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Synaptopodin expression in idiopathic nephrotic syndrome of childhood

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Synaptopodin expression in idiopathic nephrotic syndrome of childhood.

Background. Synaptopodin is a proline-rich protein intimately associated with actin microfilaments present in the podocytes’ foot processes. We investigated for synaptopodin expression in children with idiopathic nephrotic syndrome (INS), including minimal change disease (MCD), diffuse mesangial hypercellularity (DMH), and focal segmental glomerulosclerosis (FSGS); in children with congenital nephrotic syndrome of the Finnish type (CNF); and in normal kidney tissue. In particular, we examined whether an association exists between synaptopodin expression in podocyte cells and the response to steroids in INS, and whether synaptopodin expression can predict FSGS upon the initial kidney biopsy in children who progress from MCD or DMH to FSGS.

Methods. Immunohistochemistry was performed for synaptopodin expression on renal tissues from MCD (N = 18), DMH (N = 7), FSGS (N = 13), CNF (N = 9), and normal children (N = 7). Synaptopodin expression in nonsclerosed glomeruli was quantitated by computerized image analysis on the Optimas™ software for both luminance (L) and percentage of glomerular area (A).

Results. Synaptopodin expression was absent in areas of sclerosis. In nonsclerosed glomeruli, synaptopodin was significantly less expressed in all groups of INS and in CNF compared with normal (P < 0.0001 for both L and A, in each MCD, DMH, FSGS, and CNF). In INS, synaptopodin expression decreased in order from MCD to DMH to FSGS, reaching statistical significance between MCD and FSGS (P = 0.001 for L and P = 0.05 for A). Greater synaptopodin expression in podocytes was associated with a significantly better response to steroid therapy (P < 0.05 for both L and A). On the other hand, the expression of synaptopodin did not predict progression of MCD or DMH to FSGS.

Conclusion. We conclude that measurement of synaptopodin has the potential to be used as a marker to study the alteration in podocyte cell and response to therapy in INS.

Key words: idiopathic nephrotic syndrome, podocyte foot processes, steroids, sclerosed glomeruli, minimal change disease, renal disease in children.

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uria [11, 12]. MCD is characterized by minimal changes in the glomerulus. It responds well to steroid treatment and rarely progresses to renal failure. On the other hand, FSGS is characterized by segmental glomerular hyalinosis and sclerosis; it responds poorly to steroid treatment and frequently progresses to renal failure [13]. The clinical course of DMH lies between MCD and FSGS. MCD and DMH have been observed to progress to FSGS on subsequent kidney biopsies, suggesting that these lesions lie on a continuum [14–17]. The initial therapy to all forms of INS is by steroids, to which the response can be diverse, and certain children may need additional immunosuppressive therapy to which they may or may not respond.

Congenital nephrotic syndrome of the Finnish type (CNF) occurs from a genetic mutation in the nephrin protein, normally present in the slit diaphragm of the foot processes [18, 19]. The disease is characterized by massive proteinuria starting very early in life, at times in utero [19, 20]. It does not respond to steroids and needs aggressive treatment, including continuous albumin infusions, bilateral nephrectomy, and renal replacement therapy in infancy [20].

A decrease or loss of synaptopodin expression was previously observed in idiopathic FSGS, collapsing FSGS, and HIV-associated nephropathy [21–23]. On the other hand, the expression was normal in MCD, mesangio proliferative glomerulonephritis, membranous nephropathy, and IgA nephropathy [21–23]. Barisoni et al concluded that in collapsing FSGS, there is a dysregulation of the podocyte phenotype, which is characterized by lack of synaptopodin expression [21]. Based on these findings, we investigated for synaptopodin expression in INS, CNF, and normal children to examine whether it could be a key for differentiating among the various entities of INS. We also looked for a possible association between synaptopodin expression in INS and response to steroids and whether synaptopodin expression could predict FSGS on the initial biopsy in children who progress from MCD or DMH to FSGS.

