

Original Article

Morphological analyses of nephrin expression in progressive glomerulonephropathy of common marmosets

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Abstract: In this study, we focused on nephrin, one of the key molecules within the slit diaphragm of podocytes, as although there have been reports on its expression in humans and rats, their presence in common marmosets has not been reported. We investigated nephrin expression and changes in glomeruli, depending on the development of spontaneous progressive glomerulonephropathy in common marmosets. Nineteen common marmosets at two to ten years of age were evaluated. The kidney was examined by microscopy with hematoxylin and eosin and immunohistochemical staining for nephrin. The lesions were classified into three grades according to a renal lesion grading system reported previously. The nephrin-positive area was measured by morphometric analysis, and the nephrin-positive ratio was calculated. Nephrin expression was observed along the glomerular capillary loop in a continuous linear pattern in renal lesion grades 0 to 2 and either discontinuous linear or coarse granular pattern in grade 3. Nephrin expression tended to decrease significantly depending on the grade of renal lesions. Alteration in nephrin expression has been suggested to play an important role in the progression of renal lesions. (DOI: 10.1293/tox.2020-0056; J Toxicol Pathol 2021; 34: 83–88)

Key words: nephrin, marmoset, glomerulonephropathy, kidney, pathology

Introduction

Spontaneous progressive glomerulonephropathy in common marmosets (*Callithrix jacchus*) is known to occur frequently beginning at two years old, with lesion severity progressing with age. We previously reported primary and progressive lesions of nephropathy in common marmosets¹. Primary glomerular changes of an early stage nephropathy are characterized by the effacement of podocyte foot processes and partial thickening of the glomerular basement membrane, presumed to cause leakage of protein from the glomeruli, and can be observed by ultramicroscopy but not by light microscopy^{1–3}. Furthermore, immunoglobulin plays an important role in the progression of nephropathy^{1, 2}. IgM

is deposited onto the mesangium at the early stage of glomerulonephropathy, and following the first deposition of IgM, the deposit area expands to the whole glomerulus, and IgA and IgG are deposited with progression². Hyaline casts suggesting leakage of protein from the glomeruli can be observed by light microscopy with the progression of glomerulonephropathy⁴. Proteinuria exacerbates the progression of tubulointerstitial lesions in common marmosets⁴, as in humans^{5, 6}, and, in a recent study of the glomeruli, proteinuria could be induced by disorders of the slit diaphragm, thereby linking adjacent foot processes (pedicels) of podocyte. The slit diaphragm is an intercellular junctional complex present between adjacent foot processes, and some molecules related to its formation have been identified³. Decrease in the expression of these molecules or dysfunction of the slit diaphragm were thought to be related to the progression of proteinuria³. Nephrin, one of the key molecules expressed in the slit diaphragm of podocytes, is a glycoprotein consisting of 1241 amino acids and is a well-known biomarker used for monitoring the disorder of slit diaphragms^{7–10}. Molecules related to slit diaphragms have not been studied in the nephropathy of common marmosets, although the alteration of podocyte foot processes and progression of proteinuria are known to occur in them. Moreover, there have been reports

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on the expression of nephrin in humans and rats^{7–12} but not in marmosets. Therefore, we investigated nephrin expression in glomeruli and changes in nephrin expression, depending on the lesion progression, by immunohistochemical analyses.

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Central Institution for Experimental Animals (CIEA; Kawasaki, Japan) and was carried out in strict accordance with the Regulations for Animal Experimentation of the CIEA based on the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). Nineteen common marmosets (11 males and 8 females) from CLEA Japan Inc. (Tokyo, Japan) were evaluated. Their ages ranged from 2 to 10 years at the time of necropsy. The animals were housed in cages in an animal room maintained at a temperature of $26 \pm 3^\circ\text{C}$ and a humidity of $55 \pm 20\%$. They were fed with CMS-1M (CLEA Japan Inc., Tokyo, Japan) and tap water. The animals were humanely sacrificed under anesthesia with pentobarbital sodium due to various causes of moribund (such as syntexis, curvature of lumbar vertebrae, and mandibular joint dislocation etc.). Urinalysis was not performed because urine could not be sampled.

