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ORIGINAL ARTICLE

Podocyte expression of nonmuscle myosin heavy chain-IIA decreases in idiopathic nephrotic syndrome, especially in focal segmental glomerulosclerosis

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ABSTRACT

Background. Previous studies have identified significant associations between the development of idiopathic focal segmental glomerulosclerosis (FSGS) and *MYH9* encoding nonmuscle myosin heavy chain-IIA (NMMHC-IIA). However, these studies focused only on the linkage of *MYH9* polymorphisms and development of FSGS. There have been no reports on pathological changes of NMMHC-IIA in

human glomerular diseases. Here we report on the precise localization of NMMHC-IIA in podocytes and changes in NMMHC-IIA expression in pathological states in rats and humans.

Methods. Immunocytochemical (immunofluorescence and immunoelectron microscopy) studies were performed to determine the precise localization of NMMHC-IIA. Expression levels of NMMHC-IIA were investigated in puromycin aminonucleoside (PAN)-treated rats; and expression levels of NMMHC-IIA and other podocyte-related proteins were investigated in glomeruli of patients with idiopathic FSGS and other heavy proteinuric glomerular diseases.

Results. NMMHC-IIA was located primarily at the cell body and primary processes of podocytes; this localization is distinct from other podocyte-related molecules causing hereditary FSGS. In PAN-treated rat kidneys, expression levels of NMMHC-IIA in podocytes decreased. Immunohistochemical analysis revealed that expression levels of NMMHC-IIA markedly decreased in idiopathic nephrotic syndrome, especially FSGS, whereas it did not change in other chronic glomerulonephritis showing apparent proteinuria. Changes in NMMHC-IIA expression were observed in glomeruli where expression of nephrin and synaptopodin was maintained.

Conclusions. Considering previous genome-wide association studies and development of FSGS in patients with *MYH9* mutations, the characteristic localization of NMMHC-IIA and the specific decrease in NMMHC-IIA expression in idiopathic nephrotic syndrome, especially FSGS, suggest the important role of NMMHC-IIA in the development of FSGS.

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) causes steroid-resistant nephrotic syndrome, which frequently progresses to end-stage renal disease (ESRD) and is one of the most serious renal disorders in both pediatric and adult patients [1]. Over the last decade, several genes responsible for the development of hereditary FSGS, such as genes encoding podocin [2], α-actinin-4 [3], TRPC6 [4] and PLCε1 [5], have been identified using a positional cloning approach. These gene products are expressed highly and specifically in glomerular epithelial cells, i.e. podocytes. A novel nomenclature, 'podocytopathy' has been proposed, and advances in molecular knowledge on hereditary FSGS have accelerated the understanding of its pathophysiological background. Nevertheless, molecular information obtained so far has been restricted to 'familial and hereditary' FSGS. In idiopathic FSGS, most patients do not possess mutations or genetic variances of the above-mentioned genes. Therefore, the genetic background and pathogenesis of idiopathic FSGS remain to be elucidated.

In 2008, Kopp *et al.* [6] and Kao *et al.* [7] independently identified an association between idiopathic FSGS in the African American population and *MYH9* encoding nonmuscle myosin heavy chain-IIA (NMMHC-IIA). However, the SNPs identified in these studies do not change the amino acid sequence of NMMHC-IIA, and the mechanism by which these SNPs in *MYH9* cause idiopathic FSGS remains unclear.

In 2010, an association between the nonsynonymous coding variants of the *APOL1* gene located contiguous to *MYH9* and FSGS was demonstrated in the same population [8]. Several studies have reported that *APOL1* risk alleles are associated with the development of chronic kidney disease [9–11]; however, these results were derived only from African American population studies. Conversely, *MYH9* polymorphisms, but not *APOL1*, were associated with an increased risk of diabetic and nondiabetic nephropathies in European American populations [12, 13]. Discussions are therefore continuing on how these variants of the two genes cause FSGS or ESRD.

Epstein syndrome, caused by mutations in the *MYH9* gene, is characterized by congenital thrombocytopenia with giant platelets, progressive renal disease, and hearing disability [14–16]. Epstein syndrome is very rare, and renal biopsy is contraindicated owing to thrombocytopenia, which has restricted histopathological analyses of renal specimens from patients with Epstein syndrome; therefore, few studies on their renal pathology have been reported [17, 18].

We have recently analyzed the clinical and histopathological features of nine patients with R702 mutations in *MYH9* [19]. Most patients showed proteinuria in early infancy and developed ESRD before adolescence. In one patient, serial renal biopsies were performed, and the histopathological findings were consistent with FSGS. Immunohistochemical studies of the renal specimens of this patient revealed an apparently decreased NMMHC-IIA expression level in podocytes, but not in renal tubular cells and endothelial cells. Recently, three mouse lines, each with a different mutation in *Myh9* (R702C, D1424N and E1841 K) were generated [20]. Their clinical manifestations were identical to those of Epstein syndrome: macrothrombocytopenia, FSGS and mild hearing loss developed. In another study, Johnstone *et al.* [21] reported that podocyte-specific *Myh9* knock-out mice were susceptible to doxorubicin glomerulopathy. These findings reinforce the hypothesis that *MYH9* is a potential candidate for susceptibility to glomerular disease, especially FSGS, and further study of *MYH9* is warranted.

The aim of this study is to understand the role of NMMHC-IIA in the pathogenesis of nonhereditary podocyte injury. We analyzed the precise localization of NMMHC-IIA in normal kidneys and changes in expression of NMMHC-IIA in pathological states in rats and humans. The results suggest the critical role of NMMHC-IIA in physiological and pathological states specific to human FSGS.

