Expression of filtrin in human glomerular diseases

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Abstract

Background. Filtrin (NEPH3/KIRREL2) is a recently characterized member of the nephrin-like proteins of the immunoglobulin superfamily, and it has been suggested to participate in the maintenance of the glomerular filtration barrier in the kidney. In this study, the gene and protein expression of filtrin were examined in patients with acquired proteinuric diseases.

Methods. Filtrin mRNA levels in renal biopsies were measured with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in two sets of patients with proteinuria. The mRNA levels were normalized to the housekeeping gene GAPDH and also related to the podocyte-specific genes nephrin and podocin. Immunofluorescence microscopy was employed to explore changes in the glomerular distribution of filtrin.

Results. Reduced glomerular expression of filtrin mRNA was observed in all studied diagnostic groups. In focal segmental glomerulosclerosis, the filtrin mRNA level was only one-tenth of the control samples (P < 5.0 × 10−4), and this finding was confirmed in a second set of samples. The ratios of filtrin to nephrin and podocin demonstrated a marked decrease in the expression of filtrin relative to the podocyte marker genes. However, no correlation between the expression of filtrin and the levels of serum creatinine and proteinuria was observed. Immunostaining showed changes in the expression pattern of filtrin in renal biopsies. Immunoelectron microscopic studies localized filtrin at the slit diaphragm of the podocyte foot processes.

Conclusions. Down-regulation of the filtrin gene and protein expression in the renal biopsies together with the localization to the inter-podocyte filtration slit imply a potential role for this molecule in the pathogenesis of proteinuric diseases.

Keywords: gene expression; glomerulus; kidney biopsy; podocyte; proteinuria

Introduction

Podocyte foot processes surrounding glomerular capillaries form the ultimate barrier to the leakage of plasma proteins from the circulation. The unique cell junction structure, known as the slit diaphragm, bridges the extracellular aspects of the adjacent foot processes [1]. The early stages of escalating glomerular damage are reflected as disruption of the normal protein expression patterns of the slit diaphragm accompanied by the effacement of the podocyte foot processes [2,3]. While the exact pathogenetic mechanisms remain to be studied in detail, recent findings suggest a central role for the podocyte in the progressive damage finally leading to end-stage renal disease [4,5].

The knowledge of key molecules constituting the interpodocyte slit diaphragm is rapidly emerging. The discovery of nephrin at the slit diaphragm [6–8] was soon followed by identification of additional proteins which participate in the filtration barrier function. The location of CD2AP [9], podocin [10], NEPH1 [11] and NEPH2 [12] have been established at the slit diaphragm and their regulation in human proteinuric diseases has been described previously [13,14].

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Filtrin (NEPH3; official HUGO gene name KIRREL2), a recently described transmembrane protein of the immunoglobulin superfamily [11,15–17], has several properties making it a particularly interesting molecule for the glomerular filter. First, it shows homology and structural similarity to nephrin, NEPH1 and NEPH2 suggesting shared functions [11,12,15,16]. Second, the tissue distribution of filtrin is limited and it appears to participate in specific molecular interactions and signalling in parallel to NEPH1 [11,15,18]. Third, its genomic locus is only 5-kb upstream from the nephrin gene NPHS1 but, interestingly, in the opposite direction [11,15,16].

Here we examine the gene and protein expression patterns of filtrin in renal biopsies of controls and of patients with acquired proteinuric disease and attempt to define the localization of filtrin in the podocytes.

Materials and methods

Human kidney biopsies

Human renal biopsies from 69 patients with acquired proteinuric disease and seven control subjects were included in the study. These samples were obtained from the multicentre European Renal cDNA Bank (ERCB). They were divided according to their histological diagnosis into the following five groups: minimal change disease (MCD; \(n = 13\)), hypertensive nephropathy (HT; \(n = 16\)), membranous glomerulopathy (MGN; \(n = 31\)) and focal and segmental glomerulosclerosis (FSGS; \(n = 9\)). Non-affected parts of tumour nephrectomies (CON; \(n = 7\)) served as a control group. Clinical data of the patients have been described previously [14]. The glomerular compartment was manually microdissected and processed for RNA isolation [19].

