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Gene Expression Profiles of Podocyte-Associated Molecules as Diagnostic Markers in Acquired Proteinuric Diseases

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Abstract. For identifying potential diagnostic markers of proteinuric glomerulopathies, glomerular mRNA levels of molecules relevant for podocyte function (α-actinin-4, glomerular epithelial protein 1, Wilms tumor antigen 1, synaptopodin, dystroglycan, nephrin, podoplanin, and podocin) were determined by quantitative real-time RT-PCR from microdissected glomeruli. Biopsies from 83 patients with acquired proteinuric diseases were analyzed (minimal change disease [MCD; $n = 13$], benign nephrosclerosis [$n = 16$], membranous glomerulopathy [$n = 31$], focal and segmental glomerulosclerosis [FSGS; $n = 9$], and controls [$n = 14$]). Gene expression levels normalized to two different housekeeping transcripts (glyceraldehyde-3-phosphate-dehydrogenase and 18 S rRNA) did not allow a separation between proteinuric disease categories. However, a significant positive correlation between α-actinin-4, glomerular epithelial protein 1, synaptopodin, dystroglycan, Wilms tumor antigen 1, and nephrin was found in all analyzed glomeruli, whereas podocin mRNA expression did not correlate. Because varying amounts of housekeeper cDNA per glomerulus can confound expression ratios relevant for a subpopulation of cells, an "in silico" microdissection was performed using a podocyte-specific cDNA as a reference gene. Expression ratio of podocin to synaptopodin, the two genes with the most disparate expression, allowed a robust separation of FSGS from MCD and nephrosclerosis. Segregation of FSGS from MCD via this ratio was confirmed in an independent population of formaldehyde-fixed archival biopsies (MCD, $n = 5$; FSGS, $n = 4$) after glomerular laser capture microdissection. In addition, the expression marker was able to predict steroid responsiveness in diagnostically challenging cases of MCD *versus* FSGS ($n = 6$). As the above approach can be performed as an add-on diagnostic tool, these molecular diagnostic parameters could give novel information for the management of proteinuric diseases.

Proteinuric glomerular diseases still pose a formidable challenge to nephrology. Recent studies have highlighted the importance of proteinuria both as a clinical prognostic marker and as a factor predicting progressive loss of renal function (1–3). Alteration of the glomerular filtration barrier leads via damage of the glomerular podocyte to leakage of proteins into the ultrafiltrate (4). The filtration barrier consists of a specialized fenestrated endothelium, the fibrillar, hydrated meshwork of the glomerular basement membrane (GBM), and interdigitating podocyte foot processes with intervening filtration slits as the final filtration barrier (5). Identification of mutations in podocyte-associated molecules in various hereditary nephrotic disorders has highlighted the key role of the podocyte for the pathophysiology of proteinuria. The gene product of NPHS1, nephrin, mutated in congenital nephrotic syndrome of the Finnish type, has been localized to the podocyte slit diaphragm (6). Mutations in a gene termed NPHS2, encoding the novel protein podocin, are associated with an autosomal-recessive familial steroid-resistant nephrotic syndrome (7). Mutations in the gene for α-actinin-4 (ACTN4) have been identified by Kaplan *et al.* (8) to be causative in an autosomal-dominant form of familial focal and segmental glomerulosclerosis (FSGS). Thus mutations of various podocyte-specific genes in hereditary proteinuric diseases have identified novel components of the glomerular filtration barrier.

Gene expression analysis of the aforementioned molecules might elucidate common or distinct regulatory pathways in nonhereditary proteinuria and provide valuable diagnostic and prognostic information. This additional diagnostic information could be of particular relevance, as proteinuric diseases not only have a highly variable cause but also follow distinctly different clinical courses. Furthermore, similar clinical presentation, lack of specific clinical or laboratory parameters, and histologically indistinguishable lesions can complicate the diagnostic process (9) and the clinical management of the glomerulopathies. For example, minimal change glomerulopathy (MCD), although often protracted, does not lead to terminal renal failure, whereas FSGS, if not controlled by aggressive therapy, frequently progresses to ESRD.

The present study focuses on the glomerular mRNA expres-

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sion of a comprehensive series of podocyte-associated molecules in a large number of renal biopsies from adult patients with acquired proteinuric glomerulopathies. mRNA expression of the podocyte markers ACTN4, Glomerular epithelial protein 1 (GLEPP-1), Wilms tumor antigen 1 (WT-1), synaptopodin, dystroglycan, nephrin, podoplanin, and podocin was evaluated by real-time RT-PCR on RNA isolated from microdissected glomeruli. A systematic analysis of the relationship between the expression levels revealed a stringent positive correlation of most podocyte-specific cDNA. From the expression data, a marker set could be extracted, allowing a robust separation of the histologic entities FSGS from MCD and benign nephrosclerosis (NS). These findings could be confirmed in formaldehyde-fixed archival renal tissues after laser capture microdissection (LCM), making routine clinical application technically feasible.

Material and Methods

Kidney Biopsies: Patients and Control Groups

Human kidney biopsies from a total of 83 patients, obtained in a multicenter study for gene expression analysis in renal biopsies (the European Renal cDNA Consortium; see appendix for participating centers) were included. From diagnostic renal biopsies, a segment not required for diagnostic evaluation was processed for gene expression analysis after informed consent was obtained according to the guidelines of the respective local ethical committees.

