (ml/s)</i>	1.4 (0.7; 2.9)	1.3 (0.7; 2.3)	0.877
<i>Proteinuria (g/day)</i>	0.4 (0; 6.6)	0.9 (0.3; 3.1)	0.438
<i>Transplantation/Dialysis by 2 years (n, %)</i>	0	0	

* p values for categorical data were calculated by *Fisher's test* and for continuous variables by *Mann-Whitney test*.

RT-qPCR

Gene expression profile of 90 targets (Human

Immune Panel, Applied Biosystems CA, USA) known to have implications in immune response (cytokines,

costimulatory molecules and growth factors chemokines, immune regulators, apoptosis markers, ischemia markers) was determined using RT-qPCR ($2^{-\Delta\Delta C_t}$) method with GAPDH as internal control and cDNA from a control kidney serving as the calibrator in 61 renal biopsy

specimens. All evaluated genes are shown in Table 3. Each immune profiling TLDA contains lyophilized gene expression reagents (primers and probes [FAM labelled]) in a preconfigured 384 well format.

Table 3. Genes evaluated by RT-qPCR.

<i>IL1A</i>	<i>CD40</i>	<i>GZMB</i>	<i>IL9</i>
<i>18S</i>	<i>CD40LG</i>	<i>HLA-DRA</i>	<i>LRP2</i>
<i>ACE</i>	<i>CD68</i>	<i>HLA-DRB1</i>	<i>LTA</i>
<i>ACTB</i>	<i>CD80</i>	<i>HMOX1</i>	<i>MYH6</i>
<i>AGTR1</i>	<i>CD86</i>	<i>ICAM1</i>	<i>NFKB2</i>
<i>AGTR2</i>	<i>CD8A</i>	<i>ICOS</i>	<i>NOS2A</i>
<i>BAX</i>	<i>COL4A5</i>	<i>IFNG</i>	<i>PGK1</i>
<i>BCL2</i>	<i>CSF1</i>	<i>IKBKB</i>	<i>PRF1</i>
<i>BCL2L1</i>	<i>CSF2</i>	<i>IL10</i>	<i>PTGS2</i>
<i>C3</i>	<i>CSF3</i>	<i>IL12A</i>	<i>PTPRC</i>
<i>CCL19</i>	<i>CTLA4</i>	<i>IL12B</i>	<i>REN</i>
<i>CCL2</i>	<i>CXCL10</i>	<i>IL13</i>	<i>RPL3L</i>
<i>CCL3</i>	<i>CXCL11</i>	<i>IL15</i>	<i>SELE</i>
<i>CCL5</i>	<i>CXCR3</i>	<i>IL17</i>	<i>SELP</i>
<i>CCR2</i>	<i>CYP1A2</i>	<i>IL18</i>	<i>SKI</i>
<i>CCR4</i>	<i>CYP7A1</i>	<i>IL1B</i>	<i>SMAD3</i>
<i>CCR5</i>	<i>ECE1</i>	<i>IL2</i>	<i>SMAD7</i>
<i>CCR7</i>	<i>EDN1</i>	<i>IL2RA</i>	<i>STAT3</i>
<i>CD19</i>	<i>FAS</i>	<i>IL3</i>	<i>TBX21</i>
<i>CD28</i>	<i>FASLG</i>	<i>IL4</i>	<i>TFRC</i>
<i>CD34</i>	<i>FN1</i>	<i>IL5</i>	<i>TGFB1</i>
<i>CD38</i>	<i>GAPDH</i>	<i>IL6</i>	<i>TNF</i>
<i>CD3E</i>	<i>GNLY</i>	<i>IL7</i>	<i>TNFRSF18</i>
<i>CD4</i>	<i>GUSB</i>	<i>IL8</i>	<i>VEGF</i>

Gene symbols frequently used as housekeeping genes are in bold. According the geNorm and NormFinder softwares the GAPDH as internal control for this study was chosen.

