Expression of Toll-like receptor 9 in renal podocytes in childhood-onset active and inactive lupus nephritis

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Abstract

Background. Childhood-onset systemic lupus erythematosus (SLE) is frequently complicated with lupus nephritis (LN), which is characterized by the deposition of DNA-containing immune complex to the glomerulus. Toll-like receptor 9 (TLR9), capable of recognizing the microbially derived CpG oligonucleotide, plays a crucial role in the innate immunity. TLR9 is also assumed to be related to the aetiology of SLE in the recognition of anti-DNA antibody-containing immune complex, but this remains controversial. We conducted a study to elucidate the association between TLR9 and LN in childhood-onset SLE.

Methods. We compared the expression and localization of TLR9 and the slit membrane-related protein in the biopsied kidney sample by immunostaining in four children with active or inactive LN. We also evaluated their laboratory findings, such as anti-DNA antibody, complement and proteinuria at biopsy, to assess the correlation to the findings of the immunostaining.

Results. TLR9 is not expressed in a normal control kidney. However, TLR9 develops in podocytes only in active LN but disappears in remission. Meanwhile, the slit membrane-related proteins such as nephrin, podocin and synaptopodin in podocytes express clearly and uniformly in remission, but their expression is markedly diminished in active LN, which results in podocyte injury. When TLR9 is expressed in podocytes, all the patients simultaneously showed hypocomplementaemia, high titre of anti-double-stranded DNA (dsDNA) antibody and proteinuria.

Conclusion. Injured podocytes in active LN express TLR9. This expression could be associated with proteinuria and increased anti-dsDNA antibody. This is the first report indicating that TLR9 is involved in the aetiology of LN and that it may play some role in podocyte injury.

Keywords: childhood-onset systemic lupus erythematosus; lupus nephritis; podocyte; slit membrane; Toll-like receptor 9

Introduction

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease characterized by production of a number of antinuclear antibodies such as double-stranded DNA (dsDNA) antibody. Lupus nephritis (LN) is associated with a high incidence of childhood-onset SLE, and it affects prognosis [1,2].

Toll-like receptors (TLRs) play a major role in innate immunity. Human TLRs have 10 family members, each recognizing distinct pathogen-associated molecular patterns (PAMPs) [3,4]. When TLRs recognize PAMPs, MyD88 in the cytoplasmic compartment is activated, and nuclear localization of nuclear factor-kappa B is promoted, which leads to the production of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon-γ (IFN-γ) [3]. Of the TLRs, TLR9 specifically recognizes CpG motif-containing DNA (CpG DNA), a hypomethylated form of DNA typical of bacteria and DNA viruses [5]. TLRs that recognize the pathogen-derived nucleic acid such as TLR7 and TLR9 can also respond to host-derived nucleic acid [6]. Immune complexes (IC) containing DNA or CpG DNA isolated from the sera of patients with active LN can cause aberrant TLR9 activation and the release of Th1-like cytokines or chemokines, or both, from plasmacytoid dendritic cells and autoreactive B cells [6-8]. An increase in serum levels of TNF-α and IL-6 are observed in active SLE [9,10]. IFN-γ and TNF-α are expressed in glomeruli in human active LN [11].

Recently, an association between TLR9 and LN has been reported in a murine lupus model and in human lupus, which indicates the possibility of a crosstalk between innate immunity and autoimmunity. For instance, TLR9 mRNA was detected in the kidneys of MRL1pr1pr mice but not in the non-nephritic kidneys of wild-type MRL mice [12,13]. Furthermore, anti-DNA autoantibody production was impaired in TLR9 gene-knockout lupus-prone mice [14]; and CpG DNA exacerbated glomerulonephritis in a murine lupus model through activation of TLR9.
Recently, Papadimitraki et al. reported that TLR9-expressing cells in the glomeruli in adult patients with LN, but identification of TLR9-expressing cells has not been performed [17]. As above, many reports suggest that TLR9 might have a strong association to the pathogenesis of SLE. However, it is still unclear how, when and where TLR9 is involved in the development of LN. We conducted a study to elucidate the association between TLR9 and development of LN in childhood-onset SLE.

