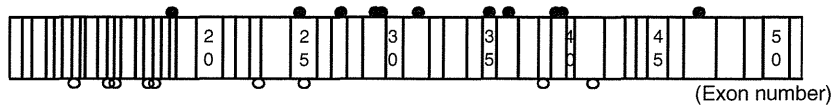


Figure 2 | Distribution map of *COL4A5* missense mutations. Black circles define the positions of missense mutations in the negative group, and open circles show the positions of missense mutations in the positive group.

positive group thus showed a significant tendency to be located before exon 25.

Phenotypes

Renal. Regarding phenotypes, the age at first proteinuria detection was significantly younger (4.0 ± 3.2 and 6.3 ± 4.8, respectively; $P = 0.036$, Table 1) and the urinary protein/creatinine ratio at the time of mutational analysis was significantly higher in the negative group than in the positive group (1.7 ± 2.4 g/g Cr and 0.78 ± 1.0 g/g Cr, respectively; $P = 0.027$, Table 1), although the ages at mutational analysis were similar in both groups. Only six patients developed ESRD, because of their relatively young age. We therefore compared the age at onset of ESRD in patients and their affected male family members. Five patients and 9 affected male family members in the negative group, and 2 patients and 13 affected male family members in the positive group developed ESRD (Supplementary Table 1 and 2 online). The age at onset of ESRD was significantly lower in the negative group than in the positive group (24.00 ± 14.2 and 37.53 ± 16.30 years, respectively; $P = 0.018$, Figure 3).

Two patients in the positive group demonstrated somatic mosaicism; both showed a mosaic pattern of α5(IV) expression in the GBM and BC, one of which we have recently reported on elsewhere.[3]

Cochlear. Previous reports have suggested a probability of hearing loss of 50% by age 15 in men with X-linked AS.[4] Our results showed that 54.3% of patients in the negative group developed hearing loss, with a median age of 13 years; however, no patients in the positive group developed hearing loss (Table 1 and Figure 4).

Ocular. It has also been reported that about 15% of X-linked AS men exhibit anterior lenticonus or other eye lesions.[4] However, there was no significant difference in ocular abnormalities between the two groups in this study (15.6% in the negative group and 6.7% in the positive group; $P = 0.65$; Table 1).

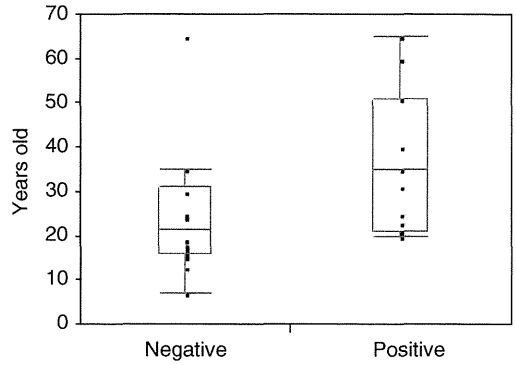
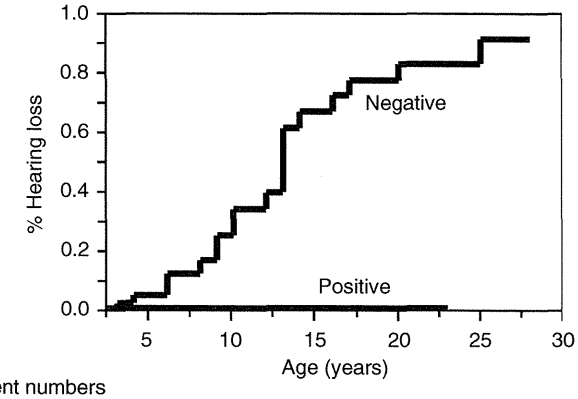


Figure 3 | Age at onset of end-stage renal disease (ESRD) in negative ($n = 14$) and positive group ($n = 15$) patients and their affected male family members. The onset age was significantly lower in negative group compared with positive group patients (24.00 ± 14.2 and 37.53 ± 16.30 years, respectively; $P = 0.018$).



Patient numbers						
Negative	2	9	15	18	19	19
Positive	0	0	0	0	0	0

Figure 4 | Probability of hearing loss in the type IV collagen α5 (α5(IV))-positive and -negative groups. The median age for developing hearing loss was 13 years in the negative group, whereas no patients in the positive group developed hearing loss.

DISCUSSION

This study provides the first report of the genetic and clinical backgrounds of male XLAS patients with atypical immunohistological findings of α5(IV) in the kidney. The results show valuable and novel information on the renal outcome and genetic background of these patients based on immunostaining patterns.