Two samples in duplicate were analyzed per card. Each loading port was filled with a 100 µl volume containing cDNA, nuclease free water and TaqMan® universal PCR master mix (2×). Following centrifugation, cards were sealed with a TLDA sealer (Applied Biosystems, CA, USA) to prevent cross contamination. Real time RT-PCR amplification was performed on an ABI Prism® 7900 H.T. Sequence Detection system (Applied Biosystems, CA, USA).

TLDA cards were analyzed as relative quantification (RQ) and RQ manager 1.2. software for automated data analysis was used (Applied Biosystems, CA, USA).

Laser-capture microdissection

In a validation cohort, laser-capture microdissection a small portion (~2 mm) of renal tissue was embedded into Tissue-Tec immediately after biopsy or after surgery in case of nephrectomy, frozen with liquid nitrogen and stored at -80 °C. To ensure the similarities between sample takings, the biopsy needle was used also to take the samples from kidneys after nephrectomy. The samples were cut to 10-µm-thick sections on a cryotome at -20 °C. Sections were mounted onto RNase free MMI Membrane Slides (MMI AG, Glattbrugg, Switzerland) and immediately fixed in 96 % ethanol for 45 s. After removal of Tissue Tec in 70 % ethanol, slides were stained in haematoxylin for 45 s, rinsed in RNase free water, counterstained by eosin for 30 s, rinsed in RNase

free water and fixed in 96 % ethanol for 45 s (H&E staining kit plus (MMI AG, Glattbrugg, Switzerland). Slides were then air-dried for 30 s and immediately microdissected on MMI CellCut® (Molecular Machines&Industries, Zürich, Switzerland). Microdissection time did not exceed 30 min to avoid the action of RNAases. Glomeruli (for each patient the area 1.08 mm² [range 0.37-3.16] corresponding to 9-96 glomeruli) or interstitium (for each patient the area of 2.9 mm² of tissue [range 0.65-4.10]) were marked, cut automatically using an UV-Laser, collected on the adhesive cap, put directly into the lysis buffer for RNA isolation (RLT, RNeasy Micro Kit, Qiagen) and stored at -20°C until isolation. RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined using Agilent RNA 6000 Pico Kit on the Agilent 2100 BioAnalyzer.

RNA was reverse transcribed into cDNA using Superscript Reverse Transcriptase II (Invitrogen). Quantitative RT-PCR was performed using Taqman gene expression assays with a fast protocol as described elsewhere (Viklicky *et al.* 2013). Genes that were identified as differentially expressed between IgAN and control (BAX-Hs00180269_m1, HMOX1-Hs00157965_m1, PTPRC-Hs04189704_m1, SELP-Hs00174583_m1, WTI-Hs01103751_m1 (as gene with glomerulus specific expression) and GAPDH-Hs99999905_m1 (as endogenous control) were measured for each sample in triplicate, separately for glomeruli and interstitium. As a calibrator, one sample of glomeruli from control group with good expression profile on all of target genes was chosen. The samples with less than 50 % measurable genes were excluded from analysis.

Statistical analysis

Differences in mRNA expression or clinical parameters between groups were analyzed using the Mann-Whitney or Kruskal-Wallis statistic with Dunn's Multiple Comparison test. The area below the receiver operating characteristic (ROC) curve was performed to estimate the cut-off value of different transcripts for disease progression. A $p < 0.05$ was considered to be statistically significant.

Supervised hierarchical clustering was performed with the MeV (V.3) software in order to visualize the results. Statistical analyses were performed using SPSS v.20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad InStat v. 3.05 for Windows (GraphPad software, San Diego, CA, USA).

Results

Differences in gene expression profiles of IgAN, FSGS and control

Hierarchical clustering (Fig. 1) indicated that IgAN, FSGS group and control group differ in immune gene expression profiles. Clustering showed gene expression profiles of normal controls and IgAN group are similar and form a cluster; by contrast gene expression profile of FSGS group differs. Gene patterns forming a cluster are more closely related to one another than objects assigned to different cluster.