Microdissected glomeruli from 69 patients with five different proteinuric diseases and 14 control subjects were analyzed. Patients were stratified according to their histologic diagnosis by the reference pathologist of the European Renal cDNA Consortium into the following five groups: MCD ($n = 13$), benign NS ($n = 16$), membranous glomerulopathy (MGN; $n = 31$), and FSGS ($n = 9$). For control biopsies, renal tissue was derived from pretransplantation kidney biopsies during cold ischemia time from four cadaveric and four living donors ($n = 8$). Histologic nonaffected parts of tumor nephrectomies ($n = 6$) served as an additional control group.

Verification of relevant mRNA expression data was performed on formaldehyde-fixed archival renal tissues of 15 patients (MCD, $n = 5$; FSGS, $n = 4$; diagnostically challenging cases of MCD *versus* FSGS, $n = 6$). Clinical data of analyzed control and disease groups (age, gender, serum creatinine [mg/dl], and proteinuria [g/24 h]) are listed in Table 1.

Microdissection, LCM, and RNA Isolation

For a detailed description of the protocol used, see reference 10. In brief, cortical tissue segments were manually microdissected in glomeruli and tubulointerstitial compartments. Glomerular microdissection was confirmed by detection of the glomerulus-specific cDNA for WT-1. Total RNA was isolated from the microdissected glomeruli using a commercially available silica gel–based isolation protocol (RNeasy-Mini; Qiagen, Hilden, Germany) followed by random primed reverse transcription. For confirmation experiments on formaldehyde-fixed archival renal tissues, glomeruli were microdissected by a laser beam and harvested by laser pressure catapulting (PALM Laser Micro Beam System; P.A.L.M, Wolfratshausen, Germany), and total RNA was isolated as described in detail (11).

Real-Time RT-PCR

Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold; Applied Biosystems). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. Target gene forward and reverse primers and probes were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA). Commercially available predeveloped TaqMan assay reagents were used for the internal standards human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18 S ribosomal RNA (18 S rRNA). All primers and probes were obtained from Applied Biosystems. The primers for GAPDH, ACTN4, GLEPP-1, WT-1, synaptopodin, dystroglycan, nephrin (NPHS1), podoplanin, and podocin (NPHS2) were cDNA-specific, not amplifying genomic DNA. The following sequences of oligonucleotide primers (300 nM) and probes (100 nM) were used: ACTN4: sense GAGGCCCAGAGGATCGCT, antisense ACTTGGAGTTGATGATTTGCGG, internal probe CAACCACATCAAGCTGTCGGGCAG; GLEPP-1: sense TCACTGTGGAGATGATTTCAGAGG, antisense CGTCAGCATAGTTGATCCGGA, internal probe AGCAGGACGACTGGGCCTGTAGACAC; dystroglycan: sense ACAGGGACCCTGAGAAGAGCA, antisense AATGATGCCAGCAATGAGCAG, internal probe CACACAGTCATTCCGGCCGTGG; nephrin (NPHS1): sense CAACTGGGAGAGACTGGGAGAA, antisense AATCTGACAACAAGACGGAGCA, internal probe TCCACAATGCACTGGTAAGCGCCA; podoplanin: sense TTGACAACTCTGGTGGCAACA, antisense GCTGTGGCGCTTGGACTT, internal probe TTCAGAAAGCACAGTCCACGCGCA; podocin (NPHS2): sense AA-

Table 1. Clinical characteristics of analyzed diagnostic groups[a]

Diagnostic group	*N*	Age (years ± SD)	Gender (f/m)	Serum creatinine (mg/dl ± SD)	Proteinuria (g/24 h ± SD)
CON Tu-N	6	64 ± 6	4/2	1.03 ± 0.15	NA
CON Dx	8	49 ± 11	3/5	0.93 ± 0.19	0.12 ± 0.22
MCD	13	36 ± 16	5/8	1.14 ± 0.49	6.39 ± 4.72
NS	16	56 ± 10	6/10	1.95 ± 1.70	0.97 ± 0.90
MGN	31	55 ± 23	10/21	1.46 ± 1.21	6.24 ± 4.65
FSGS	9	47 ± 16	3/6	1.44 ± 0.51	3.31 ± 3.23

[a] CON Tu-N, Histologic nonaffected parts of tumor nephrectomies; CON Dx, pretransplantation kidney biopsies during cold ischemia time; MCD, minimal change disease; NS, benign nephrosclerosis; MGN, membranous glomerulopathy; FSGS, focal and segmental glomerulosclerosis; NA, not available.

GAGTAATTATATTCCGACTGGGACAT, antisense TGGTCACGATCTCATGAAAAGG, internal probe TCCTGGAAGAGCCAAAGGCCCTG; synaptopodin: sense CCCAAGGTGACCCCGAAT, antisense CTGCCGCCGCTTCTCA, internal probe ACTTGCTGGATCTGGTACAGACAGCGG; WT-1: sense AAATGGACAGAAGGGCAGAGC, antisense GGATGGGCGTTGTGTGGT, internal probe ACCACAGCACAGGGTACGAGAGCGA.