MATERIALS AND METHODS

Antibodies

Rhodamine (TRITC)-conjugated donkey anti-rabbit IgG F (ab′)₂ fragment, TRITC-conjugated donkey anti-mouse IgG F (ab′)₂ fragment, fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG F(ab′)₂ fragment, FITC-conjugated donkey anti-mouse IgG F(ab′)₂ fragment and FITC-conjugated anti-guinea pig IgG F(ab′)₂ fragment were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Anti-NMMHC-IIA antibodies (PRB440P),

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obtained from Covance Research Products (Berkeley, CA, USA), were used for immunofluorescence and immunoelectron microscopic studies in human glomerular diseases. Anti-NMMHC-IIA antibodies [BT561, from Biomedical Technologies, Inc. (Stoughton, MA, USA)], which were raised against purified human platelet myosin heavy chain, were used for the other experiments. Platelets contain solely NMMHC-IIA isoform, and BT561 antibodies have been shown not to cross react with NMMHC-IIB or IIC [22–24]. Anti-NMMHC-IIB antibodies (G650) were a gift from Dr. Tomoaki Shirao [25]. Mouse monoclonal anti-synaptopodin antibodies were obtained from Progen (Heidelberg, Germany). Mouse monoclonal anti-ZO-1 antibodies were obtained from Zymed Laboratories (South San Francisco, CA, USA). Mouse monoclonal anti-WT1 antibodies and mouse monoclonal anti-desmin antibodies (clone D33) were obtained from Dako (M3561; Carpinteria, CA, USA). Mouse monoclonal anti-GLEPP1 antibodies were obtained from BioGenex (MU336-UC; San Ramon, CA, USA). Rabbit polyclonal anti-nephrin antibodies [26], rabbit polyclonal anti-Neph1 antibodies [27], and rabbit polyclonal anti-podocin antibodies were prepared as previously described [28]. Mouse monoclonal anti-rat podocalyxin antibodies (clone N3) were prepared as previously described [29]. Mouse monoclonal anti-intercellular adhesion molecule (ICAM)-2 antibodies (clone D-12) were prepared as previously described [30]. Gold-conjugated goat anti-rabbit antibodies were obtained from British BioCell (Essex, UK).

Animals

All procedures performed on laboratory animals were approved by the Institutional Animal Care and Use Committee of Juntendo University School of Medicine and were carried out in compliance with the Guidelines for Animal Experimentation of Juntendo University School of Medicine. Male Wistar rats (6 weeks old) were obtained from Charles River Japan (Kanagawa, Japan). Puromycin aminonucleoside (PAN) nephrosis was induced by a single intraperitoneal injection of PAN (Sigma) at a dose of 100 mg/kg. Rats were sacrificed under anesthesia with pentobarbital 2, 5, 7 and 11 days after receiving a PAN injection.

Immunofluorescence studies of rat glomeruli

Rat kidneys were perfused with periodate-lysine-paraformaldehyde fixative buffered with 0.1 M phosphate-buffer (PB) (pH 7.4) and immersed in the same fixative. Cryosections (thickness, 5 μm) were incubated for 2 h at room temperature with primary antibodies. Sections were then incubated with FITC or TRITC-labeled secondary antibodies (diluted 1:100) for 1 h at room temperature. All sections were examined with a confocal laser scanning microscope LSM510 (Carl Zeiss, Oberkochen, Germany).

Western blot analysis

Isolated glomeruli from normal or PAN-treated rat kidneys (Days 2, 5, 7 and 11) were solubilized in phosphate-buffered saline (PBS) containing protease inhibitors and 1 mM sodium orthovanadate, 1% SDS, and 5 mM EDTA, electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Three rats were subjected to the analysis for the control and Days 2, 5 and 7. For Day 11, four rats were used. Blots were incubated with the primary antibodies and then with HRP-conjugated goat anti-mouse IgG, and detected using the ECL western blotting detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Protein content was determined by the bicinchoninic acid assay (Pierce). The immunoblots were quantified using Quantiscan (Biosoft, Cambridge, UK). One-way ANOVA, followed by Tukey's multiple comparison test was used for statistical analysis.

Immunohistochemical and immunofluorescence studies of human glomeruli

All procedures performed on human kidney specimens were approved by the Ethics Committee of the Tokyo Women's Medical University Hospital (Approval No. 201). Sections of paraffin-embedded samples (3 μm thick) were prepared for all patients for immunohistochemical analysis. Renal specimens derived from a 51-year-old and a 38-year-old renal transplantation 0-h donor kidneys, which were excised from the donors with immediate perfusion, were used as controls after written informed consent was obtained. The renal specimen of a patient with Epstein syndrome in whom NMMHC-IIA expression was remarkably decreased (published in our previous study [19]) was used as the negative control after written informed consent was obtained. Each renal section was autoclaved for 15 min at 121°C in a citrate buffer (pH 6.0). After washing with water and PBS, sections were incubated with anti-NMMHC-IIA antibodies (1:100) for 2 h at room temperature. After washing, each section was further incubated with secondary antibodies (ENVISION, Dako) for 20 min. Subsequently, each section was treated with streptavidin-HRP and diaminobenzidine. Sections were then counterstained with hematoxylin. All sections were stained during the same run. Glomerular expression of NMMHC-IIA was graded semiquantitatively by two researchers (H.Y. and S.T.) who were masked to all the clinical data and the pathological diagnoses. The intensity levels of NMMHC-IIA staining in all glomeruli of all patients were graded according to the following scale: 0 = no or trace staining, 1 = weak, segmental staining, 2 = moderate staining, 3 = strong, diffuse staining. The representative staining level is shown in Supplementary Figure S1. Wilcoxon rank sum test was used for statistical analysis.