Patients suffering from FSGS had reduced gene expression of apoptotic molecule BCL2-associated x protein (*BAX*) ($p < 0.001$) and cytoprotective factor with anti-inflammatory and antioxidant effects heme oxygenase 1 (*HMOX-1*) ($p < 0.001$) compared with control group. On the other hand there was increased expression of another regulator of apoptosis BCL2-like 1 (*BCL2L1*) ($p < 0.01$), product of macrophages interleukin 18 (interferon-gamma-inducing factor, *IL18*) ($p < 0.05$) and adhesion molecule selectin P (*SELP*, antigen CD62) ($p < 0.05$) in a group with FSGS (Fig. 2). Similarly, patients with IgAN have also reduced intrarenal gene expression of *BAX* ($p < 0.01$) and *HMOX-1* ($p < 0.01$) compared to the control group. Furthermore, these patients have a significantly increased protein-tyrosine phosphatase, receptor-type, C (*PTPRC*) ($p < 0.05$) and *SELP* ($p < 0.05$) gene expression (Fig. 2) which shows a strong mononuclear infiltration of the tissue.

RT-qPCR of RNA from microdissected glomeruli and interstitium

In the validation cohort, genes with significantly different expression were selected and analyzed separately for glomeruli and interstitium using laser-capture microdissection.

When compared gene expression between glomeruli and interstitium in all samples, significant higher expression in glomeruli was found for *WTI* ($p < 0.001$), *PTPRC* ($p = 0.02$) and significant higher expression in interstitium for *HMOX-1* ($p = 0.036$) (Fig. 3A). *WTI* gene was selected as a molecular marker of glomerular podocytes. When compared IgAN patients and controls trends to higher expression of *PTPRC* and *HMOX-1* in glomeruli were observed in IgAN group ($p = 0.053$, $p = 0.068$, respectively) (Fig. 3B). The expression of two remaining genes found to be differentially expressed between IgAN patients and controls (*BAX*, *SELP*) did differ neither in glomeruli nor in interstitium.

Fig. 1. Supervised hierarchical clustering of 90 immune related genes expression profiles in controls, IgAN and FSGS group. Spearman's rank correlation was used as the distance measure for average linkage clustering. Controls and IgAN group are similar and form a cluster, by contrast gene expression profile of FSGS group differs.

Progression associated genes

For determination of the risk for IgAN and FSGS progression in dependence of renal gene expression during the 12 months follow-up after kidney biopsy the ROC analysis were designed and the critical cut-off points were adjusted. A higher mRNA level of endothelin 1 (*EDN1*) (RQ>0.93; p=0.02) and Fas ligand (TNF superfamily, member 6; *FASLG*) (RQ>22.4; p=0.01) were associated with the risk for IgAN progression (RR 7.4 for *EDN1*, 6.7 for *FASLG*). In contrast, reduced risk of IgAN progression was observed in patients with up-regulation of the gene for vascular endothelial growth factor A (*VEGF*) (RQ>0.51; p=0.01; RR 13.3) (Fig. 4).

Using the GeneGO Metacore software (<http://www.genego.com/>) the analytical pathway map illustrating possible mechanisms of action of our markers in the IgAN progression was designed. Network illustrates possible role of *EDN1*, *FASLG* and *VEGF* in IgAN progression *via* routes leading to NF-κB (map is not shown).

In the FSGS group, patients at risk of progression had lower expression of chemokine (C-C motif) ligand 19 (*CCL19*) mRNA (RQ<0.12, p=0.04; RR 7.5) (Fig. 4).

There were no differences in clinical data between progressor and nonprogressor groups in both IgAN or FSGS groups at the time of biopsy, neither at 1 or 2 years follow-up. However, at 2 years, 4 out of 7 progressors in IgAN group developed end stage renal disease (Table 2).

Discussion

FSGS and IgAN represent the frequent glomerulopathies which may progress to ESRD. In the present study gene expression profiling of 90 immune related genes in patients with IgAN and FSGS was performed and potential molecular biomarkers for the progression of both glomerulopathies were identified.