Histopathology

The kidneys were perfused with saline, followed by 4% paraformaldehyde, and then fixed in 10% neutral-buffered formalin for microscopic examination. The specimens were embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin (HE). The lesions were classified as glomerular, tubular, and interstitial lesions based on published criteria¹. Nephropathy grades were divided into four groups: grade 1 (3 males and 1 female), grade 2 (3 males and 5 females), grade 3 (1 male and 3 females), and grade 4 (3 males) (Table 1).

For immunohistochemistry, a monoclonal antibody against the extracellular domain of human-origin nephrin (1:1,000, Nephrin g-8, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the primary antibody. The 5- μm sections were deparaffinized, rehydrated, treated with 3% hydrogen peroxide at room temperature for 5 min, and then heated in an autoclave at 121°C for 20 min in target retrieval solution (pH 6.0, Dako, Glostrup, Denmark). After cooling, the sections were incubated with the primary antibody at 4°C overnight. Peroxidase-conjugated anti-mouse and anti-rabbit IgG polymer (Histofine Simple Stain Rat MAX-PO(MULTI), Nichirei Bioscience Inc., Tokyo, Japan) was used as the secondary antibody for the polymer-based method¹³. The sections were incubated with the secondary polymer antibody at room temperature for 30 min and the reacting products visualized using 3,3'-diaminobenzidine as a chromogen. Counterstaining was performed with hematoxylin. For the negative control, the primary antibody was omitted.

Table 1. Renal Lesion Grade and Nephrin-positive Ratio of Each Individual Animal

Animal number	Age	Sex	Glomerular lesion score	Hyalin cast score	Interstitial fibrosis	Regeneration tubuli	Interstitial cell infiltration	Total score	Renal lesion grade	Number of glomeruli examined	Nephrin positive ratio
3	2	M	1	0	0	0	0	1	0	19	6.84
2	4	M	1	1	0	0	1	3	0	17	6.44
4	2	F	1	0	0	0	0	1	0	20	5.08
1	6	M	1	0	0	0	0	1	0	18	4.27
7	4	M	2	1	0	0	2	5	1	18	5.69
11	2	F	2	1	0	0	1	4	1	16	5.46
12	2	F	2	1	0	0	1	4	1	26	5.08
10	2	F	2	1	0	0	1	4	1	24	4.92
6	3	F	2	1	1	0	1	5	1	19	3.78
9	2	F	2	1	0	1	0	4	1	24	3.56
5	8	M	2	1	0	0	1	4	1	19	2.26
8	4	M	2	1	0	0	2	5	1	24	1.19
16	2	M	2	2	1	0	2	7	2	20	6.71
14	4	F	3	2	0	0	1	6	2	19	4.36
15	2	F	3	1	0	0	2	6	2	22	2.61
13	7	M	2	2	1	1	1	7	2	20	1.58
18	10	M	3	2	1	2	1	9	3	28	3.67
17	7	M	2	2	2	1	1	8	3	27	2.17
19	6	M	2	2	1	2	2	9	3	24	1.72

Total score 1–3, renal lesion grade (RLG) 0; total score 4–5, RLG 1; total score 6–7, RLG 2; total score 8–10, RLG 3. The glomeruli with the hilum portion were submitted for examination, and 16 to 28 glomeruli were captured on each slide. The nephrin-positive ratio is calculated as nephrin-positive area over the glomerular area.

Morphological analysis

For morphological analysis, the glomeruli with the glomerular hilum were submitted for examination, and 16 to 28 glomeruli were captured on each slide (Table 1) using a whole slide scanner (Aperio AT2, Leica Microsystems Inc., Buffalo Grove, IL, USA) and a corresponding pathology slide viewing software (Aperio ImageScope, Leica Microsystems Inc.) at the same resolution and image size. Nephryn-positive and whole glomerular areas were calculated with an image analysis software (Image Pro V10, Mediacybenetics Inc., Rockville MD, USA) by drawing a region of interest around the glomerulus, according to a previous report¹⁴. The whole glomerular area was defined as the area that can be distinguished from the white-colored background. The color thresholds for calculating nephryn-positive areas were determined according to each staining condition. The thresholds for red, green, and blue were 37–67, 31–77, and 32–88, respectively. The nephryn-positive ratio was calculated by dividing the measurements of the nephryn positive area over the glomerular area. Statistical analysis was performed using the Jonckheere test (significance level: 5%, one-side test) to evaluate the trend between nephropathy grade and expression of nephryn.