Sections of frozen kidney samples (3 μm thick) were prepared for immunofluorescence studies. A renal specimen derived from a 51-year-old renal transplantation 0-h donor kidney was used as the control. Each renal section was fixed with acetone. After washing with PBS, each section was incubated with primary antibodies for 1.5 h at room temperature. After washing, each section was further incubated with secondary antibodies (Alexa-Fluor 555 and 488) for 1 h. 4′,6-diamino-2-phenylindole (DAPI) was used for staining nuclei. Images were obtained with an inverted microscope (model IX71; Olympus, Tokyo, Japan).

Electron microscopy immunogold labeling analysis

Using specimens of a normal human control, ultrathin cryosections were cut with a Leica Ultracut UCT microtome

equipped with the FCS cryoattachment (Vienna, Austria) at −110°C following the techniques of Tokuyasu [31]. A renal specimen derived from a 51-year-old renal transplantation 0-h donor kidney, which was excised from the donor with immediate perfusion, was used. Sections were transferred to nickel grids (150 mesh), which had been coated with Formvar and carbon. Subsequent incubation steps were carried out by floating the grids on droplets of the filtered solution. After quenching free aldehyde groups with PBS-0.01 M glycine, sections were incubated overnight with primary antibodies. They were then incubated with secondary antibodies coupled to 10 nm gold particles (diluted 1:100 with PBS containing 10% fetal calf serum) for 1 h. After immunostaining, sections were fixed with 2.5% glutaraldehyde buffered with 0.1 M PB (pH 7.4). Sections were then contrasted with 2% neutral uranyl acetate solution for 30 min, absorption-stained with 3% polyvinyl alcohol containing 0.2% acidic uranyl acetate for 30 min, and observed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan). For control specimens, ultrastructural localization images were analyzed without anti-NMMHC-IIA antibodies.

Patients with FSGS, minimal change disease and chronic glomerulonephritis manifesting heavy proteinuria

Renal biopsy specimens were obtained from 14 patients with steroid-resistant idiopathic FSGS, minimal change disease (MCD) and heavy proteinuric glomerulonephritis from the Tokyo Women's Medical University Hospital between 2000 and 2008, after written informed consent from each patient was obtained. We adhered to the Declaration of Helsinki throughout this study. Renal specimens were subjected to immunohistochemical and immunofluorescence studies. The clinical profile at the time of biopsy and pathological findings are described in Table 1. The enrolled patients included six patients with idiopathic FSGS (four were steroid-resistant and two were steroid-sensitive tip variant), two with MCD, two with IgA nephropathy (IgAN), two with membranous nephropathy (MN), one with Henoch-Schönlein purpura nephritis (HSPN) and one with membranoproliferative glomerulonephritis (MPGN). Most patients manifested heavy proteinuria at the time of renal biopsy. Renal functions were normal in all patients (Table 1). The diagnosis of idiopathic (primary) FSGS was supported by prominent edema and hypoalbuminemia in all cases and recurrence of FSGS after renal transplantation in three of four patients with steroid-resistant FSGS, which occurred later in their clinical courses. Patients with small kidneys and/or obesity, which would support the diagnosis of adaptive FSGS, were excluded in this study.

RESULTS

### NMMHC-IIA is localized in the podocyte cell body and primary processes

Figure 1A shows the immunofluorescence study of NMMHC-IIA and other podocyte-related proteins in glomeruli of the normal rat kidney. Signals for NMMHC-IIA were located outside the capillary lumen, but showed a distinct localization

Table 1. Clinical profile and pathological diagnosis of the patients

No.	Age/gender	Pathological findings	Urinary protein (g/day)	Serum albumin (g/dL)	eGFR(mL/min/1.73 m^2)	Treatment
1	9/F	FSGS (NOS)	1.0	2.4	98.2	PSL, ACEI
2	5/M	FSGS (NOS)	11.6	2.2	110	PSL
3	4/M	FSGS (NOS)	9.1	2.0	111	PSL, CsA, ACEI
4	3/F	FSGS (collapsing variant)	5.5	2.0	111	PSL
5	18/M	FSGS (tip variant)	0	4.9	136	PSL, CsA
6	8/F	FSGS (tip variant)	0.48	2.6	130	PSL
7	15/M	MCD	1.6	2.6	152	PSL, CsA, ARB
8	3/F	MCD	2.4	1.7	108	PSL
9	14/F	MN	1.2	2.8	130	PSL
10	6/F	MN	3.0	2.0	109	No therapy
11	12/M	IgAN	0.18	4.1	131	No therapy
12	9/M	IgAN	6.4	1.4	98.9	No therapy
13	13/F	HSPN	1.3	3.3	110	No therapy
14	15/M	MPGN	10.7	2.3	121	PSL, MZR, ARB

eGFR, estimated glomerular filtration rate determined by the Schwartz formula; NOS, not otherwise specified; PSL, prednisolone; ACEI, angiotensin converting enzyme inhibitor; CsA, cyclosporine; ARB, angiotensin receptor blocker; MZR, mizoribine.