The risk of IgAN progression at one year was associated with higher intrarenal expression of *EDN1* and *FASLG* mRNA and lower expression of *VEGF* mRNA. The progression of FSGS was associated with lower

intrarenal expression of *CCL19*.

The role of *EDN1* in the progression of chronic kidney diseases is well-known (Dhaun *et al.* 2012). It participates in podocyte dysfunction, inflammation and fibrosis and promotes mesangial cell proliferation and sclerosis. Increased staining for *EDN1* in endothelial cells of glomerular and peritubular capillaries was observed in patients with diabetic nephropathy and IgAN compared with controls (Zanatta *et al.* 2012). In our study, using the GeneGO Metacore software the possible involvement of NF- κ B signaling in glomerular injury in patients with IgAN progression was proposed. NF- κ B signaling pathway in association with *EDN1* was also described on a mice model of diabetic glomerulosclerosis (Lenoir *et al.* 2014).

The decreased *VEGF* expression in renal biopsies of various primary and secondary glomerulopathies was described in the sclerotic area, the area of marked amyloid deposition, and the area of crescent formation using immunohistochemistry and *in*

situ hybridization. (Shulman *et al.* 1996) Lower *VEGF* expression in association with proteinuria was found in all investigated types of glomerulonephritides. (Donderski *et al.* 2013) This corresponds to our finding of lower *VEGF* expression in renal tissue of (IgAN) patients with more rapid disease progression.

FASLG is involved in apoptotic cascades and probably participates in renal injury (Lorz *et al.* 2000). *In situ* hybridization and immunohistochemistry showed that proximal tubular epithelium is the main site of FasL expression in the normal kidney (Lorz *et al.* 2000). Up-regulation of mesangial FasL was observed by immunohistochemistry in proliferative lupus nephritis (Tsukinoki *et al.* 2004). The association of *FASLG* expression with IgAN has not been described yet. However, up-regulation of *FASLG* as risk factor of future IgAN progression may reflect the undergoing apoptosis. The association of *CCL19* with FSGS progression has not been described yet as well.

Fig. 2. Significant differences in gene expression profile among control group, IgAN and FSGS group. Differences between groups were calculated by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 3. (A) Significant differences in gene expression between glomeruli and interstitium in all patients regardless of diagnosis (n=32) (IgAN, n=15; controls, n=12, FSGS, n=2). **(B)** Gene expression of *BAX*, *HMOX-1*, *PTPRC* and *SELP* in microdissected glomeruli or in interstitium of patients with IgAN or controls. * $p < 0.05$, ** $p < 0.01$.

In our study we also evaluated the intrarenal expression profiles of the same set of genes in unaffected kidney in control group without glomerular and tubulointerstitial diseases. Significantly higher expression of *HMOX-1* and *BAX* and lower expression of *SELP* were observed in controls compared to IgAN or FSGS. *HMOX-1* is an enzyme up-regulated during oxidative stress with anti-inflammatory effects. Higher *HMOX-1* levels were found in urine samples of IgAN patients with

interstitial cellular infiltration (Yokoyama *et al.* 2011). *BAX* is involved in regulation of apoptosis and was found to be associated with progressive glomerular injury (Qiu *et al.* 2004). The elevated expression of *HMOX-1* and *BAX* have been reported in renal carcinomas (Balan *et al.* 2015, Gobe *et al.* 2002), however, the effect of cancer on the expression of both genes was minimized as our control samples were taken from a healthy part of kidney clearly unaffected by a tumor. P-selectin (*SELP*) belongs

Fig. 4. Receiver operating characteristic (ROC) curves for progression of IgAN and FSGS disease according to gene expression. Regulation of these markers significantly determined the progression of IgAN or FSGS patients one year after diagnosis. (RQ cut-off point for EDN1 up-regulation=0.93; sensitivity=86 %, specificity=63 %, area under the curve (AUC)=0.77, SE=0.10; RQ cut-off point for FASLG up-regulation=22.4; sensitivity=86 %, specificity=84 %, AUC=0.82, SE=0.11; RQ cut-off point for VEGF down-regulation=0.55; sensitivity=86 %, specificity=79 %, AUC=0.8, SE=0.10; RQ cut-off point for CCL19 down-regulation=2.59; sensitivity=75 %, specificity=89 %, AUC=0.86, SE=0.11).