METHODS

Kidney biopsies from children with INS were included in the study only if (1) the histology was consistent with MCD, DMH, or FSGS as described by the International Study of Kidney Disease in Children [24]; (2) patients had a minimum of two years of follow-up from the primary diagnosis of INS to ascertain the response to steroid therapy; and (3) the tissue in the paraffin block had at least five nonsclerosed glomeruli. Control tissue (normal) from nephrectomy specimens from children with Wilm’s tumor and kidney tissue from children with CNF 9, and normal 7. The age at the time of biopsy was noted. Children with INS were divided into two groups based on clinical response to steroid therapy. The standard definition for response to steroid in INS was used [14]. Children in group A (N = 19) went into remission with steroid therapy with/without an eight-week course of cyclophosphamide. Eleven children had received steroids only, while eight had received in addition an eight-week course of cyclophosphamide. Children in group B (N = 19) were either steroid resistant (N = 12) or steroid dependent (N = 7) needing additional immunosuppressive therapy in different combinations, such as cyclophosphamide (N = 5), chlorambucil (N = 5), cyclosporine (N = 12), azathioprine (N = 1), or FK 506 (N = 1). No further subgrouping was done, as the lack of adequate numbers in various subgroups made statistical analysis superfluous. An additional four children who had progressed from MCD or DMH to FSGS were analyzed separately.

Serial 3 µm sections were obtained from the cases listed previously in this article and were air dried and heat fixed on slides. The sections were deparaffinized with xylene and iodine and were rehydrated in graded series of alcohol. The sections were treated with Target Retrieval Solution (Dako #S1700; Dako Corp., Carpinteria, CA, USA) in a steamer at 90 to 95°C for 20 minutes and then cooled for 15 minutes. The endogenous avidin and biotin activity was blocked by egg white and skimmed milk as described by Miller et al [25]. The sections were stained by immunohistochemistry on the automated Dako Autostainer 3400 using Dako’s LSAB + immunoperoxidase kit with streptavidin conjugated to horseradish peroxidase. Anti-synaptopodin, a mouse monoclonal antibody (ARP Inc., Belmont, MA, USA) was used in 1:1 dilution for synaptopodin staining. Anti-human WT-1, a mouse monoclonal antibody (Dako Corp.), was used in 1:100 dilution for WT-1 staining. The staining for WT-1 was performed for tissue quality control for fixation and processing of the archived tissue. Both positive and negative controls for synaptopodin and WT-1 were run with each run on the Dako Autostainer 3400. Diaminobenzidine was used as the chromogen.

Kidney biopsies stained by immunohistochemistry for WT-1 and synaptopodin were first examined by light microscopy. On light microscopy, WT-1 expression was scored from 0 to 2+. The characteristics of synaptopodin expression in each group were initially evaluated under light microscopy and were then further analyzed by computerized image analysis using Optimas™ software. Optimas™ is a standard image analysis program used for quantitative immunohistochemistry [26–28]. Synaptopodin stands out as a distinct brown stain against the light background of the remaining glomerulus. In each biopsy, the five maximally stained nonsclerosed glomeruli were analyzed for both luminance and percentage
glomerular area of synaptopodin staining. Five glomeruli are considered an adequate sample for diagnosis [29, 30]. In each glomerulus, for luminance, an average gray value (GV) from 0 to 255 was obtained from 320 data points, with 0 being equivalent to black and 255 equivalent to white. The lower the luminance GV, the higher is the intensity of staining. The percentage glomerular area of staining was calculated by drawing a region of interest around the glomerulus and calculating the percentage of area stained with synaptopodin within the glomerulus at a fixed color threshold. For each biopsy, the average for luminance GV and percentage glomerular area from the five glomeruli was used. To compare luminance GV and percentage glomerular area among the five groups of MCD, DMH, FSGS, CNF, and normal children, univariate analysis of variance (ANOVA) was used. Box plots show the distribution of data in each group: the minimum, 25th percentile, 50th percentile, 75th percentile, and maximum value. Two-tailed unpaired Student’s t test was used to compare groups A and B and mean age among the study groups, and paired t test for analysis of the group, which progressed to FSGS.

RESULTS

On light microscopy, WT-1 and synaptopodin expression were absent in areas of sclerosis. In nonsclerosed glomeruli, WT-1 expression was 2+ in all normal, MCD, DMH, FSGS, and CNF tissue specimen. In normal children, the morphology of synaptopodin expression was intense, linear along the glomerular basement membrane, thick with very minimal segmental interruption and minimal granulation (that is, granular pattern in staining; Fig. 1A). In MCD, the qualitative expression of synaptopodin was similar to normals but was weaker in intensity, and like in normals, it was linear along the glomerular basement membrane; however, at times attenuated in thickness with minimal segmental interruption and minimal granulation (Fig. 1B). In FSGS, in nonsclerosed glomeruli, synaptopodin expression was very weak with occasional glomeruli showing no expression, and the linear expression changed to more segmental pattern with marked interruption (“patchy”) and coarse granulations (Fig. 1C). In segmentally sclerosed glomerulus, the synaptopodin expression was absent in areas of sclerosis and was patchy in the rest of the glomerulus. The pattern for DMH was in between MCD and FSGS. In CNF, the synaptopodin expression was extremely weak (Fig. 1D).