Materials and methods

To elucidate the association between TLR9 and human LN, we have conducted an immunohistochemical investigation of the expression and localization of TLR9 in renal samples biopsied from patients with childhood-onset lupus. We also analyzed the changes in the expression of TLR9 in both active and inactive LN. Additionally, we examined the expression of slit membrane-related proteins, such as nephrin, podocin and synaptopodin, because damage to the slit membrane is a cause of proteinuria or haematuria in acquired glomerular diseases [18,19].

Tissues

Four patients (two males and two females) included in this study with LN were followed up at Yokohama City University between September 1995 and December 2008. Their onsets of SLE were between 11 years and 1 month and 13 years and 11 months. Initial renal biopsies were performed on three patients within 1 month from onset, but one patient was biopsied (Patient 4) at 3 months from onset because of necessity of intensive care. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication. Patients were chosen for this study because they were diagnosed as having diffuse proliferative LN, the severest form of LN. All patients had the second biopsy at remission of LN to evaluate the therapeutic effect of remission induction therapy and possibly taper the steroid medication.

Immunofluorescence assay

The renal biopsy samples from the four patients summarized in Tables 1 and 2 were stained with mouse anti-nephrin antibody and rabbit anti-podocin antibody. Monoclonal anti-nephrit 50A9 antibody and polyclonal anti-podocin antibody 2191 were kind gifts from Dr. Yan K (Kyorin University, Tokyo, Japan). Double-staining immunofluorescence assay was performed with anti-synaptopodin antibody (mouse monoclonal IgG1, G1D4, Progen, Heidelberg, Germany) and anti-TLR9 antibody (mouse monoclonal IgG2a, 565, Abeam, Cambridge, UK). For immunofluorescence microscopy, a renal tissue was quickly frozen in n-hexane and cooled at −70°C. Histologically, each frozen section from the biopsied tissue sample contained at least four glomeruli (range: 4–7, average: 5). The 10-μm-thick sections were cut by cryostat and fixed with chilled acetone for 10 min. Next, the sections were washed twice with PBS and incubated with anti-TLR9 antibody diluted 1:100 with 1% bovine albumin for 30 min at room temperature. After being washed twice with PBS, the sections were incubated for 30 min at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG2a antibody (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1:200 with 1% bovine albumin. They were washed twice again with PBS and incubated with anti-synaptopodin antibody diluted 1:100 with 1% bovine albumin for 30 min at room temperature. After being washed twice with PBS, the sections were incubated for 30 min at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG2a antibody (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1:200 with 1% bovine albumin for another 30 min at room temperature. Finally, they were washed once with PBS and mounted. The sections were examined with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

We additionally assessed the relation between TLR9 expression and disease activity. For distribution and quantification purposes, a semiquantitative score was assigned for glomerular TLR9, synaptopodin, nephrin and podocin expression according to the following patterns: −, negative; +, weakly positive; ++, strongly positive.

Results

Immunohistochemistry of TLR9 and synaptopodin in childhood-onset LN

In the normal kidney (n = 3), TLR9 was almost undetectable in either the tubular or the glomerular cells (Figure 1A). By contrast, in active LN, expression of TLR9 was observed in some glomerular cells (n = 7) (Figure 1B). Some TLR9-positive cells were found inside the glomerular capillaries, but most were outside and along the glomerular capillaries. These TLR9-positive cells were assumed from their localization to be glomerular epithelial cells (podocytes). Some tubular cells were weakly positive with TLR9 in LN (Figure 1B).

A normal kidney, used as a control (n = 3), showed uniformly strong staining of synaptopodin in podocytes (Figure 1D). In contrast, synaptopodin was stained weakly and inconsistently in active LN (n = 7) (Figure 1E). The