to adhesion molecules involved in leukocyte accumulation within the glomerulus. Association of SNP polymorphism in selectin genes and IgAN has been described (Takei *et al.* 2002). In addition, higher expression of *PTPRC* in IgAN and higher expression of *IL18* and *BCL2L1* in FSGS compared to controls were found. The higher age of control group clearly limits generalization of our findings.

In this study, we further validated our observations using laser capture microdissection to focus

separately on the glomerulus and interstitium. It is known, that primary injury both in IgAN and FSGS is localized to glomerulus. Nevertheless, glomeruli represent only a small part of biopsy and gene expression changes might be influenced by the expression in the surrounding tissue. For genes that are differentially expressed between glomeruli and interstitium, the changes between compared groups might be greatly influenced by their proportions in analyzed sample. Higher expression of *HMOX-1* was confirmed by laser

capture microdissection in interstitium which is in line with *HMOX-1* staining within tubular epithelial cells (Morimoto *et al.* 2001). Two genes *PTPRC* and *CD40* had significantly higher expression in glomeruli compared to interstitium. Validation of 4 differentially expressed genes between IgAN and controls in another patient's cohort by laser capture microdissection confirmed only the higher expression of *PTPRC* (CD45 surface marker of leukocytes) in glomeruli of IgAN patients. Higher expression of *PTPRC* probably reflects neutrophil infiltration into glomeruli.

In conclusion, a higher intrarenal expression of *EDN1* and *FASLG* mRNA, lower intrarenal expression of *VEGF* mRNA in IgAN patients and lower intrarenal expression of *CCL19* mRNA in FSGS patients may help to identify patients at risk for future disease progression.

This observation might be used in the therapy modification and more intensive monitoring in patients at risk.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by Ministry of Health, Czech Republic-conceptual development of research organization (Institute for Clinical and Experimental Medicine-IKEM, IN 00023001 and by grant LH 15168 from the Ministry of Education, Youth and Sports of the Czech Republic. The authors thank nurses and patients for their cooperation and help and Romana Polackova for expert technical assistance.