Quantification of the given templates was performed according to the standard curve method. Serial dilutions of standard cDNA from a human nephrectomy were included in all PCR runs and served as standard curve. This method minimizes the influence of interassay and inter-run variability (12). All measurements were performed in duplicate. Controls consisting of bidistilled H_2O were negative in all runs.

Statistical Analyses

Statistical analysis was performed using the SPSS software (version 10.0; SPSS Inc., Chicago, IL). Data are given as absolute values, mean ± SD. Correlation including correlation coefficients and confidence intervals were assessed by linear regression analysis. A multivariate one-way ANOVA with a Bonferroni *post hoc* correction was used for analysis of differences between the groups. $P < 0.05$ was considered to be statistically significant.

Results

mRNA Expression of Podocyte-Associated Molecules in Acquired Proteinuric Diseases

For identifying potential molecular diagnostic markers of proteinuric glomerular diseases, glomerular mRNA expression was determined for ACTN4, GLEPP-1, WT-1, synaptopodin, dystroglycan, nephrin, podoplanin, and podocin. Initially, gene expression levels were normalized to two different ubiquitously expressed housekeeping transcripts (GAPDH and 18 S rRNA), producing comparable results. Using this approach, expression levels of ACTN4, GLEPP-1, WT-1, synaptopodin, dystroglycan, and nephrin showed a high variability in each group. There was a nonsignificant trend to increased mRNA levels in diseased glomeruli compared with controls (Figure 1a, Table 2). None of the expression levels revealed a stringent correlation with proteinuria or serum creatinine at biopsy, and no significant patterns could be recognized by multivariate analysis (data not shown). In contrast to the above molecules, the podocin/18 S rRNA ratio showed a trend toward downregulation in MGN and FSGS glomeruli compared with controls, MCD, and NS (Figure 1b). Taken together, mRNA expression profiling of podocyte-associated molecules in microdissected glomeruli presented as a ratio of target gene/housekeeper gene enabled no clear distinction between the analyzed disease categories. Conventional gene expression analysis of these markers does not seem to be a useful diagnostic tool.

Gene Expression Ratio of Two Podocyte-Associated Molecules Allows Diagnostic Separation of Glomerular Diseases

The gene expression analysis described above relates the expression level of a podocyte-specific cDNA to the expression of a "housekeeper-gene" found ubiquitously in the glomerulus. With this commonly used procedure, different

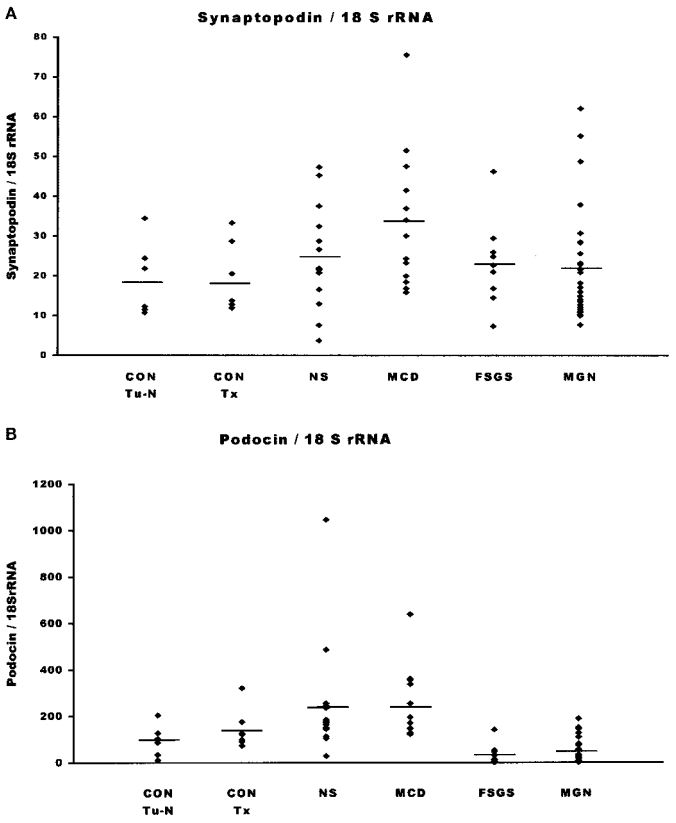

Figure 1. Glomerular mRNA expression of podocyte-associated molecules in acquired proteinuric diseases. (a) Synaptopodin/18 S rRNA. (b) Podocin/18 S rRNA. mRNA levels of podocyte-associated molecules (synaptopodin and podocin depicted) were measured in microdissected glomeruli by real-time RT-PCR. Distribution of expression values related to the mRNA-level of 18 S rRNA as housekeeper is shown for each diagnostic group. Mean values are marked as crossbars. Using this approach, expression levels revealed a high degree of variability in each group.

amounts of housekeeper cDNA per glomerulus can confound the expression ratios particularly for these genes, which are selectively expressed by cells that represent a subpopulation of the glomerulus. This could lead to a skewed profile of gene expression levels. Alteration in the proportion of podocyte cell number per total glomerular cells will alter the expression ratios of podocyte-specific mRNA to housekeeper even with stable cellular gene expression levels. As an example, a decrease in podocytes per glomerulus or an increase in mesangial cell number would result in reduction of the podocyte-specific mRNA/housekeeper ratios. This confounding factor for expression profiling of podocytes can be circumvented if ratios of podocyte-specific cDNA are used to establish ratios. Using this approach, only RNA from the podocyte compartment of the glomerulus are integrated in the analysis and thus may not be influenced by alterations in other compartments.