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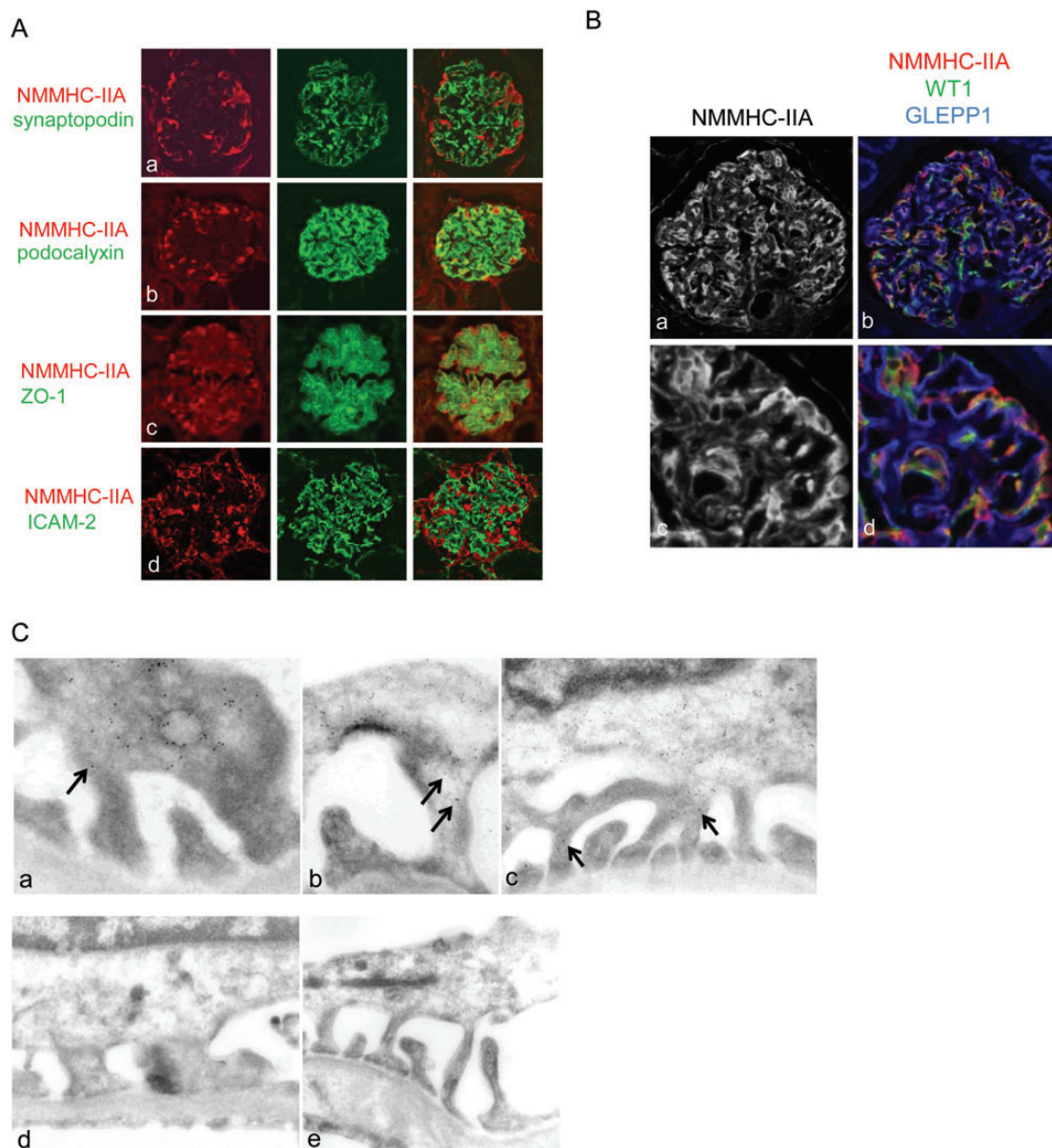
FIGURE 1: Localization of NMMHC-IIA. (**A**). Cryostat sections (5 μm thick) of the normal rat kidney were co-stained with an antibody specific for NMMHC-IIA (red) and other podocyte-associated molecules (green), synaptopodin (*a*), podocalyxin (*b*) and zonula occludens (ZO)-1 (*c*). Intercellular adhesion molecule (ICAM)-2 (green), which is localized in endothelial cells, was also co-stained with NMMHC-IIA (red) (*d*). Merged images showing the extent of co-localization are on the right. NMMHC-IIA is located in the podocytes, but it is not co-localized with synaptopodin, podocalyxin and ZO-1, which are all foot process proteins, nor with ICAM-2. (**B**). Immunofluorescence study of NMMHC-IIA, WT1 and GLEPP1 in glomeruli of the normal human kidney. Panels *a* and *c* denote NMMHC-IIA expression and panels *b* and *d* denote triple immunostaining of NMMHC-IIA, WT1 and GLEPP1. Localizations of these molecules are almost distinct. (**C**). Ultrathin cryosections derived from a normal human kidney labeled with anti-NMMHC-IIA antibodies following 10 nm gold particle-conjugated secondary antibodies (*a–c*). NMMHC-IIA is localized mainly at the primary processes and scaffolding region of foot processes. Arrows indicate NMMHC-IIA signals located at the scaffolding region of foot processes. Nonspecific signals were not seen in the control specimens, where anti-NMMHC-IIA antibodies were not applied (*d* and *e*).

from synaptopodin, podocalyxin and ZO-1, all of which are exclusively located in podocyte foot processes. NMMHC-IIA also showed a distinct localization from ICAM-2, which is specifically located in endothelial cells. In the normal human kidney, NMMHC-IIA was located in the cell bodies of podocytes, outside the capillary loop demarcated by GLEPP1 staining (Figure 1B). This is consistent with analysis of the normal rat kidney. Expression of NMMHC-IIA was also distinct from that of WT1, and they were only partially co-localized. Weak signals for NMMHC-IIA were observed in endothelial cells.

We further analyzed the precise localization of NMMHC-IIA in podocytes by electron microscopy immunogold analysis. NMMHC-IIA is localized mainly at the primary processes and in the scaffolding region of foot processes of podocytes in

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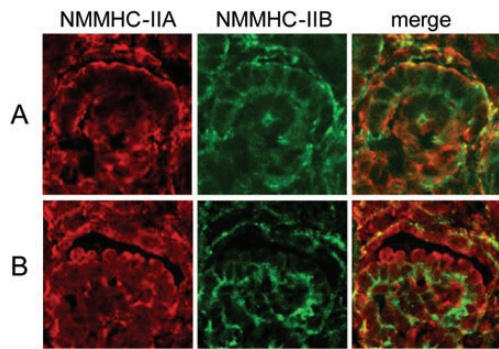
FIGURE 2: Localization of NMMHC-IIA and NMMHC-IIB in neonatal rat glomeruli. Cryostat sections at the S-shaped stage (**A**) and at the capillary loop stage (**B**) were co-stained with an anti-serum specific for NMMHC-IIA and NMMHC-IIB. The merged image showing the extent of co-localization is on the right.

the normal human kidney (Figure 1C). Small amounts of NMMHC-IIA were also expressed in endothelial cells (data not shown).