Results

The pathological scores and grades of individual renal lesions are shown in Table 1. Same as reported in our first report¹, the lesion grade tended to increase with age and there was no obvious sex difference. The results of immunohistochemical staining of nephryn are shown in Fig. 1, and the nephryn-positive ratio is shown in Fig. 2 and Table 1. Among the scores and grades in Table 1, the renal lesion grade, derived from the total score of each renal lesion, and hyaline cast score were used for comparison with the nephryn-positive ratio (Fig. 2 and 3). Individual cases in Table 1 are shown in ascending order of renal lesion grade. Nephryn expression was observed along the glomerular capillary loop in a continuous linear pattern in grades 0 to 2 (Fig. 1A–C) and a discontinuous linear or coarse granular pattern in grade 3 (Fig. 1D). In the negative control sample,

no positive reaction for nephryn was observed in any site.

The comparison of the nephryn-positive ratio to renal lesion grade is shown in Fig. 2. In renal lesion grade 0 cases, the mean \pm standard deviation (SD) of nephryn-positive ratio was $5.66 \pm 1.18\%$, and the maximum, minimum, and median values were 6.84%, 4.27%, and 5.76%, respectively. In grade 1 cases, the mean \pm SD was $3.99 \pm 1.61\%$, and the maximum, minimum, and median values were 5.69%, 1.19%, and 4.35%, respectively. In grade 2 cases, the mean \pm SD was $3.82 \pm 2.25\%$, and the maximum, minimum, and median values were 6.71%, 1.58%, and 3.49%, respectively. In grade 3 cases, the mean \pm SD was $2.52 \pm 1.02\%$, and the maximum, minimum, and median values were 3.67%, 2.17%, and 2.17%, respectively. Collectively, individual differences in nephryn expression were small in cases of grade 0, but large in cases of grade 1 or higher, and low positive expression was sporadically detected. Nephryn expression tended to decrease depending on the grade of renal lesions (Fig. 2). There was a significantly lower trend between renal lesion grade and nephryn-positive ratio according to the Jonckheere test ($p=0.0166$). The hyaline cast score in comparison to the renal lesion grade is shown in Fig. 3. In cases with a hyaline cast score of 0, the mean \pm SD of nephryn-positive ratio was $5.40 \pm 1.32\%$, in score 1 cases, it was $4.10 \pm 1.70\%$, and in score 2 cases, it was $3.37 \pm 1.98\%$. There was a significantly lower trend between the hyaline cast score and nephryn positive ratio according to the Jonckheere test ($p=0.0654$).

Discussion

A decrease in the nephryn was correlated with the progression of renal lesions during the development of marmoset glomerulonephropathy. The anti-nephryn antibody used in this study recognized the extracellular domain at the N-terminal side. This extracellular domain interacts with NEPH1 molecules and forms a zipper-like structure within the slit diaphragm¹⁵. Localization of nephryn in normal glomeruli is along the glomerular capillary loop and the staining pattern a linear pattern of continuous fine granules both in rats and humans^{11, 12, 16–18}. The staining performed