The results of synaptopodin expression by quantitative computerized image analysis, for both luminance gray value and percentage glomerular area of staining in nonsclerosed glomeruli in the five groups, and the mean ± SD for age are shown in Table 1. Children with FSGS were significantly older, and those with CNF were significantly younger than controls. The distribution of data of synaptopodin expression in normal, MCD, DMH, FSGS, and CNF children is shown as box plots for luminance GV in Figure 2A and percentage glomerular area in Figure 2B. The results of univariate ANOVA of the data presented in Figure 2 are shown in Tables 2 and 3, respectively. Synaptopodin was significantly less expressed in MCD, DMH, FSGS, and CNF children compared with normal (P < 0.0001 for both luminance and area). Among the INS entities, synaptopodin expression decreased in order for both luminance and percentage glomerular area from MCD to DMH to FSGS, reaching statistical significance between MCD and FSGS (P =
Table 1. Age of patients and synaptopodin expression on computerized image analysis using Optimas® software for luminance on a gray scale (GV) and percentage glomerular area stained for synaptopodin

<table>
<thead>
<tr>
<th></th>
<th>Normal (N = 7)</th>
<th>MCD (N = 18)</th>
<th>DMH (N = 7)</th>
<th>FSGS (N = 13)</th>
<th>CNF (N = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years</td>
<td>3.5 ± 1.9</td>
<td>5.2 ± 3.5</td>
<td>5.3 ± 3.6</td>
<td>9.2 ± 3.8</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Normal vs. (P value)</td>
<td>—</td>
<td>0.22</td>
<td>0.26</td>
<td>0.001</td>
<td>0.0008</td>
</tr>
<tr>
<td>Luminance GV</td>
<td>78.8 ± 12.2</td>
<td>129.9 ± 16.8</td>
<td>143.6 ± 23.9</td>
<td>155.8 ± 13.4</td>
<td>148.5 ± 4.6</td>
</tr>
<tr>
<td>Area percentage</td>
<td>45.7 ± 6.6</td>
<td>15.8 ± 9.6</td>
<td>11.4 ± 12.0</td>
<td>6.4 ± 6.5</td>
<td>5.6 ± 3.5</td>
</tr>
</tbody>
</table>

The lower the luminance GV, the higher is the intensity of staining. The results are expressed as mean ± SD. Abbreviations are: MCD, minimal change disease; DMH, diffuse mesangial hypercellularity; FSGS, focal segmental glomerulosclerosis; and CNF, congenital nephrotic syndrome of the Finnish type.

Table 2. Univariate analysis of variance (ANOVA) for normal, MCD, DMH, FSGS and CNF for luminance GV based on data presented in Figure 2A

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean difference</th>
<th>95% Cl of mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>MCD</td>
</tr>
<tr>
<td>Mean</td>
<td>−50.63</td>
<td>−66.91</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lower</td>
<td>−70.26</td>
<td>−90.47</td>
</tr>
<tr>
<td>Upper</td>
<td>−30.99</td>
<td>−43.34</td>
</tr>
</tbody>
</table>

Table 3. Univariate analysis of variance (ANOVA) for normal, MCD, DMH, FSGS and CNF for percent glomerular area based on data presented in Figure 2B

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean difference</th>
<th>95% CI of mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>MCD</td>
</tr>
<tr>
<td>Mean</td>
<td>29.53</td>
<td>34.99</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lower</td>
<td>18.78</td>
<td>22.09</td>
</tr>
<tr>
<td>Upper</td>
<td>40.28</td>
<td>47.89</td>
</tr>
</tbody>
</table>

Abbreviations are in Table 1.