NMMHC-IIA expression is altered in developing glomeruli and in the podocyte injury model of rats

We next analyzed NMMHC-IIA expression in developing rat glomeruli. Expression of NMMHC-IIA and NMMHC-IIB in the neonatal rat glomeruli is shown in Figure 2. Glomeruli in the upper panels are at the S-shaped stage, and glomeruli in the lower panels are at the capillary loop stage. NMMHC-IIA is strongly expressed in the apical membrane of immature podocytes at the S-shaped stage. In contrast, NMMHC-IIB is mainly expressed in the adhesion sites of podocytes, and weakly at the basolateral membrane of podocytes at this stage (Figure 2A). As shown in Figure 2B, the expression pattern of NMMHC-IIA in podocytes at the capillary stage changed, and the expression was distributed diffusely at the cytoplasm of podocytes. At the capillary stage, the expression of NMMHC-IIB in podocytes disappeared, and mesangial cells developed to express NMMHC-IIB, suggesting different roles of each molecule.

We then examined whether expression of NMMHC-IIA was altered in an acquired podocyte injury model (PAN nephropathy). The signals for NMMHC-IIA markedly decreased in specimens 11 days after a PAN injection, during which massive proteinuria was noted; whereas the expression levels of both podocalyxin and ZO-1 did not change (Figure 3A). Western blot analysis revealed that NMMHC-IIA protein levels markedly decreased at Day 11 after a PAN injection compared with the control (P = 0.059), while the protein levels of desmin, a marker for podocyte damage, significantly increased after a PAN injection as previously described [32] (Figure 3B–D). Western blot analysis in each run is shown in Supplementary Figure S2.

NMMHC-IIA expression is decreased in idiopathic FSGS

Next, we analyzed the expression of NMMHC-IIA in patients with FSGS and other proteinuric nephropathy. Figure 4A shows representative images of glomeruli of the two

normal subjects (*a* and *b*), the patient with Epstein syndrome (*c*), steroid-resistant FSGS (*d–g*), FSGS tip variant (*h* and *i*), MCD (*j–l*), MN (*m* and *n*), IgAN (*o* and *p*), HSPN (*q*) and MPGN (*r*). Each sample contained 2–10 glomeruli (median 7 glomeruli). Intensity scores for NMMHC-IIA staining in each patient are shown in Figure 4B. Intensity scores of steroid-resistant FSGS were significantly lower than those of chronic glomerulonephritis (P = 0.016) and showed a tendency to be lower than steroid-sensitive tip variant and MCD (Table 2).

NMMHC-IIA change is distinct from other podocyte-related molecules in human glomerular diseases

We further analyzed the expression levels of nephrin, Neph1, synaptopodin, podocin, ZO-1 and GLEPP1 by immunofluorescence study in idiopathic FSGS (Patient 2), MCD (Patient 7 when in relapse), and MN (Patient 10). As shown in Figure 5, expression levels of these proteins did not significantly change. In contrast, the expression levels of NMMHC-IIA markedly decreased in idiopathic FSGS and moderately decreased in MCD, while NMMHC-IIA expression did not change in MN. As shown in Figure 6, NMMHC-IIA expression exclusively decreased in podocytes in FSGS and MCNS, whereas nephrin and synaptopodin were well preserved. In MN, the expression levels of NMMHC-IIA did not change.

DISCUSSION

Recent molecular studies have indicated that FSGS is primarily podocytopathy. Although the relationship between mutated podocyte molecules and development of familial FSGS is apparent, it does not necessarily mean that these proteins play primary roles in the development of idiopathic FSGS. In fact, so far there has been no definite evidence supporting the notion that any genes expressed in podocytes are related to the development of idiopathic FSGS. In contrast, genetic variations of *MYH9* and *APOL1* genes have been shown to be associated with increased susceptibility to idiopathic FSGS or progressive kidney disease in the African American population [6–11]. These findings and our recent clinical and pathological findings on Epstein syndrome with mutations in *MYH9* [19] have prompted us to perform the present study.