References

- BALAN M, MIER Y, TERAN E, WAAGA-GASSER AM, GASSER M, CHOUHEIRI TK, FREEMAN G, PAL S: Novel roles of c-Met in the survival of renal cancer cells through the regulation of HO-1 and PD-L1 expression. *J Biol Chem* **290**: 8110-8120, 2015.
- BARBOUR SJ, ESPINO-HERNANDEZ G, REICH HN, COPPO R, ROBERTS IS, FEEHALLY J, HERZENBERG AM, CATTRAN DC: The MEST score provides earlier risk prediction in IgA nephropathy. *Kidney Int* **89**: 167-175, 2016.
- CHANDRAMOHAN K, MATHEWS A, KURIAN A, THOMAS S, RAMACHANDRAN K: Squamous cell carcinoma arising from perineal lesion in a familial case of Hidradenitis suppurativa. *Int Wound J* **6**: 141-144, 2009.
- COPPO R, TROYANOV S, BELLUR S, CATTRAN D, COOK HT, FEEHALLY J, ROBERTS IS, MORANDO L, CAMILLA R, TESAR V, ET AL.: Validation of the Oxford classification of IgA nephropathy in cohorts with different presentations and treatments. *Kidney Int* **86**: 828-836, 2014.
- COPPO R, TROYANOV S, CAMILLA R, HOGG RJ, CATTRAN DC, COOK HT, FEEHALLY J, ROBERTS IS, AMORE A, ALPERS CE, ET AL.: The Oxford IgA nephropathy clinicopathological classification is valid for children as well as adults. *Kidney Int* **77**: 921-927, 2010.
- DHAUN N, WEBB DJ, KLUTH DC: Endothelin-1 and the kidney--beyond BP. *Br J Pharmacol* **167**: 720-731, 2012.
- DONDESKI R, SZCZEPANEK J, DOMAGALSKI K, TRETYN A, KORENKIEWICZ J, MARSZALEK A, SZYMANSKI A, WOLSKI Z, ODRÓWAZ-SYPNIEWSKA G, MANITIUS J: Analysis of relative expression level of VEGF (vascular endothelial growth factor), HIF-1alpha (hypoxia inducible factor 1alpha) and CTGF (connective tissue growth factor) genes in chronic glomerulonephritis (CGN) patients. *Kidney Blood Press Res* **38**: 83-91, 2013.
- GOBE G, RUBIN M, WILLIAMS G, SAWCZUK I, BUTTYAN R: Apoptosis and expression of Bcl-2, Bcl-XL, and Bax in renal cell carcinomas. *Cancer Invest* **20**: 324-332, 2002.
- KOOP K, EIKMANS M, BAELEDE HJ, KAWACHI H, DE HEER E, PAUL LC, BRUIJN JA: Expression of podocyte-associated molecules in acquired human kidney diseases. *J Am Soc Nephrol* **14**: 2063-2071, 2003.
- KRONBICHLER A, LEIERER J, OH J, MEIJERS B, SHIN JI: Immunologic changes implicated in the pathogenesis of focal segmental glomerulosclerosis. *Biomed Res Int* **2016**: 2150451, 2016.
- LENOIR O, MILON M, VIRSOLVY A, HENIQUE C, SCHMITT A, MASSE JM, KOTELEVTSSEV Y, YANAGISAWA M, WEBB DJ, RICHARD S, THARAUX PL: Direct action of endothelin-1 on podocytes promotes diabetic glomerulosclerosis. *J Am Soc Nephrol* **25**: 1050-1062, 2014.