A second set of patients was used to verify the results. Biopsies taken for routine diagnostic purposes at St Vincent’s Hospital (Fitzroy, Australia) were examined [MGN, \(n = 4\); diabetic nephropathy (DN), \(n = 7\); MCD, \(n = 5\); and FSGS, \(n = 11\)]. Renal samples from the tumour-free part of nephrectomy specimens were used as control material (\(n = 6\)). Clinical data are provided in Table 1. Biopsies were immediately snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analysed. Total RNA was extracted from whole biopsies using the RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with random hexamers (Roche Diagnostics, Mannheim, Germany) using AMV reverse transcriptase (Roche Diagnostics).

Human kidney biopsies or control samples were obtained from patients after informed consent and with permission from the local ethics committees.

Real-time quantitative RT-PCR

Real-time the reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix, No AmpErase® UNG and Assays-on-Demand™ Gene Expression Assay Mix according to the manufacturer’s instructions. Commercially available pre-developed TaqMan gene expression assays were used for human filtrin (Assay ID: Hs00375638_m1) and GAPDH (Hs99999905_m1). Nephrin and podocin mRNA quantitation has been described earlier [14].

After the initial two minutes at 50°C and 10 min at 95°C, the reaction mixtures were cycled 40 times at 95°C for 15 s and 60°C for 60 s. Measurements were performed in duplicates. After the run was concluded, the threshold cycle (Ct) for each individual reading was determined and \(A_{Ct}\) was calculated by subtracting the corresponding mean GAPDH Ct from each mean filtrin Ct. The ratio of filtrin relative to GAPDH was defined as \(2^{\Delta Ct}\) as described earlier [19]. Serial dilutions of a nephrectomy sample served as a standard cDNA in all PCR runs and appropriate non-template controls were included in each experiment.

Statistical analyses

Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>n</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Serum creatinine (µmol/l)</th>
<th>Proteinuria (g/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranous glomerulo-nephropathy</td>
<td>4</td>
<td>65±4</td>
<td>2/2</td>
<td>125±50</td>
<td>4.5±2.8</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>7</td>
<td>51±19</td>
<td>4/3</td>
<td>173±80</td>
<td>4.9±7.5</td>
</tr>
<tr>
<td>Minimal change disease</td>
<td>5</td>
<td>31±12</td>
<td>3/2</td>
<td>86±21</td>
<td>9.5±3.4</td>
</tr>
<tr>
<td>Focal segmental glomerulosclerosis</td>
<td>11</td>
<td>56±19</td>
<td>6/5</td>
<td>145±42</td>
<td>5.0±4.4</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

Antibodies

The polyclonal antibody against the intracellular part of filtrin (#1217) was generated in rabbits immunized with the peptide CWR HSK ASA SFS EQK N (amino acids 533–548). The peptide was synthesized and purified by Alpha Diagnostics International Inc. (San Antonio, TX, USA). Two rabbits were immunized in parallel with 500 mg of KLH-coupled peptide in Freund’s complete adjuvant (Difco Laboratories, Detroit, MI, USA). Two booster injections with 400 µg antigen in Freund’s incomplete adjuvant were given at intervals of 4 weeks and the sera were collected. The antiserum was protein-A-purified

Table 1. Clinical characteristics of the patients from St Vincent’s Hospital
according to the manufacturer’s instructions (Amersham Biosciences, Uppsala, Sweden).

The affinity-purified antibody against the extracellular part of filtrin (#1155) was used in immunoelectron microscopy. In immunoblotting, this antibody specifically recognized a protein of 107 kDa from human and rat glomerular extracts [15]. Mouse monoclonal anti-synaptopodin antibody (Progen, Goettingen, Germany) was used as a podocyte-specific marker.

Immunofluorescence

A total of 15 renal biopsies from patients with a proteinuric disease were examined (MCD, n = 4; MGN, n = 6; and FSGS, n = 5). In addition, control kidney samples were included in the analysis (CON, n = 3). After diagnostic routine procedures were accomplished, 5 μm thick frozen sections were cut on albumin-coated slides, followed by cold-acetone fixation and staining by an indirect immunofluorescence method. Double stainings were conducted using a monoclonal anti-synaptopodin followed by Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Invitrogen, S. Giuliano Milan, Italy), and the polyclonal anti-filtrin antibody (#1217) revealed by Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes). The slides mounted with an anti-fading aqueous medium (Fluorsave; Chemicon, Prodotti Gianni, Milan, Italy) were examined under a fluorescence microscope equipped with appropriate filters (Zeiss Axioscope 40 FL; Zeiss, Jena, Germany), and the images were taken with a digital camera (AxioCam MRc5; Zeiss) connected to the image software AxioVision 4.3 (Zeiss). As a control, the primary filtrin antibody was either substituted with control immunoglobulins (rabbit primary antibody isotype control; Zymed, Invitrogen) or immunodepleted by pre-incubation for 1 h at 37°C with the polypeptide used as the original immunogen. Negative secondary antibody controls were processed in parallel.