The distribution of data in group A and B is shown as box plots in Figure 3. The luminance GV for synaptopodin expression in group A was 132.3 ± 18.8 versus group B 149.0 ± 18.9 (P = 0.01), and the percentage glomerular area for synaptopodin expression in group A was 15.5% ± 9.0% versus group B, 8.8% ± 10.3% (P = 0.039). In group A, the predominant diagnosis was MCD (N = 14), and in group B, it was FSGS (N = 12). The sensitivity and specificity for synaptopodin expression and good steroid response at luminance GV of <140 and percentage glomerular area of >12% were 68.4 and 0.001 for luminance GV and P = 0.05 for percentage glomerular area.)
DISCUSSION

Synaptopodin is a linear proline-rich protein that is intimately associated with actin microfilaments present in the foot processes of the podocytes. In a puromycin model of nephrotic syndrome, the actin cytoskeletal structure is disaggregated with onset of proteinuria and is completely restored on resolution of proteinuria [11, 12]. One would expect synaptopodin to be altered in INS because of its association with the actin cytoskeletal apparatus. We did observe a decrease in synaptopodin expression in INS with a decrease in order from MCD to DMH to FSGS. Barisoni et al reported loss of synaptopodin in collapsing FSGS and HIV nephropathy, but found no difference between normal and MCD [21]. Kemeny et al observed decrease in synaptopodin even in early stages of idiopathic FSGS but preserved in MCD [22]. In both studies, synaptopodin was reported as either absent or present, but no attempt was made to quantitate the expression [21, 22]. Indeed, in our study on light microscopy, the qualitative morphological appearance of synaptopodin in MCD was almost similar to that of normal (Fig. 1). However, the expression of synaptopodin was significantly different on quantitative computerized image analysis (Tables 1–3 and Fig. 2). It thus seems that the loss of synaptopodin expression is not an “all or none phenomenon,” as suggested in earlier studies, but may vary quantitatively from one entity to another, as well as among children with the same histology (Figs. 1 and 2). In support of our observation of the importance of quantitative analysis was the finding of intermediate expression of synaptopodin in DMH biopsies, which has long been regarded histologically and clinically as intermediate between MCD and FSGS [17]. Indeed, the synaptopodin expression was between MCD and FSGS (Table 1). Furthermore, our observation of much attenuation of synaptopodin expression in FSGS rather than complete disappearance is supported by the report by Kihara et al [23]. As in other studies, synaptopodin and WT-1, which are exclusively expressed in podocytes, were absent in areas of sclerosis following replacement of podocytes and other glomerular cells by hyaline and fibrous tissue [21, 22].

Could the observed difference in synaptopodin expression between INS and normal children be age related (Table 1)? Although children with FSGS were older, there was no significant difference between the ages of children with MCD or DMH and normal children. There was no correlation between luminance GV or percentage glomerular area and age of the children (data not shown). Furthermore, despite the difference in ages between children with FSGS and those with CNF, the expression of synaptopodin in both groups was very similar (Table 1 and Fig. 2). There was no difference between Caucasian and African American children (data not shown). Hence,
it seems unlikely that either age or race played a factor in the expression of synaptopodin.

Was the observed difference in synaptopodin expression between INS and normal children the result of loss of podocytes from the glomerulus or possibly an artifact? The loss of podocytes alone cannot explain the loss of synaptopodin expression in children with INS, as unlike children with inflammatory glomerulonephritis, loss of podocytes in the urine is not observed in children with MCD [31]. Using double staining technique, Kemeny et al showed a decrease in synaptopodin expression in FSGS without loss of podocytes [22]. Synaptopodin and WT-1 are exclusively expressed in podocytes, the former in the foot processes and the latter in the nucleus. In our study, the WT-1 expression was abundant in all five categories, suggesting that the loss of synaptopodin in INS was not the result of disappearance of podocytes. Therefore, we do not believe that the observed loss of synaptopodin expression in INS could have been an artifact.