One gene (podocin) showed the most disparate expression relative to the other seven genes. Upon comparison with these seven genes, podocin showed the lowest correlation coefficient when compared with synaptopodin. We hypothesized that al-

Table 2. Gene expression levels of various podocyte-associated molecules expressed as ratios to 18 S rRNA[a]

Target gene/18 S rRNA	CON Tu-N	CON Dx	MCD	NS	MGN	FSGS
α-actinin-4	4.00 ± 2.12	7.46 ± 4.93	8.20 ± 6.34	8.47 ± 7.88	7.15 ± 6.97	5.01 ± 2.39
(range)	1.24–6.97	2.34–15.9	2.81–20.1	0.74–29.5	0.81–34.6	0.32–8.00
GLEPP-1	53.2 ± 30.4	85.2 ± 31.7	131 ± 69.4	155 ± 150	146 ± 128	90.6 ± 46.1
(range)	25.7–100	46.5–129	55.8–317	10.1–646	30.4–565	10.9–157
WT-1	10.9 ± 6.58	23.7 ± 17.0	52.7 ± 72.5	7.79 ± 7.22	36.2 ± 48.9	19.8 ± 19.8
(range)	5.24–23.0	5.79–52.4	3.66–244	0.58–30.0	2.98–208	0.83–63.6
synaptopodin	19.2 ± 9.43	19.0 ± 8.81	33.5 ± 17.2	24.3 ± 12.4	21.8 ± 13.6	23.1 ± 10.9
(range)	10.7–34.4	11.9–33.3	15.8–75.5	3.71–47.3	7.6–62.0	7.34–46.5
dystroglycan	5.59 ± 4.22	6.27 ± 1.97	20.7 ± 19.2	8.99 ± 6.12	19.4 ± 12.3	9.81 ± 4.95
(range)	1.91–12.1	2.35–9.67	3.18–75.8	3.33–25.5	2.25–49.2	0.41–16.2
nephrin	168 ± 167	146 ± 69.1	350 ± 338	605 ± 831	449 ± 607	178 ± 135
(range)	18.5–463	66.9–243	121–1357	10.1–3340	1.15–2602	15.4–471
podoplanin	17.1 ± 14.4	29.8 ± 19.4	60.4 ± 69.5	71.0 ± 58.4	121 ± 141	41.2 ± 48.6
(range)	2.12–33.9	10.9–65.0	4.45–195	2.26–187	1.81–505	3.31–160
podocin	94.4 ± 69.1	155 ± 86.1	239 ± 150	240 ± 235	103 ± 323	35.5 ± 44.9
(range)	10.7–204	73.1–320	122–638	28.8–1045	2.20–1792	0.38–141

[a] Data are mean ± SEM and range. GLEPP-1, glomerular epithelial protein 1; WT-1, Wilms tumor antigen 1.

though any one gene was unable to distinguish adequately among the biopsy samples, by combinatorial analysis of two podocyte-specific genes relative to each other, one could enhance the prognostic character of podocyte gene expression analysis.

To this end, the expression of these two genes was studied as a ratio to each other. The ratio of podocin relative to synaptopodin mRNA allowed a clear separation between MCD and FSGS with no overlap (MCD mean ratio, 7.77 ± 3.04; FSGS mean ratio, 0.92 ± 0.79; $P = 0.03$; Figure 2a). In addition, the same ratio separated benign NS from FSGS glomeruli. In contrast, biopsies with the histologic diagnosis of MGN revealed a highly variable podocin/synaptopodin ratio. No significant correlation with histopathologic classification (*e.g.*, Churg and Ehrenreich classification) or clinical parameters could be identified (data not shown). Ratios of, for example, WT-1/synaptopodin and dystroglycan/synaptopodin mRNA showed no selectivity for the analyzed glomerulopathies; neither did the ratio of synaptopodin/GLEPP-1 mRNA (data not shown).

For confirmation of these findings and for testing the applicability of this approach on routine renal biopsies, glomeruli of formaldehyde-fixed archival renal tissues (MCD, $n = 5$; FSGS, $n = 4$; diagnostically challenging cases of MCD *versus* FSGS, $n = 6$) were analyzed after LCM. In this different set of biopsies, the podocin to synaptopodin ratio determined by real-time RT-PCR allowed a distinct and significant separation between MCD and FSGS samples (MCD mean ratio, 0.33 ± 0.09; FSGS mean ratio, 0.13 ± 0.03; $P = 0.01$; Figure 2b, left). In FSGS, the podocin to synaptopodin ratio grouped both sclerosed and nonsclerosed glomeruli in the FSGS category (podocin/synaptopodin ratio for sclerosed glomeruli, 0.15 ± 0.05; for nonsclerosed glomeruli, 0.14 ± 0.04; 197 and 84 glomerular cross-sections from 6 and 3 patients, respectively). In addition, a retrospective study on six biopsies with diagnostically challenging histologies was performed. These samples showed no or minimal glomerular abnormality, as seen in MCD, and isolated sclerosed glomeruli consistent with FSGS and were in conjunction with clinical data thus classified as focal and segmental sclerosis and hyalinosis superimposed on minimal change nephrotic syndrome (13). Analysis of the clinical follow-up data (follow-up period 3 to 14 mo; Table 3) allowed segregation of the patients into two groups: steroid-responsive and steroid-resistant patients. Using the podocin to synaptopodin ratio, the clinical course of the patients could be predicted from the biopsy (Figure 2b, right). Steroid-responsive patients were grouped in the MCD group, and steroid-resistant patients were grouped in the FSGS group.