Immunoelectron microscopy

Immunoelectron microscopy was performed using indirect immunogold labelling on ultra thin frozen sections of adult rat kidney fixed in 4% freshly prepared paraformaldehyde as described earlier [20]. The sections were incubated with the
anti-filtrin antibody (#1155) before incubation with 10-nm gold-conjugated goat anti-rabbit IgG (1:50; Amersham Biosciences).

**Results**

**Messenger RNA expression of filtrin is decreased in human glomerular diseases**

Filtrin expression levels in renal biopsies representing different diagnostic groups were determined in two sets of patients with quantitative RT-PCR. In ERCB patients, a significant decrease of filtrin was observed in microdissected glomeruli in all analysed diagnostic groups when compared with control samples (Table 2A, Figure 1A). Most remarkably, the mean expression levels were decreased in MGN ($P \approx 0.3 \times 10^{-5}$) and in FSGS ($P \approx 4.5 \times 10^{-5}$).

Verification of the mRNA results was performed in a second group of patients. In these samples, the cDNAs were prepared from whole biopsies. A trend to decreased filtrin expression was observed in all studied diagnostic groups (Table 2B; Figure 1B). The decrease was statistically significant in the samples from FSGS ($P \approx 1.6 \times 10^{-3}$) and DN ($P \approx 5.1 \times 10^{-3}$). Taken together, the mRNA expression of filtrin is decreased in acquired proteinuric diseases in two individual patient cohorts.

**Table 2. Messenger RNA levels of filtrin in the kidney biopsies**

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>n</th>
<th>Filtrin/GAPDH</th>
<th>% P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) ERCB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>0.30 ± 0.30</td>
<td>100</td>
</tr>
<tr>
<td>Minimal change disease</td>
<td>13</td>
<td>0.11 ± 0.08</td>
<td>37</td>
</tr>
<tr>
<td>Hypertensive nephropathy</td>
<td>16</td>
<td>0.09 ± 0.05</td>
<td>30</td>
</tr>
<tr>
<td>Membranous</td>
<td>31</td>
<td>0.04 ± 0.03</td>
<td>13</td>
</tr>
<tr>
<td>glomerulonephropathy</td>
<td>9</td>
<td>0.03 ± 0.03</td>
<td>10</td>
</tr>
<tr>
<td>Focal segmental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glomerulosclerosis</td>
<td>9</td>
<td>0.34 ± 0.33</td>
<td>20</td>
</tr>
<tr>
<td>(B) St Vincent’s Hospital</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1.74 ± 0.84</td>
<td>100</td>
</tr>
<tr>
<td>Membranous</td>
<td>4</td>
<td>1.33 ± 1.18</td>
<td>76</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>7</td>
<td>0.31 ± 0.28</td>
<td>18</td>
</tr>
<tr>
<td>Minimal change disease</td>
<td>5</td>
<td>0.91 ± 0.54</td>
<td>52</td>
</tr>
<tr>
<td>Focal segmental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glomerulosclerosis</td>
<td>11</td>
<td>0.34 ± 0.33</td>
<td>20</td>
</tr>
</tbody>
</table>

Two sets of patients were included in the expression analysis: (A) ERCB, where the glomerular compartment was microdissected for the analysis, and (B) biopsies taken at St Vincent’s Hospital, where whole renal biopsies were processed as such for the RNA isolation. Quantitative RT-PCR data are presented as a filtrin/GAPDH ratio and given as mean ± SD. The mean expression in each diagnostic category is related to the mean of the controls and given as percentages (%). Dunnett 2-sided t-test was applied to indicate significantly different mean values from the controls.

**Relating the expression of filtrin to podocyte markers confirms the reduced expression in proteinuria**

Normalization of gene expression data to housekeeping genes (e.g. GAPDH) has been standard in expression studies. In our study, this approach was problematic since podocytes form only a part of the glomerular volume and the changes in housekeeping gene levels might also reflect alterations in other glomerular cell types such as the mesangial cells and endothelia. Therefore, the glomerular expression ratios of filtrin to nephrin and filtrin to podocin were calculated. We have previously published the glomerular mRNA expression data of nephrin and podocin from the ERCB patients [14].