Minimal change disease and DMH have been shown to progress to FSGS, and the three entities are believed to lie along a continuum [14–17]. In most children, MCD responds well to steroid therapy and has a benign clinical course, while FSGS responds poorly to steroid therapy and carries a guarded prognosis for chronic renal failure, while DMH has a clinical course in between the two. Thus, on scale of severity, the severity in INS increases in order from MCD to DMH to FSGS. The treatment of all three entities starts with corticosteroids. Some children have a good response to steroids or may need an additional short course of cyclophosphamide to enter remission. They are also regarded to have a better prognosis (group A). In other children who do not respond to the previously mentioned protocol, aggressive treatment with additional immunosuppressive drugs is required. The prognosis in that group is more guarded (group B). We found that children in group A had better expression of synaptopodin than those in group B (Fig. 3). Not surprisingly, group A was comprised mainly of MCD and group B of FSGS children. However, in spite of the statistical significance between groups A and B, the data could not well predict the steroid responsiveness for the individual patient. This could partially be explained by the fact that biopsies of MCD were biased toward more severe cases of MCD, as children with MCD with immediate response to steroids do not undergo a biopsy. It is thus possible that the difference between groups A and B would have been stronger if we had biopsy material from all children with MCD. The response to immunosuppression treatment in INS lies along a continuum, and hence, it is difficult to categorize them clearly. The clinical relevance and statistical strength were lost on attempts to subgroup the data further, and thus, the biopsies were grouped empirically into two groups, as discussed previously in this article. The purpose was to evaluate for any association between steroid response and synaptopodin expression. Unlike adults with INS, 24-hour proteinuria quantitation is not performed on a routine basis in children with INS, and thus, we did not have the data on 24-hour proteinuria in all children at the time of biopsy to estimate a correlation between proteinuria and synaptopodin expression. There was no difference between onset of nephrotic syndrome and timing of renal biopsy between the different groups (data not shown). Only four children in the described case series have either elevated creatinine or have progressed to end-stage renal failure, and therefore, we did not correlate synaptopodin expression and renal outcome as the sample size was too small to make any statistical analysis.

Children with INS may progress from MCD or DMH to FSGS. It would be advantageous to have a marker by which one could predict FSGS as early as the initial biopsy that still shows findings of MCD or DMH. In four children who upon their renal biopsies progressed from MCD or DMH to FSGS, synaptopodin expression failed to predict FSGS on their initial biopsy, as its expression on those biopsies was closer to that observed in MCD rather than FSGS. The sample size was too small to reach a definite conclusion, and therefore, the predictive accuracy of this method cannot be ruled out completely and needs to be investigated further.

As discussed previously in this article, the severity in INS increases in order from MCD to DMH to FSGS. In our study, we observed the following: (1) synaptopodin expression decreased significantly in order from normal to MCD to DMH to FSGS; (2) synaptopodin expression decreased significantly in order from normal to group A to group B; and (3) in children who progressed from MCD or DMH to FSGS, synaptopodin expression was lower on final biopsy with FSGS compared with the initial biopsy. These findings cumulatively suggest that synaptopodin expression decreases with increasing severity of INS. The disease process in MCD, DMH, and FSGS is believed to have an immunologic basis, which has still not been well elucidated. The characteristic changes of INS are seen in the podocyte cell, and both albuminuria and FSGS can be produced in vivo by isolated podocyte injury in experimental animals [32, 33]. The decrease in synaptopodin expression in podocytes indirectly reflects the magnitude of damage suffered by the podocyte cell with increasing severity in INS.

To test the hypothesis of whether the alteration in synaptopodin expression is a generalized podocyte cell phenomenon and not necessarily related to an immunologic injury in INS or to immunosuppressive treatment, we evaluated synaptopodin expression in CNF, a nonimmune podocyte disease, which is due to mutation in the nephrin protein and is not treated with immunosuppressive medications [18, 19]. We found synaptopodin expression to be decreased also in CNF. Shih et al have
recently described a CD2-associated protein (CD2AP) that anchors nephrin to the actin cytoskeleton in the foot process in mice [34]. In CD2AP knockout model, the mice die of renal failure with changes resembling CNF in the glomerulus [34]. In addition, a decrease in mRNA for nephrin has been observed in MCD and puromycin model [35, 36]. We hypothesize that nephrin, CD2AP, and actin cytoskeletal apparatus are interlinked; changes in one protein leads to a cascade of changes in associated proteins in the podocyte foot process leading to loss of synaptopodin in CNF.

In summary, synaptopodin expression declines with increasing severity of INS, as suggested by the decrease in expression from MCD to DMH to FSGS as well as its lesser expression associated with a worse response to steroid therapy. Synaptopodin expression could not predict FSGS on the initial biopsy in children who progressed from MCD or DMH to FSGS. The loss of synaptopodin expression is not specific for INS alone but also is seen in CNF. Thus, it seems that synaptopodin expression is not a part of primary pathophysiological mechanism, but rather is a secondary phenomenon that reflects the magnitude of damage. We conclude that synaptopodin has a potential to be used as a marker to study the alteration in podocyte cells and possibly to predict steroid response in INS, although larger studies are needed to confirm our observations. Furthermore, our study indicates that quantitative analysis of synaptopodin expression may yield different results than qualitative-only analysis.

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