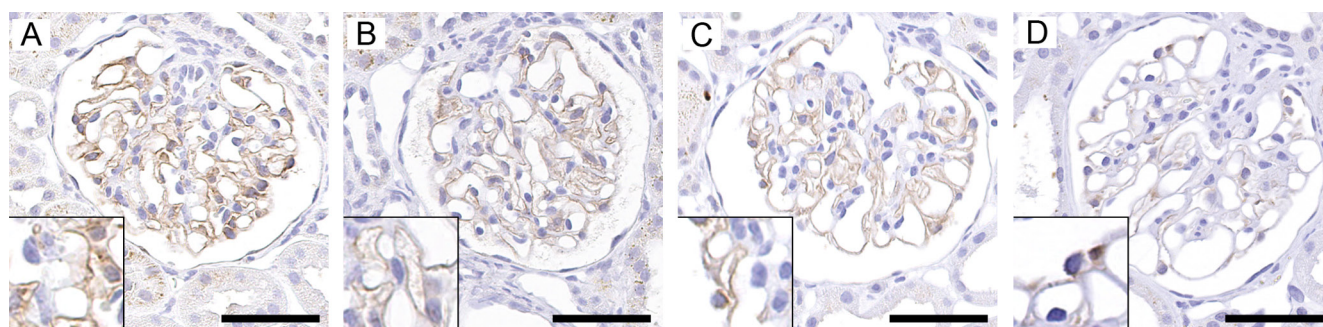


Fig. 1. Immunohistochemical stain of nephryn in the glomerulus. Nephryn expression is shown along the glomerular capillary loop in a continuous linear pattern in renal lesion grade 0 to 2 (A–C) and discontinuous linear or coarse granular pattern in renal lesion grade 3 (D). A: Renal lesion grade 0. B: Renal lesion grade 1. C: Renal lesion grade 2. D: Renal lesion grade 3. Bars = 50 μ m.

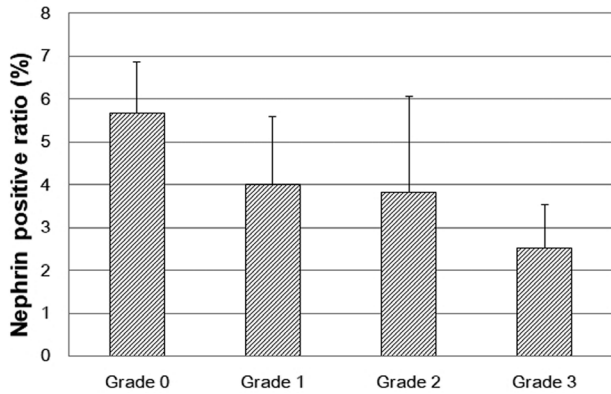


Fig. 2. Comparison of nephrin-positive ratio and renal lesion grade. Values are presented as mean and standard deviations. Nephrin expression tends to decrease depending on the renal lesion grade. There is a significantly lower trend between the renal lesion grade and nephrin-positive ratio according to the Jonckheere test ($p=0.0166$).

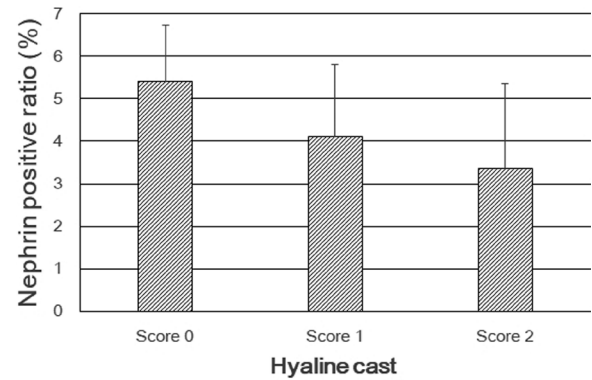


Fig. 3. Comparison of nephrin-positive ratio and hyaline cast score. Values are presented as mean and standard deviations. Nephrin expression tends to decrease depending on the hyaline cast score; however, there is no significant difference between the hyaline cast score and nephrin-positive ratio by Jonckheere test ($p=0.0654$).

in this study is appropriate; which is confirmed by the observation that the capillary walls in contact with the mesangial region were not stained, while those in contact with the podocytes were. Under abnormal conditions, such as nephritic syndrome in humans or puromycin aminonucleoside nephrosis in rats, the nephrin staining pattern in glomeruli is discontinuous and coarse granular, and the intensity of immunohistochemical staining is weaker than in normal glomeruli^{11, 16, 17}. In the present study, nephrin expression pattern in grade 0 to 2 was normal; however, the staining pattern in grade 3 was comparable to that of abnormal conditions. In a previous investigation of glomerular nephrin expression in Crl:CD(SD) rats with minimal change disease, nephrin expression was intact, although podocyte foot processes showed effacement under electron microscopy with no apparent morphological changes observed under light microscopy¹⁸. In the common renal disease of rats, chronic progressive nephropathy (CPN), it has been demonstrated that albuminuria accompanied by early-stage CPN may result in less changes in glomerular permeability from the failure to reabsorb albumin from proximal tubules¹⁹. In humans, there have been some investigations on the reduction of nephrin expression under minor changes¹⁰ or under other kidney diseases^{14, 16}. On the other hand, there has been a report on intact nephrin expression in minimal change nephrosis²⁰. The presence or absence of a change in the expression of nephrin in minimal change nephrotic disease is still being discussed. In actuality, it was difficult to confirm the increase or decrease of the antibody reaction of nephrin by visual observation in marmoset nephropathy, but it was possible to detect the change in the whole kidney by image analysis. Our investigation suggests that the abnormality of slit diaphragms of glomerulus may be involved in the progression of nephropathy in common marmosets due to a decrease in nephrin. The transformation of the positive staining pattern of nephrin, from linear to granular pattern,