We determined the genotypes of patients with immunohistochemical evidence of α5(IV) expression, and confirmed that this staining pattern was associated with a milder renal course. We demonstrated that 29% of male XLAS patients were positive for α5(IV) staining; 60% of them had missense mutations, 27% had an in-frame deletions, and 13% had somatic mosaic mutations. In contrast, all patients with truncating mutations showed negative staining for α5(IV). Several groups have described genotype–phenotype correlations in XLAS.[5] Large rearrangements and all mutations that change the reading frame of the gene were associated with

severe types of AS,[6] whereas patients with in-frame mutations tended to have relatively mild phenotypes.[4,6,7] Jais *et al.*[4] reported normal GBM incorporation of defective α5(IV) and the related α3(IV) and α4(IV) chains in two patients, indicating that normal GBM expression of α5(IV) did not preclude a diagnosis of XLAS. Mazzucco *et al.*[8] also reported three female XLAS patients with normal α3(IV) and α5(IV) staining patterns and proven *COL4A5* mutations, including two patients with small in-frame mutations and one with a missense mutation. In addition, two patients showed positive α3(IV) staining patterns, despite negative staining for α5(IV) chain, and these authors hypothesized that very low levels of α5(IV) expression that were undetectable by immunohistochemical study may still be sufficient to allow the correct folding of the triple helix comprising the α3-α4-α5 chains.[9] Massella *et al.*[10] recently reported that 3 out of 22 patients (14%) showed α5(IV) positivity (one diffuse and two segmental), and 5 of 22 patients (23%) showed diffuse α3(IV) staining. Type IV collagen, which is a component of the GBM, is a triple helix composed of three α chains. We hypothesized that some missense and in-frame mutations might affect the structure of this triple helix, but its rate of degradation is low. The α3-α4-α5(IV) triple helix network in GBM may thus sometimes be present in reduced amounts, and low, rather than absent expression levels may lead to the milder phenotype of XLAS.[11] The apparent discrepancies in α5(IV) positivity between the current and previous reports may be the result of the use of different antibodies with different sensitivities, associated with the methods of antibody production.[12–14] These factors should be taken into account when interpreting the results of these studies.

We also examined the correlation between mutation positions and staining patterns in patients with missense mutations. Missense mutations located in exons 1–25 were more common in α5(IV)-positive patients. The three α-chains comprising the triple helix of type IV collagen consist of triple helical protomers with different compositions.[15] Each protomer has a 7S triple helical domain at the N-terminal, a collagenous domain in the middle of the molecule of Gly-X-Y repeats, and a non-collagenous trimer (NC1) at the C-terminal. The repetitive Gly sequence in the collagenous domain is required for proper assembly of the collagen triple helix and the amino-acid residues in the X–Y positions are located on the outside of the triple helix.[16] The NC1 domain has an important role in heterotrimer formation,[6,17] because the zipper-like folding mechanism of the triple helix of type IV collagen is believed to start from the C-terminal end.[7,18,19] We used receiver operating characteristic analysis to evaluate the distance from the NC domain of the missense mutations affecting α5(IV) expression, and found a cutoff point at exon 25 that distinguished between the two groups; mutations in the positive group were significantly more likely to be located before exon 25. A previous study analyzed the effect of mutation position on disease severity by comparing 98 glycine-substituting missense mutations between two groups with mutations located in exons 1–20 and 21–47 of *COL4A5*, respectively. They found that patients with mutations in exons 1–21 had less severe disease in terms of ESRD.[7] Dividing our patients into the same categories in terms of mutation locations showed that patients with missense mutations located in exons 1–21 were more likely to be positive ($P = 0.05$). The results of this study suggest that mutations located between exons 1 and 25 may lead to a less critical disruption of triple helix-forming process. Naito *et al.*[13] reported that a point mutation, such as a Gly substitution, within the collagenous domain had no effect on the construction of the NC1 domain. Predicted minimal changes in protein structure cause late onset of ESRD. Our study indicated that positivity was related to less severe effects on urinary protein levels and older age at onset of ESRD.

We also demonstrated the incidence of somatic mosaic mutations in male XLAS patients with mild phenotype. We previously reported a somatic mosaic mutation in *COL4A5* in a male XLAS sufferer,[3] and the current report showed two patients with this pattern, including a previously reported case, both of whom showed relatively mild phenotypes. This suggests that somatic mosaic mutations should be considered in male XLAS patients with mild phenotypes and mosaic α5(IV) expression.