Gene Expression Levels of Podocyte-Associated Molecules Are Closely Related in Microdissected Glomeruli

The above analysis of gene expression ratios between various podocyte-associated cDNA showed an extremely low interdisease variability for most markers. A tight correlation of the gene expression levels in each glomerulus is one potential explanation for this. A systematic analysis of the relationship between expression levels revealed a highly significant positive correlation between ACTN4, GLEPP-1, synaptopodin, dystroglycan, WT-1, nephrin, and, to a lesser extent, podoplanin (Figure 3a, Table 4). Podocin did not correlate with the above cDNA (Figure 3b, Table 4). Podocin compared with synaptopodin, the ratio used as a diagnostic marker, showed the lowest correlation coefficient of all molecules studied. The strong correlation of most podocyte-specific cDNA in acquired

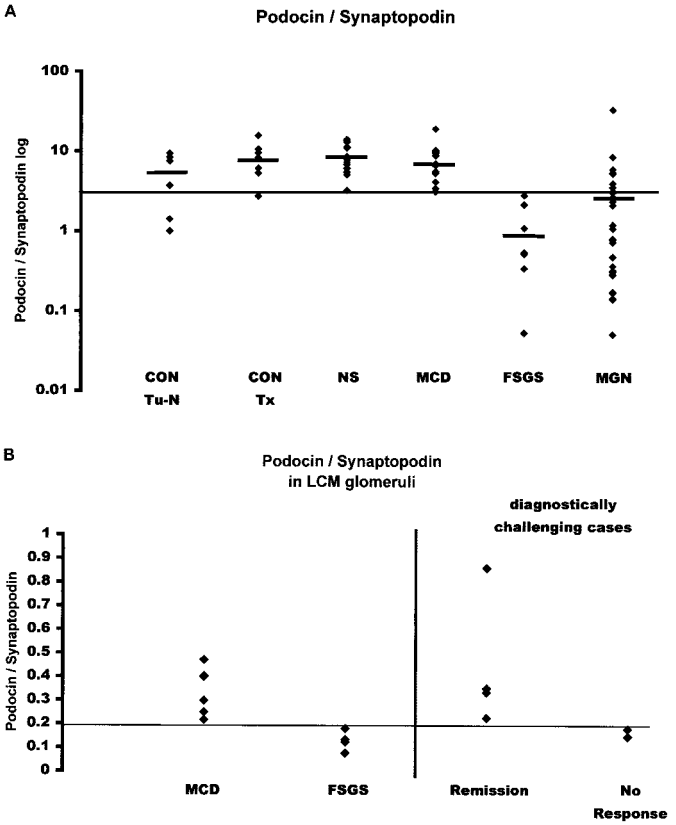

Figure 2. Gene expression ratio of two podocyte-associated molecules (podocin/synaptopodin) allows diagnostic separation. (a) Podocin/synaptopodin mRNA expression ratio in manually microdissected glomeruli. (b) Confirmation in formaldehyde-fixed samples after laser capture microdissection. The podocyte-associated cDNA levels obtained from microdissected glomeruli were related to each other, with the podocyte-specific molecule synaptopodin serving as the denominator. The ratio of podocin to synaptopodin mRNA showed significant differences between minimal change disease (MCD), nephrosclerosis (NS), and focal and segmental glomerulosclerosis (FSGS; $P = 0.0341$ and 0.0486, respectively) and allowed a clear separation of FSGS from MCD and NS (a; the diagnostic cut-off is marked as a continuous line). To evaluate this marker in an independent population with a different technique, glomeruli of formaldehyde-fixed archival renal tissues (MCD, $n = 5$; FSGS, $n = 4$; diagnostically challenging cases of MCD *versus* FSGS, $n = 6$) were analyzed after laser microdissection. The podocin to synaptopodin ratio determined by real-time RT-PCR allowed a clear separation between MCD and FSGS samples (b, left; $P = 0.008$). In addition, the same podocin to synaptopodin cut-off separated biopsies with diagnostically challenging histology into two groups (b, right): Patients assigned by the molecular marker to the MCD group showed a steroid-responsive course. Patients attributed to the FSGS group proved to be steroid resistant (see Table 3).

human diseases may indicate a common mechanism determining the observed cDNA levels.

Discussion

A considerable challenge in the differential diagnosis of proteinuric glomerulopathies is the differentiation between FSGS and MCD. MCD is characterized by no visible alterations on light microscopy and by foot process fusion on electron microscopy. It responds well to steroid treatment and rarely progresses to renal failure. In contrast, FSGS is characterized by focal and segmental glomerular hyalinosis and sclerosis. It responds poorly to steroid treatment and frequently progresses to chronic renal failure (14). As the prevalence of FSGS in the Western world has increased steadily (15), it now represents the leading cause of chronic renal failure in North America. Treatment of FSGS is limited, and the disease frequently recurs in the renal transplant with massive proteinuria (16). As treatment and prognosis of MCD and FSGS are fundamentally different, a clear diagnostic distinction between these entities is of central importance.