Expression levels of several podocyte-associated proteins in acquired human nephrotic syndrome have been reported. Expression levels of CD2-associated protein and α-actinin-4 did not change in MCD and FSGS [33]. Expression levels of nephrin varied among reports [34–36]. Altered expression and subcellular localization of podocin in nephrotic syndrome were demonstrated [37, 38]. In the present study, we observed no significant alteration of slit diaphragm molecules in idiopathic nephrotic syndrome. However, expression of NMMHC-IIA exclusively decreased in FSGS, as is the case in Epstein syndrome with *MYH9* mutations. It should be noted that the change in expression levels of NMMHC-IIA was not related to the level of urinary protein excretion (Table 1). We could not clearly discriminate idiopathic FSGS from MCD by NMMHC-IIA expression levels. This might be partially explained by the
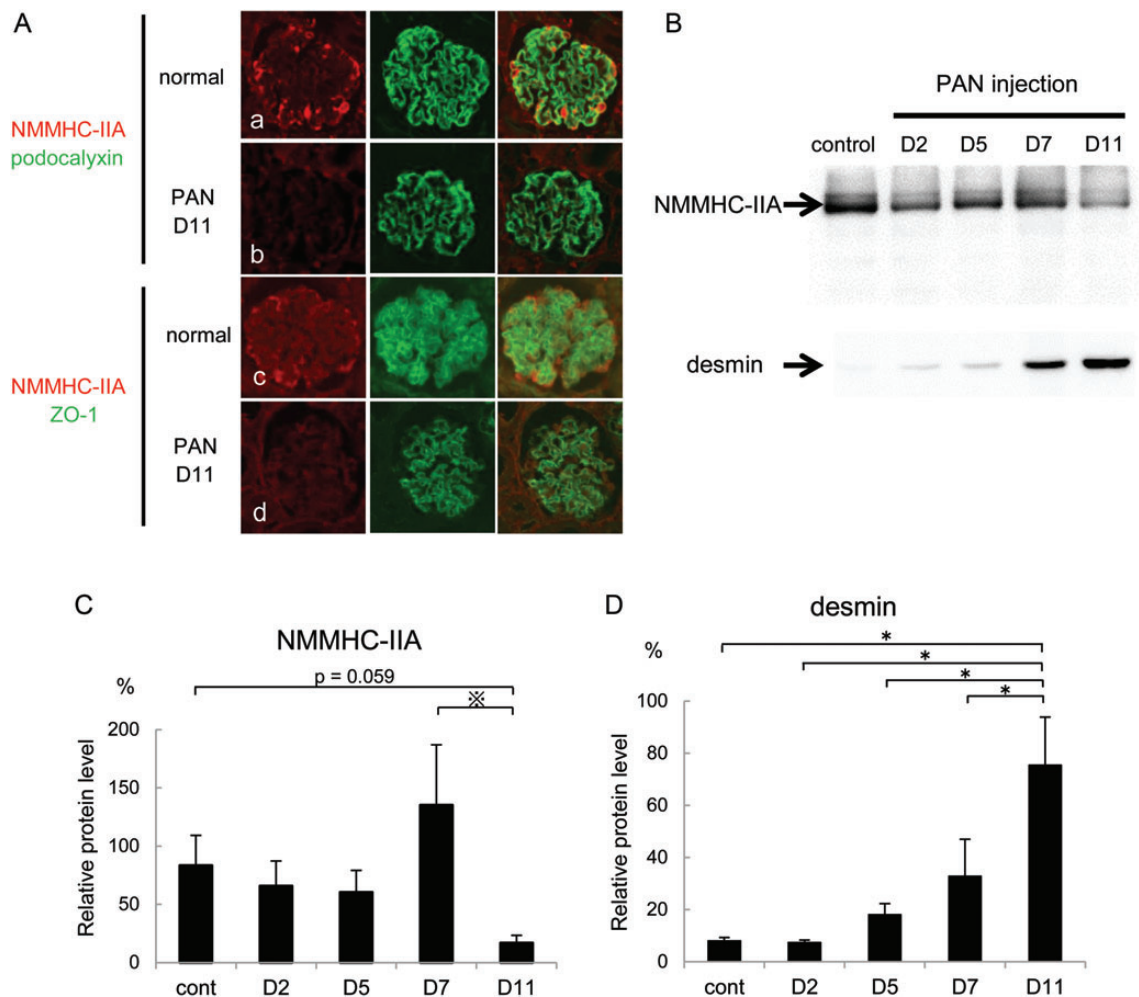
FIGURE 3: Expression of NMMHC-IIA in puromycin aminonucleoside (PAN)-treated rat kidney. (**A**) Immunofluorescent study of NMMHC-IIA in PAN-treated rats. Cryostat sections (45 µm thick) were co-stained with an anti-serum specific for NMMHC-IIA (red) and other podocyte-associated molecules (green), podocalyxin (*a* and *b*) and ZO-1 (*c* and *d*). Merged images showing the extent of co-localization are on the right. Panels *a* and *c* show expressions of these molecules in a normal rat glomerulus, and panels *b* and *d* show those in a rat glomerulus at Day 11 after a PAN injection. The signal for NMMHC-IIA is lost in PAN-treated rat glomerulus, whereas those of podocalyxin and ZO-1 do not change. (**B**) Representative images of western blot analysis of NMMHC-IIA and desmin in lysates from glomeruli in the normal and PAN-treated kidney. Expression of NMMHC-IIA protein was markedly decreased in the specimen on Day 11 after a PAN injection, while expression of desmin was gradually increased after a PAN injection. (**C**) Quantitative analyses of western blotting using glomerular samples. NMMHC-IIA protein levels decreased on Day 11 after a PAN injection, compared with control and Day 7 (P = 0.059, 0.001, respectively). NMMHC-IIA protein levels were not significantly increased on Day 7 when compared with the control (P = 0.22). (**D**) Quantitative analyses of western blotting using glomerular samples revealed a significant increase in desmin protein levels after a PAN injection. D, day; ※P = 0.001; ✳P < 0.005.

fact that the clinical course of the patient with MCD (Patient 7) was also refractory at the time of biopsy, if it was eventually steroid-sensitive.

We also demonstrated that signal intensity of NMMHC-IIA decreased in PAN-treated rats in immunofluorescence study, while those of podocalyxin and ZO-1 did not change. Western blot analysis revealed a marked decrease in expression levels of NMMHC-IIA in PAN-treated rats. Although PAN nephrosis is generally considered to be a model of minimal change nephrotic syndrome since the pathological change is reversible, it is accompanied by podocyte detachment and apoptosis, which are typical features of FSGS [39]. Considering the specific decreases in the expression levels

of NMMHC-IIA in both human idiopathic FSGS and an animal model of PAN nephrosis, the pathophysiological role of NMMHC-IIA in the development of FSGS appears to be highly possible.