None of the α5(IV)-positive patients developed hearing loss in this study, compared with more than half of the negative-group patients (54%). There was no difference in the incidence of ocular lesions between the two groups, although this could have been because of the relatively small number of patients in this study. These results suggest that α5(IV)-positive patients exhibit milder renal and cochlear phenotypes.

This study had several limitations related to its retrospective nature and the small number of patients who developed ESRD. A previous study reported a median renal survival rate of 25 years.[4] Patients in this study were too young to permit differences in clinical severity to be detected by estimated glomerular filtration rate. In addition, the sample size in this study was relatively small, and further studies with more patients should be conducted to confirm the current findings.

In conclusion, male XLAS patients with positive α5(IV) chain expression had milder clinical manifestations than those with no α5(IV) expression. All α5(IV)-positive patients had non-truncating or somatic mosaic mutations. Furthermore, the location of missense mutations was related to differences in α5(IV) expression.

MATERIALS AND METHODS

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine, and consent for the study was obtained from the patients or their parents.

Inclusion criteria

Clinical and laboratory findings for patients with XLAS were obtained from their medical records. Patients were referred to our

hospital for clinical evaluation or genetic analysis. Most of the patients were followed in various local hospitals in Japan. DNA and data sheets were sent to our lab after acceptance of the request for mutational analysis. All patients in this study were identified with disease-causing mutations in the *COL4A5* gene and satisfied at least one of the following criteria: (1) male patients with proteinuria and hematuria or ESRD, whose renal pathology showed thickening and thinning with lamellation (basket-weave changes) or thin GBM by electron microscopy and total absence of α5(IV) in the GBM and BC. (2) Male patients with proteinuria and hematuria or ESRD whose renal pathology showed basket-weave changes or thin GBM by electron microscopy and positive α5(IV) expression in the GBM and BC.

The degree of urinary protein excretion was evaluated by the urinary protein/creatinine ratio. Estimated glomerular filtration rate was calculated using Schwartz's formula[20,21] or glomerular filtration rate-estimating equations for Japanese,[22] for patients under and over 21 years, respectively. All clinical, laboratory, and pathological data were collected when the request for mutational analysis was accepted. Images of kidney α5(IV) staining were sent to us for evaluation of the staining patterns and assessed by the same person (KN). Estimated glomerular filtration rate was measured based on the data in the data sheets. In Japan, mass urinary proteinuria screening is available for children aged 3 years, and every year from 6 to 18 years old. Information on the age at first detection of proteinuria is thus reliable. Hearing screening by audiometry is also available for children aged 6, 7, 8, 10, 12, 14, and 15 years, and information on age at detection of hearing loss is thus also very reliable.

Mutational analyses

Mutational analyses of *COL4A5* were carried out using the following methods: (1) PCR and direct sequencing of genomic DNA for all exons and exon–intron boundaries; (2) reverse transcription–polymerase chain reaction of mRNA and direct sequencing to detect abnormal splicing, and (3) multiplex ligation-dependent probe amplification to detect copy number variations. Genomic DNA was isolated from peripheral blood leukocytes from patients and family members using the Quick Gene Mini 80 system (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's instructions. For genomic DNA analysis, all specific 51 exons of *COL4A5* were amplified by PCR, as described previously.[23] The PCR-amplified products were then purified and subjected to direct sequencing using a Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) with an automatic DNA sequencer (model ABI Prism 3130; Perkin Elmer Applied Biosystems, Foster City, CA). Total RNA was extracted from blood leukocytes and/or urine sediments. RNA from leukocytes was isolated using a Paxgene Blood RNA Kit (Qiagen, Chatsworth, CA) and was then reverse-transcribed into complementary DNA using random hexamers and the Superscript III Kit (Invitrogen, Carlsbad, CA). RNA from urine sediment was isolated as described previously.[24] Complementary DNA was amplified by nested PCR using primer pairs for *COL4A5* as described previously[25,26] with slight modifications (sequences available on request). The PCR-amplified products were purified and subjected to direct sequencing.

Immunohistochemical analyses

Immunohistochemical analyses were performed using either frozen- or paraffin-embedded sections of kidney tissue. The immunohistochemical procedure has been described previously.[12,13,26] The mixture of fluorescein isothiocyanate-conjugated rat monoclonal antibody against human α5(IV) chain (H53) and Texas red-conjugated rat monoclonal antibody against human α2(IV) chain (H25) was purchased from Shigei Medical Research Institute (Okayama, Japan). Their epitopes were EAIQP at position 675–679 of the α2(IV) chain, and IDVEF at position 251–255 of the α5(IV) chain.[14] Patients showing complete negativity for α5(IV) staining were classified as the negative group; all other patients were classified as the positive group, including those with normal and mosaic expression patterns.