In FSGS, as well as in MCD, podocyte damage seems to be the primary cause of disease. Histopathologic parameters including electron microscopy for morphologic assessment of podocyte and foot process do not allow a separation of these entities in all patients. Diagnosis of FSGS is dependent on the extent of disease and the number of glomeruli in the biopsy section. Problematic cases with no sclerosis on histology would greatly benefit from molecular diagnostics using a parameter differentially regulated in all FSGS but not in MCD glomeruli. Molecules mutated in hereditary FSGS are prime candidates to serve as such markers in acquired FSGS.

Podocin (NPHS2) has been found as the causative gene of an autosomal-recessive FSGS (7) and seems to be responsible for a considerable proportion of hereditary nephroses (17). Podocin expression is restricted to podocytes as shown by *in situ* RNA hybridization (7,18).

For an autosomal-dominant form of FSGS, a mutation in the ACTN4 gene has been identified as an underlying mechanism (8). ACTN-4 serves as a linker molecule between actin filaments in the cytoskeletal scaffold of podocyte foot processes. In experimental nephrotic syndrome, mRNA expression of ACTN4 is increased (19).

The congenital nephrotic syndrome of the Finnish type, the most severe nephrosis in humans, is caused by a mutation in nephrin (NPHS1). Nephrin and podocin have been colocalized to the podocyte slit diaphragm (20). Gene expression analyses of nephrin mRNA in human, rat, and murine glomeruli yielded conflicting results, with most studies showing a trend toward nephrin mRNA induction in early disease, whereas in late disease stages, glomeruli seem to exhibit lower mRNA levels (21–23).

Mutations in the Wilms tumor suppressor gene are responsible for the Denys-Drash syndrome and diffuse mesangial sclerosis (24), both presenting with failure of the glomerular filtration barrier during childhood. WT-1 is widely expressed in epithelial cells of the early nephron and becomes restricted to podocytes in mature glomeruli (25).

Podoplanin is expressed in podocyte foot processes and is downregulated in experimental nephrosis (26,27). Podoplanin antibodies induce transient proteinuria with foot processes retraction. Currently, no data on podoplanin mRNA regulation in human renal diseases are available.

Table 3. Diagnostically challenging cases of minimal change glomerulopathy with FSGS: Clinical characteristics and steroid response

Age (years)	Gender	Serum creatinine (mg/dl) at biopsy	Proteinuria (g/24 h) at biopsy	Follow-up period (months)	Serum creatinine (mg/dl) at follow-up	Proteinuria (g/24 h) at follow-up	Clinical course under steroid treatment
53	M	1.18	8.20	5	1.05	0.240	Remission
22	M	1.90	13.76	6	1.39	3.80	No Remission
68	M	1.00	8.00	4	1.00	5.10	No Remission
48	M	1.40	22.00	12	1.10	0	Remission
58	F	0.66	8.60	14	0.60	0	Remission
27	F	0.74	10.60	2	0.62	0.518	Remission

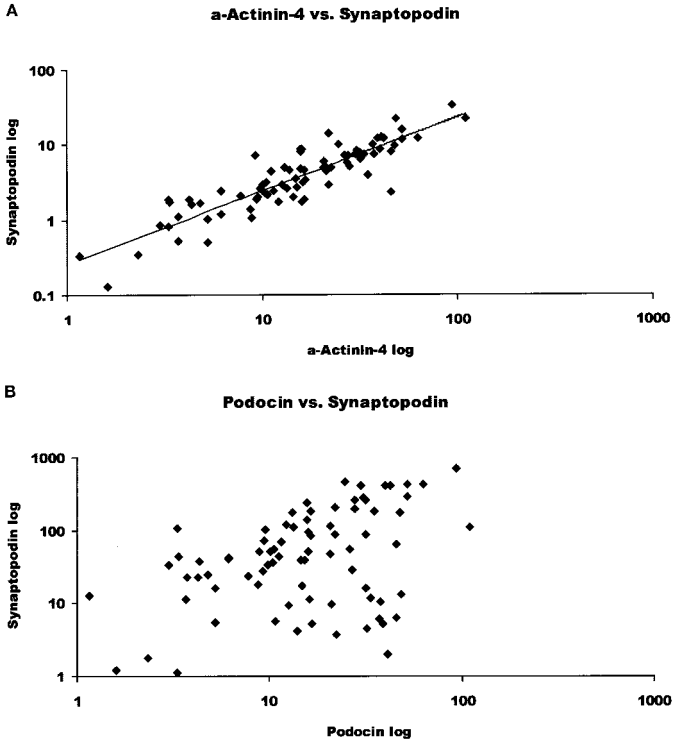

Figure 3. Correlation plots for gene expression levels of podocyte-associated molecules. A systematic analysis of the relationship between expression levels of analyzed marker molecules revealed a significant positive correlation between α-actinin-4, Glomerular epithelial protein 1, synaptopodin, dystroglycan, WT-1, nephrin, and, to a lesser extent, podoplanin. As an example, a correlation plot of α-actinin-4 *versus* synaptopodin (absolute mRNA expression levels, $\log_{10}$ transformed, $r = 0.866$, $P < 0.001$) is shown (a). Podocin did not correlate with the above cDNA as demonstrated for absolute mRNA expression levels of synaptopodin to podocin ($\log_{10}$ transformed, $r = 0.037$; b).