The glomerular expression ratio of filtrin to nephrin was significantly higher in controls (CON, 1.59 ± 1.02; Figure 1C) than in patients with a proteinuric disease (e.g. for FSGS, 0.14 ± 0.10; $P \approx 0.04 \times 10^{-6}$). Similar statistically significant difference in the expression ratios for filtrin/podocin was detected between the control and the proteinuric samples (data not shown).

**Correlation between the mRNA expression of filtrin and clinical variables**

Correlation analysis of the gene expression data and clinical variables available from the patients (Table 1) was performed. The expression ratios of filtrin/GAPDH and filtrin/nephrin did not reveal correlation with the level of proteinuria or serum creatinine at the time of biopsy (Table 3). Statistically significant correlation between the filtrin/podocin ratio and proteinuria was observed, but this was due to the direct correlation of proteinuria with the podocin/GAPDH ratio itself ($n = 57$, $r = 0.32$, $P \approx 0.01$).

**Glomerular mRNA levels of filtrin and nephrin are tightly correlated**

The surprising separation between the controls and disease categories based on filtrin/nephrin and filtrin/podocin values encouraged us to study the possible relation between the expression of filtrin and nephrin and clinical variables reflecting renal function.

**Table 3. Correlation analysis of the gene expression data with clinical variables reflecting renal function**

<table>
<thead>
<tr>
<th>Serum creatinine</th>
<th>Proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) ERCB</td>
<td></td>
</tr>
<tr>
<td>Filtrin/GAPDH</td>
<td>0.05 (0.70)</td>
</tr>
<tr>
<td>Filtrin/nephrin</td>
<td>0.12 (0.35)</td>
</tr>
<tr>
<td>Filtrin/podocin</td>
<td>0.11 (0.39)</td>
</tr>
<tr>
<td>(B) St Vincent’s Hospital</td>
<td></td>
</tr>
<tr>
<td>Filtrin/GAPDH</td>
<td>−0.29 (0.19)</td>
</tr>
</tbody>
</table>

Relative mRNA levels were correlated with the levels of serum creatinine and proteinuria at biopsy, and correlation coefficients ($r$) were calculated. Results are presented as $r$ ($P$-value). Gene expression data and clinical variables were log$_{10}$ transformed before the analysis.
correlation between the expression of these genes. Correlation coefficients between filtrin/GAPDH, nephrin/GAPDH and podocin/GAPDH values were determined. Statistically significant, positive correlation between filtrin/GAPDH and nephrin/GAPDH was seen in proteinuric samples (n = 63, r = 0.72, P = 2.9 x 10^-11). The scatter plot showed that the control samples remained outside of the cluster formed by the samples from proteinuric diseases (Figure 1D). Podocin/GAPDH values did not correlate with filtrin/GAPDH values (n = 63, r = -0.14, P = 0.28).

Glomerular distribution of filtrin in renal biopsies

Immunostaining of filtrin was performed on renal biopsies of normal tissue and of patients with MCD, MGN and FSGS (Figure 2). In control glomeruli, filtrin immunoreactivity was found throughout the glomerulus and particularly in capillary loops facing the urinary space (Figure 2; CON). Double labelling with the podocyte marker synaptopodin showed that glomerular filtrin was localized in podocytes represented by a yellow overlap (Figure 2; CON). In MCD and MGN, a moderate staining of filtrin was found in areas expressing synaptopodin. Additionally, staining in the mesangium was observed in MCD (Figure 2; MCD). In MGN, the glomerular staining of filtrin appeared granular (Figure 2; MGN). In FSGS, a profound decrease of filtrin staining was observed, that ranged from segmental (Figure 2; FSGS) to global (data not shown). The specificity of filtrin immunoreactivity was confirmed in a blocking experiment: the specific staining was abolished by pre-incubation with the antigenic peptide (data not shown).

To examine the distribution of filtrin in podocytes in detail, immunoelectron microscopy was carried out on rat kidney sections. The filtrin-specific gold particles were found at the podocyte slit membrane area (Figure 3).

Discussion

This study demonstrates a significant decrease in the gene expression of filtrin in human glomerular diseases. The immunohistochemical analysis confirmed the changes in the expression pattern at the protein level, and immunoelectron microscopic studies suggest that filtrin localizes to the podocyte slit diaphragm area.