Statistical analyses

Data were expressed as mean ± s.d. All calculations were made using standard statistical software (JMP version 8 package for Windows, SAS, Cary, NC). The genetic and clinical backgrounds of patients in both groups were compared using Fisher's exact test, Wilcoxon's test, and receiver operating characteristic analysis. A P-value of <0.05 was considered statistically significant.

DISCLOSURE

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SUPPLEMENTARY MATERIAL

Table S1. COL4A5 mutations and family history in α5(IV)-negative group.

Table S2. COL4A5 mutations, α5 expression pattern, and family history in α5(IV)-positive group.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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Japanese Dent disease has a wider clinical spectrum than Dent disease in Europe/USA: genetic and clinical studies of 86 unrelated patients with low-molecular-weight proteinuria

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ORIGINAL ARTICLE

ABSTRACT

Dent disease is an X-linked disorder characterized by low-molecular-weight (LMW) proteinuria, hypercalciuria, nephrocalcinosis, urolithiasis and renal dysfunction. Dent disease is caused by mutations in at least two genes, i.e. *CLCN5* and *OCRL1*, and its genetic background and phenotypes are common among European countries and the USA. However, only few studies on Dent disease in Japan, which was originally called 'low-molecular-weight proteinuric disease', have been reported thus far. In this study, we analysed genetic background and clinical phenotype and laboratory data of 86 unrelated Japanese Dent disease patients. The results demonstrated that the genetic basis of Japanese Dent disease was nearly identical to those of Dent disease in other countries. Of 86 unrelated Japanese Dent patients, 61 possessed mutations in *CLCN5* (Dent-1), of which 27 were novel mutations; 11 showed mutations in *OCRL1* (Dent-2), six of which were novel, and the remaining 14 patients showed no mutations in *CLCN5* or *OCRL1* (Dent-NI). Despite the similarity in genetic background, hypercalciuria was detected in only 51%, rickets in 2% and nephrocalcinosis in 35%. Although the patients were relatively young, six patients (8%) showed apparent renal dysfunction. Japanese Dent disease has a wider clinical spectrum than Dent disease in Europe and the USA.

Keywords: *CLCN5*, dent disease, Japanese Dent disease, low-molecular-weight proteinuria, Lowe syndrome, *OCRL1*

INTRODUCTION

Dent disease is an X-linked disorder characterized by low-molecular-weight (LMW) proteinuria, hypercalciuria, nephrocalcinosis, urolithiasis and renal insufficiency [1–4]. In 1964, Dent and Friedman described the first two cases with hypercalciuric rickets associated with renal tubular dysfunction [1]. In the early 1990s, Wrong *et al.* reported detailed data of similar cases including Dent's report, and designated it as Dent disease [2]. Other than Dent disease, several similar disorders, such as 'X-linked recessive nephroliasis with renal failure' in North America and 'X-linked recessive hypophosphatamic rickets' in Italy, had been reported [3]. After the identification of the most common responsible gene, *CLCN5*, these disorders have been collectively called 'Dent disease' [4, 5]. As described below, the same is true for Japanese Dent disease. Thus far, two genes responsible for Dent disease have been identified [4, 6]. The first is *CLCN5*, which encodes voltage-dependent chloride channel 5 (CLC-5) [4]. CLC-5 is localized in the proximal tubular cells, thick ascending limb of Henle and collecting duct cells; CLC-5 is supposed to play critical roles in the acidification of intraendosomal compartments and endosomal recycling [7]. *CLCN5* knockout mice manifest phenotypes nearly identical to those in Dent disease [8, 9]. In 2005, Hoopes *et al.* identified *OCRL1* as the second causative gene for Dent disease [6]. Thereafter, we also identified mutations in *OCRL1* in Japanese Dent disease patients [10]. *OCRL1* was originally identified as the causative gene for Lowe syndrome, which is characterized by congenital cataract,

moderate-to-severe mental retardation and generalized solute transport dysfunction of proximal tubular cells [11].