Dystroglycan seems to be relevant for the dynamic attachment of podocytes to the GBM, with a loss of dystroglycan staining in MCD (28). In animal models, dystroglycan expression seems to be reduced in adriamycin nephropathy, but no significant changes of dystroglycan have been reported in rat models of puromycin aminonucleoside nephrosis or passive Heymann nephritis (29,30).

GLEPP-1 and synaptopodin show a podocyte-specific expression starting at the capillary loop stage of glomerular formation. GLEPP-1 immunohistochemical staining is reduced or lost in most proteinuric diseases (31). Synaptopodin mRNA has been shown to be differentially regulated in a small sample of pediatric nephroses (32). In collapsing forms of FSGS, including idiopathic FSGS and HIV-associated nephropathy, marked reduction of synaptopodin expression was noticed (33).

Differential diagnosis of MCD *versus* FSGS using molecular approaches was attempted in two studies to date: Glomerular protein expression of dystroglycans, determined by counting immuno-gold particles, seems to be reduced in MCD but not in FSGS (28). Increased mRNA expression of TGF-β1 has been associated with proliferative and fibrotic lesions in glomerulopathies and has been reported to be indicative for progressive renal damage typical of FSGS but not of MCD (34).

Despite the continuous characterization of podocyte-associated molecules, there is limited progress in understanding their regulation and differential expression in proteinuric diseases. Data available from small single-center populations are often conflicting. Therefore, we studied the glomerular-specific mRNA expression of podocyte-associated molecules in renal biopsies from adult patients with various proteinuric glomerulopathies to identify differentially regulated podocyte markers as potential adjunctive molecular diagnostic tools.

The comprehensive analysis of eight podocyte-associated molecules in 83 patients collected in a multicenter study showed no significant regulation in microdissected glomeruli, as long as the mRNA expression data were related to ubiquitously expressed housekeeper cDNA. Dystroglycan mRNA expressed as a ratio to 18 S rRNA or GAPDH was not repressed in MCD (Table 2); TGF-β1 mRNA also was not significantly increased in FSGS glomeruli (data not shown). These findings indicate, that on the basis of a "conventional" housekeeper-based approach of mRNA expression analysis, no clear separation between the proteinuric disease categories

Table 4. Correlation of podocyte-associated molecules[a]

Correlation coefficient (r, *P* values)	α-actinin-4	GLEPP-1	WT-1	Synaptopodin	Dystroglycan	Nephrin (NPHS1)	Podoplanin	Podocin (NPHS2)
Podocin (NPHS2)	0.142 $P < 0.001$	0.049 $P = 0.007$	0.145 $P < 0.001$	0.037 $P < 0.001$	0.041 $P < 0.001$	0.045 $P < 0.001$	0.072 $P = 0.488$ NS	1
Podoplanin	0.569 $P < 0.001$	0.545 $P < 0.001$	0.500 $P < 0.001$	0.405 $P < 0.001$	0.645 $P < 0.001$	0.668 $P < 0.001$	1	
Nephrin (NPHS1)	0.608 $P < 0.001$	0.617 $P < 0.001$	0.487 $P < 0.001$	0.556 $P < 0.001$	0.608 $P < 0.001$	1		
Dystroglycan	0.710 $P < 0.001$	0.800 $P < 0.001$	0.572 $P < 0.001$	0.610 $P < 0.001$	1			
Synaptopodin	0.866 $P < 0.001$	0.712 $P < 0.001$	0.644 $P < 0.001$	1				
WT-1	0.706 $P < 0.001$	0.506 $P < 0.001$	1					
GLEPP-1	0.711 $P < 0.001$	1						
α-actinin-4	1							

[a] Absolute mRNA levels were correlated to each other, and correlation coefficients were determined; *P* values are quoted; ns, not significant.

(benign NS, MGN, and particularly between MCD and FSGS) could be obtained. Taken together, there are no consistent changes in examined podocyte markers in glomerulopathies, and analysis of these markers related to housekeeper genes does not seem to be a useful diagnostic tool.

For the analysis of gene expression profiles, the selection of a relevant observation unit is crucial. In our study, variability was reduced by microdissection of specific nephron segments enabling glomerulus-specific gene expression analysis. However, for evaluation of podocyte-specific cDNA, the podocyte, not the entire glomerulus, would be the optimal observation and reference unit. Single podocyte mRNA analysis is possible (35) but is not feasible with tissue collected within this multicenter study. Therefore, we performed an "in silico" microdissection by using a podocyte-specific cDNA (synaptopodin) as reference instead of ubiquitously expressed housekeeper genes. With this approach, only RNA from the podocyte compartment of the glomerulus were integrated into the analysis and thus are not influenced by alterations in, for example, mesangial or endothelial gene expression. Gene expression ratios of podocyte markers as presented in this study do not allow one to draw conclusions about absolute gene expression levels per podocyte. They are used here as a vehicle for molecular diagnostics.