- LORZ C, ORTIZ A, JUSTO P, GONZALEZ-CUADRADO S, DUQUE N, GOMEZ-GUERRERO C, EGIDO J: Proapoptotic Fas ligand is expressed by normal kidney tubular epithelium and injured glomeruli. *J Am Soc Nephrol* **11**: 1266-1277, 2000.
- MOLDOVEANU Z, WYATT RJ, LEE JY, TOMANA M, JULIAN BA, MESTECKY J, HUANG WQ, ANREDDY SR, HALL S, HASTINGS MC, ET AL.: Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. *Kidney Int* **71**: 1148-1154, 2007.
- MORIMOTO K, OHTA K, YACHIE A, YANG Y, SHIMIZU M, GOTO C, TOMA T, KASAHARA Y, YOKOYAMA H, MIYATA T, ET AL.: Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases. *Kidney Int* **60**: 1858-1866, 2001.
- MORIYAMA T, TANAKA K, IWASAKI C, OSHIMA Y, OCHI A, KATAOKA H, ITABASHI M, TAKEI T, UCHIDA K, NITTA K: Prognosis in IgA nephropathy: 30-year analysis of 1,012 patients at a single center in Japan. *PLoS One* **9**: e91756, 2014.
- PLATZER C, ODE-HAKIM S, REINKE P, DOCKE WD, EWERT R, VOLK HD: Quantitative PCR analysis of cytokine transcription patterns in peripheral mononuclear cells after anti-CD3 rejection therapy using two novel multispecific competitor fragments. *Transplantation* **58**: 264-268, 1994.
- QIU LQ, SINIAH R, I-HONG HSU S: Downregulation of Bcl-2 by podocytes is associated with progressive glomerular injury and clinical indices of poor renal prognosis in human IgA nephropathy. *J Am Soc Nephrol* **15**: 79-90, 2004.
- ROBERT T, BERTHELOT L, CAMBIER A, RONDEAU E, MONTEIRO RC: Molecular insights into the pathogenesis of IgA nephropathy. *Trends Mol Med* **21**: 762-775, 2015.
- ROBERTS IS, COOK HT, TROYANOV S, ALPERS CE, AMORE A, BARRATT J, BERTHOUX F, BONSI S, BRUIJN JA, CATTRAN DC, ET AL.: The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. *Kidney Int* **76**: 546-556, 2009.
- SHULMAN K, ROSEN S, TOGNAZZI K, MANSEAU EJ, BROWN LF: Expression of vascular permeability factor (VPF/VEGF) is altered in many glomerular diseases. *J Am Soc Nephrol* **7**: 661-666, 1996.
- SCHMITT R, STAHL AL, OLIN AI, KRISTOFFERSSON AC, REBETZ J, NOVAK J, LINDAHL G, KARPMAN D: The combined role of galactose-deficient IgA1 and streptococcal IgA-binding M Protein in inducing IL-6 and C3 secretion from human mesangial cells: implications for IgA nephropathy. *J Immunol* **193**: 317-326, 2014.
- SCHWARTZ MM, EVANS J, BAIN R, KORBET SM: Focal segmental glomerulosclerosis: prognostic implications of the cellular lesion. *J Am Soc Nephrol* **10**: 1900-1907, 1999.
- STANGOU M, ALEXOPOULOS E, PAPAGIANNI A, PANTZAKI A, BANTIS C, DOVAS S, ECONOMIDOU D, LEONTSINI M, MEMMOS D: Urinary levels of epidermal growth factor, interleukin-6 and monocyte chemoattractant protein-1 may act as predictor markers of renal function outcome in immunoglobulin A nephropathy. *Nephrology (Carlton)* **14**: 613-620, 2009.
- TAKEI T, IIDA A, NITTA K, TANAKA T, OHNISHI Y, YAMADA R, MAEDA S, TSUNODA T, TAKEOKA S, ITO K, ET AL.: Association between single-nucleotide polymorphisms in selectin genes and immunoglobulin A nephropathy. *Am J Hum Genet* **70**: 781-786, 2002.
- TESTAGROSSA L, AZEVEDO NETO R, RESENDE A, WORONIK V, MALHEIROS D: Immunohistochemical expression of podocyte markers in the variants of focal segmental glomerulosclerosis. *Nephrol Dial Transplant* **28**: 91-98, 2013.
- TSUKINOKI T, SUGIYAMA H, SUNAMI R, KOBAYASHI M, ONODA T, MAESHIMA Y, YAMASAKI Y, MAKINO H: Mesangial cell Fas ligand: upregulation in human lupus nephritis and NF-kappaB-mediated expression in cultured human mesangial cells. *Clin Exp Nephrol* **8**: 196-205, 2004.
- VIKLICKY O, KRYSTUFKOVA E, BRABCOVA I, SEKERKOVA A, WOHLFAHRT P, HRIBOVA P, WOHLFAHRTOVA M, SAWITZKI B, SLATINSKA J, STRIZ I, ET AL.: B-cell-related biomarkers of tolerance are up-regulated in rejection-free kidney transplant recipients. *Transplantation* **95**: 148-154, 2013.
- YOKOYAMA T, SHIMIZU M, OHTA K, YUNO T, OKAJIMA M, WADA T, TOMA T, KOIZUMI S, YACHIE A: Urinary heme oxygenase-1 as a sensitive indicator of tubulointerstitial inflammatory damage in various renal diseases. *Am J Nephrol* **33**: 414-420, 2011.

-
- ZANATTA CM, VERONESE FV, LORETO Mda S, SORTICA DA, CARPIO VN, ELDEWEISS MI, DA SILVA VD, LOPES TG, GROSS JL, CANANI LH: Endothelin-1 and endothelin a receptor immunoreactivity is increased in patients with diabetic nephropathy. *Ren Fail* **34**: 308-315, 2012.
- ZHAO Y, ZHU L, ZHOU T, ZHANG Q, SHI S, LIU L, LV J, ZHANG H: Urinary CXCL1: a novel predictor of IgA nephropathy progression. *PLoS One* **10**: e0119033, 2015.
-