Other than Dent and Wrong's studies, Suzuki and Okada proposed a disease entity named 'low-molecular-weight (LMW) proteinuria disease' in 1985 and 1990 [12, 13]. In Japan, annual urinary screening in school has been routinely conducted for a long time, and markedly high levels of LMW protein urine were identified in some children. In these children, renal biopsy examination did not show any glomerular or tubular lesions, and they showed no further increase in urinary protein level or renal function deterioration. Thus, the prognosis of LMW proteinuria disease in Japan had been considered benign.

In 1995, Igarashi *et al.* speculated that LMW proteinuria disease in Japan is the same as Dent disease [14]. Thereafter, Igarashi and Thakker identified mutations in *CLCN5* in Japanese patients with LMW proteinuria, and concluded that these two disorders are genetically identical [15–17]. Other groups also identified mutations in *CLCN5* in Japanese Dent disease patients [18, 19].

Although the genetic basis of patients with LMW proteinuria disease in Japan and Dent disease may be similar in limited patients, there are few actual genetic data of Japanese patients. Moreover, differences in the phenotype of Dent disease between patients in Europe/the USA and Japanese patients still remain unclear. In European countries and the USA, most of the patients with Dent disease showed hypercalciuria, renal calcification or renal stone, which appears to be the cause of the deterioration of renal function [2–5]. In particular, hypercalciuria is an essential clinical symptom in Dent disease as proposed by Hoopes *et al.* [20], although the frequency of hypercalciuria in LMW proteinuria disease is unknown. Furthermore, the prognosis of the renal function of patients with LMW proteinuria disease is also not known: in England, between the third and fifth decades of life, more than two-thirds of the affected males developed end-stage renal failure after 40–50 years of age [2].

In the present study, we conducted genetic and clinical analyses of 86 unrelated patients with LMW proteinuria. All of the patients suspected of having LMW proteinuria disease were subjected to the genetic analysis of *CLCN5*/*OCRL1*. As many clinical data as possible, including those of family members, were collected by the main attending physicians, and we carefully analysed them. The present study revealed the similarities and differences between Dent disease and LMW proteinuria disease in Japan. Hereafter, we will refer to 'LMW proteinuria disease in Japan' as Japanese Dent disease to eliminate confusion.

PATIENTS AND METHODS

Patient inclusion criteria

The criteria for inclusion of patients in this study were as follows:

(i) Extremely high-LMW proteinuria determined on the basis of the urinary level by either β2- microglobulin (β2-MG) or α1-microglobulin (α1-MG).

(ii) Absence of histories or clinical data indicative of underlying renal diseases that cause proximal tubular dysfunction, such as mitochondrial disorders, ingestion of nephrotoxic substances and other tubular diseases.

In Hoopes's criteria [20], hypercalciuria is essential for the clinical diagnosis of Dent diseases. However, because Japanese Dent disease patients with mutations in *CLCN5* or *OCRL1* are not necessarily accompanied by hypercalciura or nephrocalcinosis, we did not include hypercalciuria as an inclusion criterion.

When OCRL1 mutations were identified, the following were confirmed to exclude Lowe syndrome patients:

(i) Absence of cataract, which was confirmed by slit lamp examination by ophthalmologists.

(ii) No apparent neurological or mental abnormalities indicative of Lowe syndrome, such as mental retardation, muscular hypotonus and behavioral abnormalities.

All of the patients in this study were clinically diagnosed as having Japanese Dent disease by pediatric nephrologists, and referred to our institute for genetic examination.

Genetic analyses

Informed consent for participation in this study was obtained from the patients and their family members after the purpose, methods and potential risks were thoroughly explained to them. This study was approved by the Ethics Committee for Analysis of the Human Genome of Tokyo University Hospital. For each patient, direct sequencing of *CLCN5* was first conducted as described previously [15, 16]; when no mutation was identified, *OCRL1* was directly sequenced. The conditions of genetic analysis of *CLCN5* and *OCRL1* were the same as those described previously [10].

Clinical data

The most recent clinical data were collected from the attending pediatric nephrologists.

The clinical data including urinary β2-microgloburin, Ca/Cr ratio and urinary protein excretion are the mean of multiple measurements or single data. When the attending doctors informed us of the data of multiple measurements, we calculated the mean values, and used them for subsequent analyses. If the data of each patient were of single measurements, we used it. Approximately, two thirds of the data are the mean of multiple measurements.

RESULTS

Except for D-88 and D-48, all of the patients are males. The Patients, ages ranged from 0.3 to 66 years. The average age of patients was 12.8 years, which reflects that most Japanese Dent patients are diagnosed by the annual school urinary screening test.