The sections were thoroughly deparaffinized and rehydrated using standard protocols. Briefly, for antigen retrieval, the sections immersed in 10 mmol/L sodium citrate buffer, pH 6.0, were autoclaved at 120°C for 15 min and cooled at room temperature. The sections were then treated with 0.3% H2O2 for 30 min at room temperature to inactivate endogenous peroxidase activity. Then, they were incubated with anti-TLR9 antibody diluted 1:100 with an Antibody Diluent (Dako, Copenhagen, Denmark) for 1 h at room temperature. To stain synaptopodin, more sections were incubated with pre-diluted anti-synaptopodin antibody for 1 h at room temperature. Next, they were washed three times with phosphate-buffered saline (PBS) and incubated for 1 h with EnVisionTM Detection Reagent Peroxidase Rabbit/Mouse (Dako, Copenhagen, Denmark). After further washings with PBS, peroxidase activity was detected using H2O2/diaminobenzidine substrate solution, and the sections were counterstained with haematoxylin before dehydration and mounting.
**Table 1.** Laboratory parameters and medications of patients when renal biopsy was performed

<table>
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<th>Case</th>
<th>Patient 1 (female)</th>
<th>Patient 2 (male)</th>
<th>Patient 3 (male)</th>
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<td>17Y 9M</td>
<td>11Y 9M</td>
<td>12Y 9M</td>
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<td>18</td>
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<td>×40</td>
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<td>AZP</td>
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</table>

ESR₆₀, erythrocyte sedimentation rate of 60 min; TP, total protein; Alb, serum albumin; BUN, blood urea nitrogen; s-Cr, serum creatinine; MPT, methylprednisolone pulse therapy; PSL, prednisolone; MZB, mizoribine; AZP, azathioprine; IVCY, intravenous cyclophosphamide; PE, plasma exchange; HD, haemodialysis; APS, antiphospholipid antibody syndrome; Y, years; M, months.
degree of expression of synaptopodin in the glomeruli varied from a marked decrease to almost complete disappearance of protein expression. Based on the localization of synaptopodin-positive cells, the TLR9-positive glomerular cells observed in active LN are considered to be podocytes.

**Immunofluorescence assay of TLR9 and synaptopodin and its association to clinical condition**

To ascertain whether TLR9 is expressed in podocytes in active LN, double immunofluorescent staining with TLR9 and synaptopodin was performed \( (n = 4, \text{Tables 1 and 2}) \). In the normal kidney, expression of TLR9 was negative in both glomerular and tubular cells, as shown in Figure 1A (and Figure 2A). Synaptopodin was stained uniformly in the controls, as seen in Figure 1D (and Figure 2B). Expression of TLR9 in the glomeruli was observed at the onset of lupus (Figure 2D) in all four patients. The overlaps of TLR9 and synaptopodin indicated that TLR9 was specifically expressed in the podocytes (Figure 2F). TLR9 expression in the glomeruli disappeared on remission (Figure 2G) in all four patients but developed again when a relapse occurred in Patient 3 (Figure 2J). Meanwhile, the tubular cells showed weak but constant expression of TLR9 in all patients with SLE (Figures 2D, 2G, 2J).

The fluorescence intensity in all cases scored from − to ++ according to the following scores: −, negative; +, weakly positive; ++, strongly positive.

### Table 2. Immunohistological results

<table>
<thead>
<tr>
<th>Case</th>
<th>Patient 1 (female)</th>
<th>Patient 2 (male)</th>
<th>Patient 3 (male)</th>
<th>Patient 4 (female)</th>
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<tbody>
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<td>Condition</td>
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<td>Remission (active)</td>
<td>Onset (active)</td>
<td>Remission (active)</td>
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<td>Second biopsy</td>
<td>First biopsy</td>
<td>Second biopsy</td>
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<tr>
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<td>Synaptopodin</td>
<td>Nephrin</td>
<td>Podocin</td>
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<td>++</td>
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<td>Fluorescence intensity</td>
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<td>−</td>
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</table>

The fluorescence intensity in all cases scored from − to ++ according to the following scores: −, negative; +, weakly positive; ++, strongly positive. ISN/RPS 2003 classification, International Society of Nephrology/Renal Pathology Society classification of lupus nephritis 2003.

Fig. 1. Immunohistochemistry for TLR9 and synaptopodin in active LN and normal control. In active LN (B, C, arrow, arrowhead; Patient 2) but not in the normal control (A), some glomerular cells strongly expressed TLR9. Some tubular cells were also weakly stained in active LN (asterisk in B). Most of the TLR9-positive cells localized adherent to outside of glomerular capillaries (B and C arrow). In the normal control kidney, synaptopodin was strongly and evenly stained (D) whereas weakly and irregularly stained in active LN (E; Patient 2).
Expression of synaptopodin was slightly decreased in active LN (Figures 2E, K) but recovered in remission (Figure 2H). This change was observed in all glomeruli in all four patients (Table 2).