Although NMMHC-IIA has been reported to be expressed in podocytes [19, 40, 41], its precise localization has not been elucidated. Our study revealed that in rodent and human glomeruli, NMMHC-IIA was primarily expressed in primary processes of podocytes, and was also localized at the scaffolding region of foot processes (Figure 1C), namely the basal region where foot processes are projecting. Since myosin is a molecule which moves the actin filament and the solid structure of foot processes is composed of actin bundles, it is plausible that

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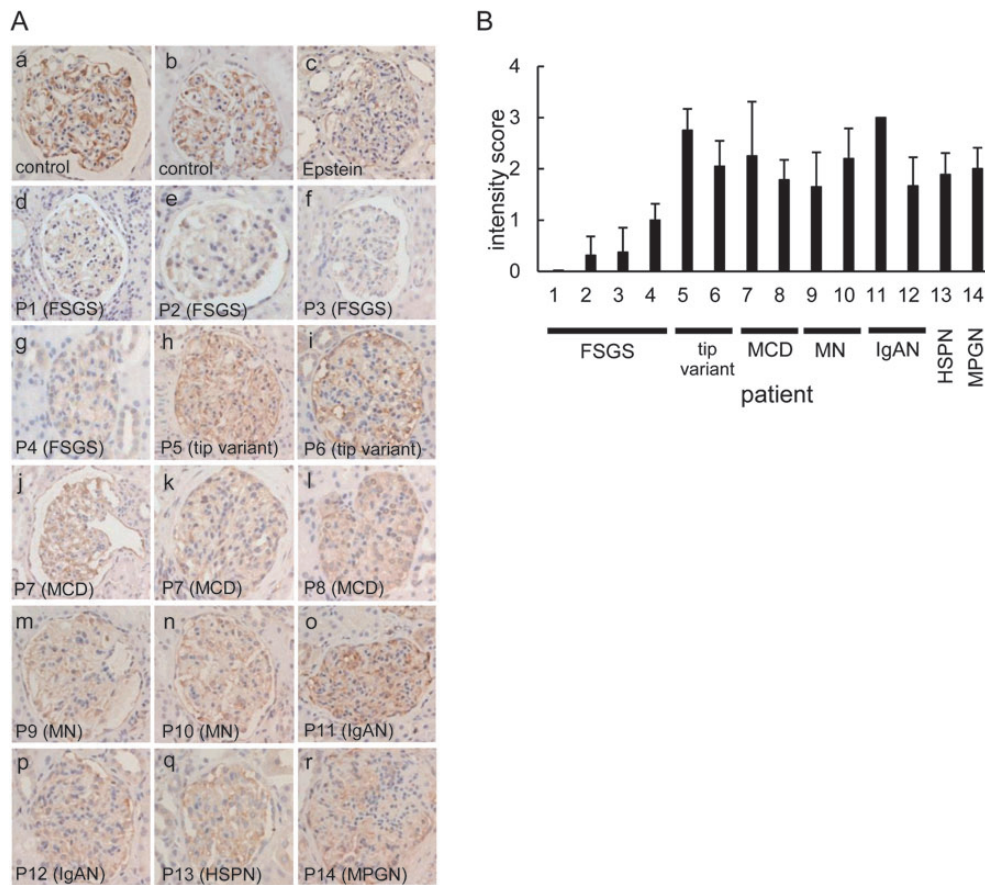
FIGURE 4: Immunohistochemical staining for NMMHC-IIA in human glomerular diseases. (**A**) The expression levels of NMMHC-IIA markedly decreased in Epstein syndrome (*c*) and steroid-resistant FSGS (*d–g*) compared with those in two control subjects (*a* and *b*). In contrast, the expression of NMMHC-IIA was preserved in the tip variant of FSGS (*h* and *i*). The expression of NMMHC-IIA did not significantly change in MCD both in relapse (*j* and *l*) and in remission (*k*). Expression of NMMHC-IIA did not significantly change in MN (*m* and *n*), IgAN (*o* and *p*), HSPN (*q*) and MPGN (*r*). P(1–14) shows each patient. (**B**) Intensity scores for NMMHC-IIA expression in each patient determined by immunohistochemical analysis. For statistic evaluation of the difference in intensity scores between diagnoses, see Table 2.

Table 2. Intensity scores of NMMHC-IIA expression determined by immunohistochemical analysis in the patients with steroid-resistant FSGS and other proteinuric nephropathy

	Steroid-resistant FSGS	Tip variant	MCD (relapse)	Chronic glomerulonephritis
Number of patients	4	2	2	6
Intensity score (mean ± SD)	0.42 ± 0.42	2.40 ± 0.49	2.02 ± 0.33	2.07 ± 0.50
P value[a]	–	0.13	0.13	0.016

Chronic glomerulonephritis includes MN, IgAN, HSPN and MPGN.
[a]Compared with steroid-resistant FSGS.

this characteristic localization of NMMHC-IIA would contribute to maintaining the unique structure of podocytes. Abnormalities of NMMHC-IIA caused by mutations in *MYH9* result in foot process effacement and development of FSGS [19]. Expression patterns of NMMHC-IIA in the capillary stage (Figure 2B) are consistent with immunofluorescence studies (Figure 1A) and electron microscopy immunogold labeling analyses (Figure 1C).

Nonmuscle myosin II has diverse functions in cell contractility, morphology, cytokinesis and migration [42]. NMMHC-IIA maintains a balance between actomyosin and microtubule systems by regulating microtubule dynamics [42]. The present result, that NMMHC-IIA is localized at the podocyte primary processes where microtubule systems maintain the cytoskeleton, predicts a perturbed interaction between NMMHC-IIA and cytoskeleton molecules in primary processes,