has been reported in a rat animal model of nephrotic disease during the development of the lesion^{11, 16, 21}. An association between abnormalities of the slit membrane and proteinuria has frequently been reported^{22, 23}; therefore, it has been speculated that proteinuria may be caused by slit diaphragm dysfunction. In our study on marmoset nephropathy, the progression of glomerular protein permeability due to slit diaphragm dysfunction might have occurred because the morphological features of hyaline casts were observed only in the cases without tubulointerstitial changes. In this study, no statistically significant difference was observed between the score of morphological changes of hyaline casts and the decrease in nephrin expression. However, nephrin expression tends to decrease depending on the hyaline cast score, which is an indicator of protein leakage from the glomerulus.

In our previous investigation, effacement of foot processes was observed in the early stage of marmoset nephropathy and the affected foot process area was expanded depending on the renal lesion grade¹. Table 2 shows the relationship between the reduction rate of nephrin in this study and the electron microscopy findings for each renal lesion grade from a previous study. Effacement is thought to be due to a breakdown in the actin cytoskeleton of the foot processes²⁴. Nephrin is dephosphorylated when the slit membrane is constructed, and it binds to actin through CD2AP and podocin^{25, 26}. When a slit diaphragm is injured, nephrin molecules become clustered, inducing nephrin phosphorylation, actin polymerization, and the effacement of foot processes²⁶. Nephrin phosphorylation also occurs rapidly following the induction of foot process effacement in the protamine sulfate model in mice²⁷. Nephrin is a glomerular adhesion protein associated with podocyte maturation, differentiation, process formation, and signaling²⁴. Our investigation showed that there was a correlation between reduction of nephrin expression, as determined by immu-

Table 2. Relationship of Morphological Changes of Podocyte Foot Processes and Reduction of Nephrin Immunoreactivities

Renal lesion grade	Electron microscopic features of GBM and foot process of podocytes*	Nephrin reduction rate (Comparison with grade 0)
Grade 0	Not examined	100%
Grade 1	The GBM partially and slightly thickened. Effacement of foot processes was observed near the thickened GBM.	70.6%
Grade 2	The GBM was much thicker than in grade 1, and the trilaminar structure was partially unclear because the lamina rara interna and lamina densa frayed and separated. Effacement of foot processes expanded wider than in grade 1.	67.4%
Grade 3	The uneven outline of the GBM was widely observed, and the lamina densa partially laminated and reticulated. Effacement of podocyte foot processes was observed near the irregular GBM more widely than in grade 2.	44.5%

*From the investigation of our previous report (Yamada *et al.* 2013). GBM, glomerular basement membrane.

nohistochemistry, and renal lesion grade progression, suggesting an expansion of effacement of podocyte foot processes. However, it is unclear whether abnormalities in the slit diaphragm protein complex cause damage to podocyte foot processes, or if podocyte damage causes changes in slit diaphragms and nephrin reduction. Since nephrin is also involved in podocyte differentiation, it is thought that this relationship will be further clarified by data on podocyte regeneration and differentiation.

In conclusion, our results provide information on nephrin expression in progressive glomerulonephropathy in common marmosets. Morphological analysis revealed a lower trend of nephrin expression in podocytes in progressive glomerulonephropathy of common marmosets, and the alteration of nephrin expression was suggested to play an important role in the progression of renal lesions.

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