At the time of onset, all patients showed high titres of anti-dsDNA antibody and hypocomplementaemia (Table 2). The nephritis of all patients was classified in class IV of the INS/RPS 2003 classification [20] (Table 2), and remarkably, the tissue of all patients showed the intracapillary cellular proliferation. In remission, a decrease of anti-dsDNA antibody and recovery from hypocomplementaemia was observed in all of the patients (Table 2).

Immunofluorescence assay of nephrin and podocin in glomeruli

In the normal kidney, both nephrin (Figure 3A) and podocin (Figure 3B) were demonstrated strongly and were distributed evenly among the glomeruli. In contrast, the immunostaining intensity with nephrin and podocin was reduced non-uniformly in all the patients’ specimens at the onset of LN (Figures 3C, D). Three of the four patients developed proteinuria at the onset (Table 1). In remission, all of the patients showed recovery of the staining intensities and patterns of nephrin and podocin in a similar manner to the normal controls (Figures 3E, F). In Patient 3, the expression of both nephrin and podocin was weak and non-uniform in the glomeruli at the time of recurrence (Figures 3G, H) (Table 2). Attenuation of the staining of nephrin and podocin was consistent to the presence of proteinuria.

Discussion

Although this report is a small cohort study about childhood-onset LN, this is the first report to show that injured
Podocytes in active LN develop TLR9 and that this expression was reversible dependent on the disease activity. TLR9 has been suspected to have some association to the aetiology of LN. Benigni et al. reported that tubular cells were positively stained with TLR9 in human LN and in the NZB × NZW mouse model, but not in glomerular cells [21]. Moreover, Papadimitraki et al. reported that TLR9 was expressed in both glomerular and tubular cells in 6 of 12 adult patients with LN [17]. However, the type of cells expressing TLR9 in glomeruli has been unknown. Five of six patients expressing TLR9 in glomeruli had a high activity index of LN and increase of anti-DNA antibody, but the six with inactive LN did not express TLR9 in glomeruli, which is consistent with our results (Table 2). These findings suggested that the presence of the anti-DNA antibody could be associated with TLR9 expression in the glomeruli and activity of LN. In addition, we ascertained TLR9 expressed to the podocyte for the first time, but they merely reported that TLR9 is expressed in cytoplasm and predominately perinuclear lesions.

In our immunohistochemistry of active LN, most of the TLR9-positive cells which were localized adherent to the outside of the glomerular capillary walls, were thought to be podocytes because of their specific localization. Double immunofluorescent staining with TLR9 and synaptopodin clearly demonstrated that podocytes express TLR9 in active LN. A few of the TLR9-positive cells in the glomeruli or tubules may be infiltrating lymphocytes, monocytes and macrophages.

Up until now, knowledge of innate immunity in podocytes is particularly limited. There have been two reports about TLR4 being expressed on the podocyte's cell surface [22,23]. In a mouse model, activation of TLR4 by lipopolysaccharide (LPS) produced chemokines and induced podocyte injury [22]. B lymphocytes and dendritic cells are activated and trigger innate immune responses by CpG

Fig. 3. Immunofluorescence staining for nephrin and podocin in LN and normal control. In active LN (C, D, G, H; Patient 3), nephrin and podocin were stained non-uniformly and weakly. In contrast, in remission (E, F; Patient 3) and normal control kidney (A, B), nephrin and podocin were strongly and uniformly distributed.
DNA through TLR9, and produce various cytokines and chemokines [4,24]. Circulating DNA-containing IC is deposited directly in the glomerular capillary wall in a mouse model [25] and in human LN, as could be recognized from the presence of TLR9 in podocytes. In the present study, we found that a high titre of anti-dsDNA antibody was observed in the patients’ sera when TLR9 was expressed in the podocytes.