Table 1 shows a summary of the mutations in the patients enrolled in this study. In 86 unrelated patients with Japanese

Table 1. Genetic background of 86 unrelated Japanese Dent Patients. (see also Tables 2–5)

	Types of mutations	Number of patients	Number of novel mutations
Dent 1 (*CLCN5* mutation)	Deletion	4	
	Frame shift	7	7
	Missense	27	15
	Nonsense	16	5
	Spice site	7	
	Total number (%)	61(71%)	
Dent 2 (*OCRL1* mutation)	Frame shift	1	
	Missense	9	4
	Nonsense	1	1
	Total number (%)	11(13%)	
Dent NI (mutation not identified)	Total number (%)	14(16%)	

Dent NI; Dent-NOT identified mutations in either *CLCN5* or *OCRL1*.

Dent disease, 61 (71%) possessed mutations in *CLCN5* (Dent-1) and 11 (13%) in *OCRL1* (Dent-2). In the remaining 14 patients (16%), no mutations in *CLCN5* or *OCRL1* were identified (Dent-NI: Dent-not identified). The mutations in each gene were divided according to the mutational type (Table 1). As is evident in Table 1, mutations in *CLCN5* (Dent-1) are diverse. In contrast, 9 of 11 *OCRL1* mutations are missense mutations, and their sites of mutations are concentrated in several codons (see also Table 3).

Table 2 shows description of the mutations identified in *CLCN5* in this study. The novel mutations that have not been reported elsewhere are indicated by #. Nearly half of the mutations identified in this study in *CLCN5* are novel (29 mutations: 48%). Mutations in *CLCN5* are scattered throughout the gene, whereas there are small numbers of hot spots of mutations in *CLCN5*, i.e. p.Arg34X, p.Arg637X, p.Arg704X, p. Ser244Leu and p.Arg516Trp. These mutations are detected in other countries and Japan.

Table 3 shows the genotypes and phenotypes of Dent-2 patients. Most mutations in *OCRL1* are missense mutations (9 out of 11, 80%). In this study, four mutations were found to be located at p.Arg318. Except p.Arg318Cys/His and p.-Arg493Trp, all of the other mutations in *OCRL1*, namely, c.46G>C, c.265266insGG, p.Leu136X, p.Lys293Glu, p.Arg493Trp, p.Phe689Ser and p.Pro829Leu are novel. Regarding p. Arg318Cys mutation in *OCRL1*, we examined the genotype of mothers of D-47 and D-111, and revealed that they do not possess this mutation. This result indicates that p.Arg318Cys mutation is *de novo* in these cases, and denies the founder effect of p.Arg318Cys. Moreover, pArg318 mutation is also reported in other countries. Taken together, p.Arg318 is the unique hot spot, whose missense mutation leads to Dent-2 phenotype.

Figure 1 illustrates the scheme of the primary structure of *OCRL1* gene, and mutations identified in Dent-2 diseases. The mutations depicted in box are those identified in the present study, and grey colored ones are novel mutations. As will be discussed later, most mutations in Dent-2 exist between exons 5 and 15. Thus far, only two missense mutations after exon 15, i.e. p.Glu737Asp and p.Pro799Leu, have been identified as the causative mutations in Dent-2. In the present study, we identified two more missense mutations at the 3′ side of exon 15; i.e. p.Phe680Ser and p.Pro829Leu, which exist in exons 18 and 23, respectively.

Table 3 shows the laboratory data and clinical phenotype of Dent-2 patients. In previous studies, it was shown that the levels of the serum muscle enzymes, creatinine phosphokinase (CPK) and lactate dehydrogenase (LDH) are relatively high. In this study, however, the level of serum CPK was not necessarily high; for example, the levels of serum CPK were very low in D-88 and D-105. Also, LDH level ranges from 205 IU to 578 IU. Thus, even in Dent-2 laboratory findings were not common, which might also reflect the phenotypical variance among *OCRL1* mutations.

Figure 2A shows the relationship between urinary excretion of β2-microglobulin (β 2-MG), and Figure 2B shows those between urinary β 2-MG and urinary Ca/Cr ratios of the patients of the present study. Since all of the clinical data, except for β2-microglobulin and α1-microglobulin, could not be collected in this study, we plotted the data of patients in whom both of these data could be analysed. As depicted in Figure 1, the urinary β2-microglobulin level is extremely high (more than several thousands, and mostly ten thousands to one hundred thousands). As shown in Figure 2B, patients with extremely high levels of LMW proteinuria do not necessarily show high calciuria. Figure 3 shows the data of urinary β2-MG among different mutations in *CLCN5* (Dent 1). We found no statistical difference among the mutational types (ANOVA, $P = 0.212$)

Table 4 shows the data of patients with renal impairment. Six patients (7%) developed definite renal dysfunction. Among these six patients, four had mutations in *CLCN5*. In the remaining two patients, D-79 and D-115, no mutations were identified in *CLCN5* or *OCRL1*. However, the extremely high level of LMW protein in the urine, and the exclusion of other renal diseases in these two patients are compatible with Dent disease.