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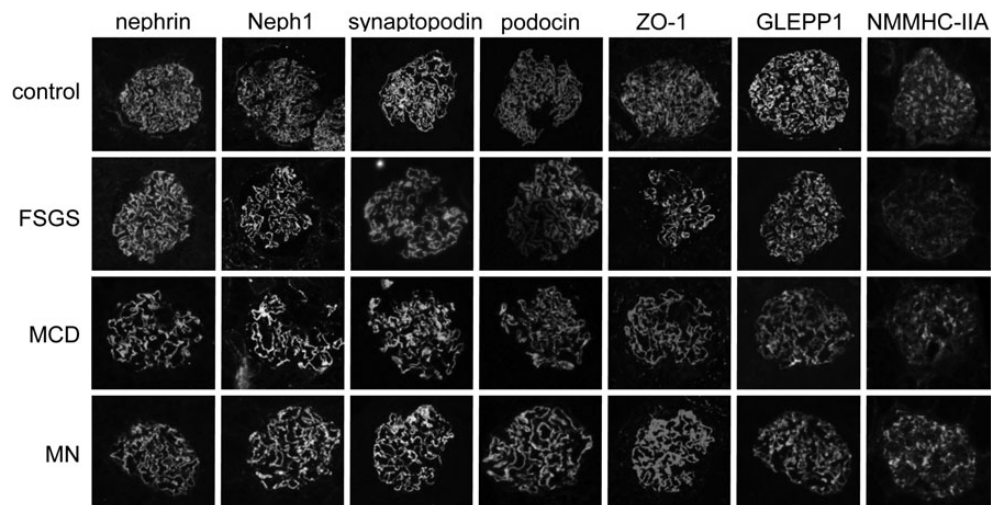
FIGURE 5: Immunofluorescence studies of podocyte-related proteins in human glomerular diseases. Cryostat sections (4 µm thick) were stained with an anti-serum specific for foot process proteins (nephrin, Neph1, synaptopodin, podocin, ZO-1 and GLEPP1) and NMMHC-IIA in control, idiopathic FSGS (Patient 2), MCD (Patient 7 when in relapse) and MN (Patient 10). No significant changes in expression of these foot process proteins were observed in glomeruli of any patients, whereas expression of NMMHC-IIA markedly decreased in idiopathic FSGS and moderately decreased in MCD.
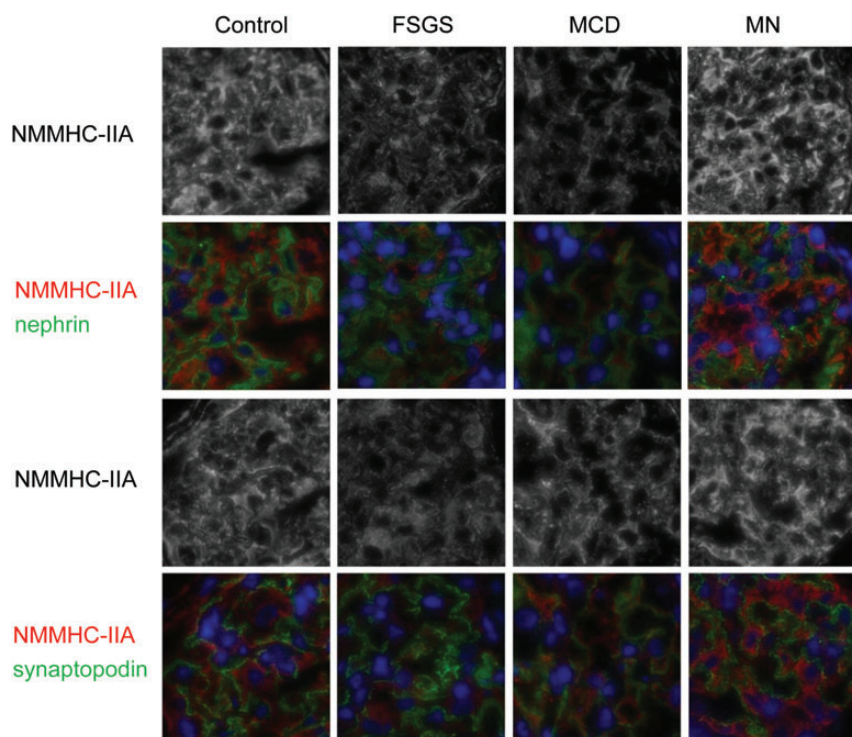
FIGURE 6: Dual immunostaining of NMMHC-IIA and other podocyte-associated proteins in human glomerular diseases. Cryostat sections (4 µm thick) were co-stained with an anti-serum specific for NMMHC-IIA (red) and foot process proteins (nephrin and synaptopodin) (green) in control, idiopathic FSGS (Patient 2), MCD (Patient 7 when in relapse) and MN (Patient 10). Nuclei were stained with DAPI (blue). NMMHC-IIA expression was distinct from nephrin and synaptopodin. Expression levels of NMMHC-IIA markedly decreased in FSGS and MCD, whereas those of nephrin and synaptopodin were preserved. Expression levels of NMMHC-IIA, nephrin and synaptopodin were preserved in MN.

particularly in the adjacent area between the primary and foot processes: this unique localization could cause morphological changes of podocytes in idiopathic FSGS and Epstein syndrome. In this regard, Babayeva *et al.* [43] showed that plasma from a patient with recurrent idiopathic FSGS rapidly decreased cultured podocyte levels of the phosphorylated

myosin light chain and perturbed the usual localization of NMMHC-IIA along actin stress fibers. Further studies are required to identify the mechanisms by which NMMHC-IIA maintains the highly specific structures of podocytes as the ultrafiltration barrier.

In conclusion, we demonstrated the decreased expression of NMMHC-IIA in human idiopathic FSGS. This phenomenon is specific to idiopathic nephrotic syndrome, especially FSGS, and not observed in other heavy proteinuric glomerulonephritis and nephropathy. NMMHC-IIA is primarily localized in podocyte primary processes. These results suggest the critical role of NMMHC-IIA in the development of idiopathic FSGS.

CONFLICT OF INTEREST STATEMENT

None declared.

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ORIGINAL ARTICLE

Decreased expression of NMMHC-IIA in idiopathic FSGS