TLR9 signalling plays a crucial role in controlling bacterial and viral infections, but recent studies using autoimmune-prone mouse models have suggested that TLR9 is associated with the development of autoimmune diseases [3,26,27]. DNA/nucleosome/anti-DNA antibody complex can bind to TLR9, with subsequent activation of B lymphocytes that favours the development of autoimmunity [8,26]. These cells produce pro-inflammatory cytokines and type I and II interferons. Increased expression of TLR9 on peripheral blood cells from patients with active SLE was significantly correlated with the decrease of the CH50 titre and the SLE Disease Activity Index (SLEDAI) to TLR9, and induced the production of anti-DNA antibody by TLR9–CpG ligation [28]. As a result, several studies were conducted to regulate or treat autoimmune diseases, especially SLE, via TLR modulation [29–31]. Blockade of TLR9 and TLR7 ameliorates LN in MRL/lpr mice and NZB × NZW mice [32,33]. Modulation of TLR9 in podocytes and lymphocytes may be a future treatment option for LN.

Proteinuria clinically reflects damage to the podocytes, and in particular, the breakdown of slit membrane function. In this study, we also confirmed that the expression of the slit membrane-related proteins nephrin, podocin and synaptopodin is attenuated in active LN. These findings suggesting podocyte injury have been reported in many acquired glomerular diseases [18,19,34–37]. We firstly demonstrate the recovery from the attenuated expression of these slit membrane-related proteins in conjunction with the remission of SLE. Our study revealed that TLR9 is expressed in the injured podocytes. From the viewpoint of innate immunity, this finding is very interesting. DNA-containing IC may be recognized as a ‘danger signal’ like other PAMPs. TLR9 could be developed by DNA-containing IC. However, further study is needed to elucidate whether the TLR9 expressed in injured podocytes causes inflammation or degrades DNA-containing IC, which may lead to a new therapeutic approach to SLE.

Acknowledgements. This work was supported by Grant-in-Aid for Scientific Research (C) (no. 19591260) from Ministry of Education, Culture, Sports, Science and Technology, Japan. We are also grateful to Mr. C.W.P. Reynolds for his careful linguistic assistance with this manuscript.

Conflict of interest statement. None declared.

References
Behavioural abnormalities in children with nephrotic syndrome

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Abstract

Background. Glucocorticoid therapy in children with nephrotic syndrome can lead to many adverse effects including behavioural problems. The present study was undertaken to assess the changes in individual behaviour among different sub-groups of patients with idiopathic nephrotic syndrome (INS) and also to find out the relationship, if any, between different behavioural problems with cumulative dose of steroid therapy.

Methods. This was a prospective hospital-based study. We assessed behavioural patterns in 131 children and adolescents with steroid-responsive INS aged 1.5–15 years. Fifty healthy children matched for age and gender were included to serve as controls. The Achenbach Child Behaviour Checklist was used to assess individual behaviour. Patients were sub-grouped according to age (1.5–5 and 6–15 years) and disease status (first attack before and after 12-week prednisolone, infrequent relapser, frequent relapser/steroid-dependent).

Results. All groups had significantly elevated mean behavioural abnormality scores for dimensions assessed in both groups. Total and individual behavioural scores showed close associations with cumulative prednisolone dose in both groups.

Conclusions. It is evident that nephrotic syndrome patients should be given due consideration in clinical practice for behavioural abnormalities especially after steroid therapy.

Keywords: behavioural problems; nephrotic syndrome; steroid therapy

Introduction

Nephrotic syndrome is one of the most common renal disorders in the paediatric population. Approximately 90% of cases are caused by an immunological dysregulation sensitive to glucocorticoid treatment. While these patients generally have a favourable long-term prognosis, 70% of cases suffer a relapsing course [1]. The therapeutic benefits of glucocorticoids are accompanied by significant side effects. Attention to steroid toxicity in nephrotic syndrome has been focused mainly on physical side effects such as growth retardation, obesity, hirsutism, cataract, etc., but the neuropsychological side effects are usually underestimated. Studies in children with cancer [2] and asthma

Behavioral changes in nephrotic syndrome


Received for publication: 12.5.09; Accepted in revised form: 22.1.10

doi: 10.1093/ndt/gfq097
Advance Access publication 2 March 2010

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