Table 5 summarizes the important clinical characteristics of the Japanese Dent disease patients. The data are presented for genotype including the total counts. The data are compared with those previously reported in a review article [22]. Regarding hypercalciuria, the number of patients with hypercalciuria (urinary Ca/Cr mg/mg >0.2) was 37 out of 73 patients (51%). The urinary Ca/Cr ratio ranged from 0.016 to 0.192 in patients without hypercalciuria, and from 0.2 to 0.75 in those with hypercalciuria. Among mutational types, calciuria is present, 46% in Dent-1, 70% in Dent-2 and 56% in Dent-NI. If we define hypercalciuria as having a Ca/Cr ratio of more than 0.25, hypercalciuria was present in only 42% of Dent-1 patients. The correlation of ages of patients and the levels of urinary β2-MG were also analysed using linear regression analysis, and the results show that the relation of these two values is not significant ($r = 0.356$). The percentage of patients with hypercalciuria was apparently lower in Japan than in other countries. Nephrocalcinosis was only identified in 35% of the patients. As has been expected, the percentage of patients with renal failure was relatively low in Japan, which is partly due to the early diagnosis

Table 2. Each mutation of CLCN5. Novel mutations are depicted as asterisk (#)

Pt. ID	Mutation type	Exon (intron)	Nucleotide changes	Amino acid changes	Codon, site or portion of mutation
D-56	del	7	c.801_803del	p.Glu267del	267
D-83	del	10	c.1566_1568del	p.Val522del	522
D-96	del large	4, 5	Deletion of exons 4 and 5	Deletion of exons 4 and exon 5	Exons 4–5
D-51	del large	1_12	CLCN5 total del	CLCN5 total del	total del
D-124	fs	3	c.165_169del	# p.Phe55Leufs*41	55
D-18	fs	3	c.191del	# p.Ile64Metfs*7	64
D-50	fs	7	c.746_752del	# p.Ala249Aspfs*3	249
D-58	fs	9	c.1526del	# p.Ala509Alafs*3	509
D-85	fs	10	c.1537del	# p.Gly513Glyfs*2	513
D-98	fs	10	c.1668del	# p.Gly556Glyfs*30	556
D-37	fs	11	c.2081_2082insC	# p.Thr694Thrfs*48	694
D-70	mis	4	c.263G>T	# p.Gly88Val	88
D-109	mis	4	c.270C>G	# p.Cys90Trp	90
D-60	mis	4	c.307T>C	# p.Trp103Arg	103
D-125	mis	6	c.527T>A	# p.Ile176Asn	176
D-81	mis	6	c.608C>T	p.Ser203Leu	202
D-106	mis	6	c.631G>C	# p.Glu211Gln	211
D-104	mis	6	c.634G>A	# p.Gly212Ser	212
D-43	mis	6	c.638C>T	# p.Pro213Leu	213
D-63	mis	7	c.731C>T	p.Ser244Leu	244
D-55	mis	7	c.731C>T	p.Ser244Leu	244
D-36	mis	7	c.796C>G	# p.Leu266Val	266
D-117	mis	8	c.814T>A	# p.Tyr272Asn	272
D-103	mis	8	c.815A>G	p.Tyr272Cys	272
D-13	mis	8	c.834G>C	p.Leu278Phe	278
D-66	mis	9	c.1403T>C	# p.Leu468Pro	468
D-100	mis	9	c.1505A>G	# p.Tyr502Cys	502
D-42	mis	9	c.1516G>A	# p.Gly506Arg	506
D-69	mis	10	c.1537G>A	p.Gly513Arg	513
D-24	mis	10	c.1546C>T	p.Arg516Trp	516
D-39	mis	10	c.1546C>T	p.Arg516Trp	516
D-102	mis	10	c.1546C>T	p.Arg516Trp	516
D-112	mis	10	c.1547G>A	p.Arg516Gln	516
D-34	mis	10	c.1571T>A	p.Ile524Lys	524
D-68	mis	11	c.2108T>C	# p.Phe703Ser	703
D-27	mis	11	c.2117T>C	# p.Leu706Pro	706
D-44	mis	11	c.2133C>G	# p.Cys711Trp	711
D-119	mis	12	c.2173A>G	p.Lys725Glu	725
D-40	non	2	c.82C>T	p.Arg28*	28
D-64	non	2	c.100C>T	p.Arg34*	34
D-99	non	2	c.100C>T	p.Arg34*	34
D-61	non	4	c.277G>T	# p.Gly93*	93
D-54	non	4	c.370C>T	# p.Gln124*	124
D-65	non	6	c.566G>A	# p.Trp189*	189
D-38	non	8	c.836G>A	p.Trp279*	279
D-74	non	8	c.1039C>T	p.Arg347*	347
D-97	non	9	c.1467G>A	# p.Trp489*	489
D-77	non	10	c.1885C>T	# p.Gln629*	629
D-35	non	10	c.1909C>T	p.Arg637*	637
D-95	non	10	c.1909C>T	p.Arg637*	637
D-12	non	11	c.1942C>T	p.Arg648*	648
D-16	non	11	c.2110C>T	p.Arg704*	704
D-33	non	11	c.2110C>T	p.Arg704*	704
D-26	non	11	c.2110C>T	p.Arg704*	704
D-86	splice	(int 3)	c.206G-1G>A		int 3
D-67	splice	(int 4)	c.393+1G>A	#	int 4
D-15	splice	(int 4)	c.394-2A>C		int 4
D-101	splice	(int 4)	c.394-2A>G		int 4
D-46	splice	(int 5)	c.516+1G>A		int 5
D-45	splice	(int 8)	c.1347+1G>T		int 8
D-17	splice	(int 10)	c.1933+2_1933+3insTGGT	#	int 10