Filtrin mRNA levels in the kidney biopsies showed a marked reduction in two individual patient cohorts (from the ERCB and St Vincent’s Hospital) with acquired proteinuric diseases. In ERCB biopsies, the glomerular compartment was separated from the tubulointerstitium with microdissection for the analysis, whereas in the second one, the biopsies were processed as such for quantitative RT-PCR. It is noteworthy that a trend towards decreased expression was observed in both sample sets, irrespective of the mode of preparation of the samples. However, results from the second set of patients should be considered with caution because of the low number of patients and variability in the expression for the controls and the patients with membranous glomerulopathy.

The gene expression of filtrin was first normalized to the GAPDH housekeeping gene. The results from both sets of patients showed that the reduction of filtrin was most pronounced in focal segmental glomerulosclerosis. The observed repression of filtrin could be a consequence of reduced podocyte number per glomerulus/renal biopsy or reduced mRNA steady-state level per podocyte or both. To adjust the
expression data for the possible alterations, we related the filtrin mRNA levels to nephrin and podocin. This showed a repression of filtrin relative to these two slit-membrane-associated molecules. The results suggest that the expression of filtrin in the kidney glomerulus is down-regulated under proteinuric conditions beyond the regulation of nephrin and podocin, consistent with an mRNA repression per podocyte and not only a consequence of loss of podocyte. Furthermore, the filtrin/nephrin expression ratio separated controls from all proteinuric diseases. This observation might reflect a disturbed balance in the expression of these genes in proteinuria.

The role of filtrin in the pathogenesis of proteinuric diseases was also assessed using the quantitative variables, serum creatinine level and proteinuria, in the analysis instead of the categorical diagnostic groups. However, this approach did not yield any statistically significant associations. Taken into account the decrease of filtrin mRNA levels in proteinuria, the results may suggest that the repression of filtrin is a marker of a general glomerular and/or podocyte injury, not directly related to the slit diaphragm dysfunction per se.

Due to the physical proximity of the filtrin (KIRREL2) and nephrin (NPHS1) genes on chromosome 19p13.3 and potentially overlapping regulatory regions [11,15], these genes could show parallel regulation in health and disease. Our results give some support to such a hypothesis: The filtrin and nephrin gene mRNA levels revealed a highly significant positive correlation in proteinuric samples suggesting a transcriptional co-regulation. Examples of homologous genes with head-to-head genomic organization contributing to similar molecular functions include the oestrogen receptor co-repressor genes SAFB1 and SAFB2 on human chromosome 19p13.3 [21] and the human CYP1A1 and CYP1A2 genes on chromosome 15q24, involved in the metabolism of drugs and toxic compounds [22]. Notably, experimental data show evidence of shared transcriptional enhancer elements in the intergenic region of the CYP genes [22]. The possibility exist that the 5-kb intergenic region between the filtrin and nephrin genes also contains common regulatory elements responsible for transcriptional activation and regulation. A positive correlation has previously been reported for e.g. the podocyte-expressed genes alpha-actinin-4, nephrin and synaptopodin in acquired proteinuric diseases [14]. On the other hand, the expression of podocin did not correlate with the genes at the mRNA level [14]. In agreement with this, we could not find any correlation between filtrin and podocin expression levels either.

We examined the distribution of filtrin in the kidney to define its localization. Most of filtrin expression within the glomerulus was confined to podocytes as indicated by immunofluorescent double labelling with the podocyte marker synaptopodin. We also examined the expression at the ultrastructural level in podocytes, and the results indicate that filtrin localizes at the slit diaphragm of the podocyte foot processes, similar to the localization of both NEPH1 and NEPH2 [12,23,24]. To further elucidate the involvement of filtrin in proteinuric diseases, we performed an immunohistochemical analysis of renal biopsies of normal controls and patients with a proteinuric renal disease. The results suggest that altered glomerular distribution of filtrin is a common feature in proteinuric renal diseases, with the general repression observed in glomeruli of patients with focal segmental glomerulosclerosis. We also detected filtrin expression in mesangial cells in glomeruli of patients with minimal change disease.

In conclusion, these results show the decreased mRNA expression and altered distribution of filtrin in glomerular disease, and demonstrate the localization of filtrin at the slit diaphragm area. In the context of the essential role of nephrin and NEPH1 for the glomerular filter, these findings suggest a potential role for filtrin in proteinuric renal disease.

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Conflict of interest statement. None declared.

References


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