The ratio podocin/synaptopodin showed a clear separation between FSGS and MCD as well as FSGS and benign NS with no overlap between FSGS and MCD or NS. Separation of FSGS from MCD by determining the podocin to synaptopodin mRNA expression ratio was confirmed in an independent population of laser captured microdissected glomeruli from formaldehyde-fixed archival renal tissues.

In contrast, MGN gave a highly variable podocin/synaptopodin ratio that revealed no correlation with histopathologic or clinical parameters. As MGN has a specific histologic picture, molecular markers to differentiate MGN from FSGS are not required.

There are several intrinsic limitations to our study. First, we analyzed a European white population showing only classic nonproliferative FSGS. There were no black patients included in the study or patients with collapsing glomerulopathy.

Second, despite using a large study population for gene expression analysis of human glomeruli with confirmation in formaldehyde-fixed archival renal tissues, sample size in each disease category is still small for the establishment of a diagnostic parameter. At least in the case of FSGS, this was a consequence of the exclusion of secondary FSGS and, compared with North America, the lower frequency of FSGS in the European biopsy population studied. Comparable gene expression analysis of independent populations of renal biopsies will be a crucial next step for validation of this marker set.

Third, on the basis of the study protocol used, no histology was available on the specific, manually microdissected glomeruli from which gene expression profiles were obtained. To circumvent this limitation, we performed an additional series of experiments using LCM of fixed biopsies in which the histology of the laser-dissected glomeruli could be evaluated. It is interesting that in the FSGS group, both glomeruli with segmental sclerosis and those without a sclerotic lesion showed an identical podocin to synaptopodin ratio. This study confirmed the results obtained with the manually dissected glomeruli from unfixed biopsies, underlining the validity of the approach. This prompted the examination of the important question: Is the observed difference in the expression ratios between FSGS and MCD also seen in diagnostically challenging cases with unclear histopathology? Therefore, the marker profile was obtained in a small retrospective study on six

archival biopsies with focal and segmental sclerosis and hyalinosis superimposed on minimal change nephrotic syndrome. According to the podocin to synaptopodin ratio, four biopsies showed the MCD and two showed the FSGS pattern. Follow-up data indicated a steroid-responsive course for the four cases that showed the MCD molecular pattern and a steroid resistance in the two cases with the FSGS pattern. These initial data may indicate a predictive therapeutic response value of the gene expression ratio but have to be confirmed in a prospective manner.

What are the potential consequences of this observation? If the repressed ratio in steroid-resistant FSGS is a consequence of reduced podocin mRNA expression in podocytes, as suggested by the trend toward repressed podocin/18 s rRNA ratio in FSGS glomeruli, then the podocin/synaptopodin ratio may help to define a set of patients with a specific pathogenesis of nephrotic disease. As podocin has been shown to be an essential part of slit diaphragm and the associated signaling (36), alterations could have deleterious effects in stressed podocytes. Perhaps as a consequence of altered podocyte signaling, these patients do not respond to steroids, the effective therapy for a majority of nephrotic syndromes. To what extend podocin is causally related cannot be concluded from this descriptive human study.

A surprising finding was the strong positive correlation for the expression levels of ACTN4, GLEPP-1, synaptopodin, dystroglycan, WT-1, and nephrin in acquired proteinuric diseases. These data are consistent with a parallel regulatory mechanism of these genes in podocytes. It is tempting to speculate that podocytes respond, in parallel to their uniform structural alterations in disease states (37), with a uniform transcriptional program to alteration in the filtration barrier. This would not hold true for all podocyte-associated molecules, as podocin and, to a lesser extent, podoplanin mRNA expression did not correlate with the above molecules. As studies on transcriptional regulation and mRNA stability become available, these questions can be addressed experimentally in the future. Strategies to target this potential common response could yield exciting options for intervention.

In conclusion, a comprehensive gene expression study of podocyte-associated molecules in acquired glomerular diseases identified the podocin/synaptopodin mRNA expression ratio as a potential molecular diagnostic parameter, which could aid in the diagnostic separation of FSGS from MCD and benign NS. In addition, a strong positive correlation of the majority of podocyte-associated cDNA indicates common regulatory mechanisms activated in proteinuric glomerular diseases.

APPENDIX: MEMBERS OF THE EUROPEAN RENAL cDNA CONSORTIUM

C. Cohen, M. Kretzler, D. Schlöndorff, Munich; F. Delarue, J.D. Sraer, Paris; M.P. Rastaldi, G. D'Amico, Milano; F. Mampaso, Madrid; P. Doran, H.R. Brady, Dublin; D. Mönks, C. Wanner, Würzburg; A.J. Rees, P. Brown, Aberdeen; F. Strutz, G. Müller, Göttingen; P. Mertens, J. Floege, Aachen; N. Braun, T. Risler, Tübingen; L. Gesualdo, F.P. Schena, Bari; J. Gerth, G. Stein, Jena; R. Oberbauer, D. Kerjaschki, Vienna; M. Fischereder, B. Krämer, Regensburg; W. Samtleben, W. Land, Munich; H. Peters, H.H. Neumayer, Berlin; K. Ivens, B. Grabensee, Düsseldorf.

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