Del, deletion mutation; fs, frame shift mutation; mis, missense mutation; non, nonsense mutation; splice, splice donor site mutation.

Table 3. Each mutation of OCRL1. Novel mutations are depicted as asterisk (#)

Pt. ID	Age (years)	Mutation type	Exon	Nucleotide changes	Amino acid changes	CPK[a] (IU/L)	LDH[a] (IU/L)	Cataract	Intelligence	Behavior
D-108	4.2	fs	5	# c.265_266insGG	p.Asp89Glyfs[a]18	298	390	None	Normal	Normal
D-88	9.1	mis	2	# c.46G>C	p.Glu16Gln	62	357		Normal	Normal
D-105	0.3	mis	10	# c.877A>G	p.Lys293Glu	39	319	None		
D-21	24.8	mis	11	c.953G>A	p.Arg318His	116	205	None	Normal	Normal
D-28	9.5	mis	11	c.952C>T	p.Arg318Cys		210	None	Normal	Normal
D-47	9.6	mis	11	c.952C>T	p.Arg318Cys		578			
D-111	15.3	mis	11	c.952C>T	p.Arg318Cys	342	257			
D-120	3.4	mis	15	c.1477C>T	p.Arg493Trp					
D-30	17.4	mis	18	# c.2039T>C	p.Phe680Ser	299	224	(+)	Normal	Normal
D-49	9.0	mis	23	# c.2486C>T	p.Pro829Leu	255	268		Normal	Normal
D-118	4.1	non	6	# c.[407T>A; 408A>G]	p.Leu136[a]	192	274	None	Normal	Normal

Abbreviations are the same as in Table 3.
[a]Normal value of CPK and LDH.
CPK < 30~ 350 IU/L (more than 1 year).
LDH < 150~380 IU/L (more than 1 year).

and young age of patients, whereas in Europe and the USA, renal insufficiency develops after middle age. One of the most distinct sets of data that differs from the European countries and USA are the rate of rickets. Only two patients (2%) showed rickets in Japanese Dent patients, whereas in other countries it is 33%. In addition, in the present study, no apparent patients with Fanconi syndrome were identified (glucosuria, aminoaciduria, metabolic acidosis and phosphaturia).

DISCUSSION

The present study is the first large-scale comprehensive analysis of Japanese Dent disease in terms of both genetic background and clinical phenotype. The first important finding of this study is that the genetic background of patients with Japanese Dent disease is nearly identical to those in Europe and the USA. In other countries, *CLCN5* mutations were identified in ~60% in patients with Dent disease, and 15% in *OCRL1*. Among 86 unrelated Japanese Dent patients, 71% (61 patients) possess mutations in *CLCN5* (Dent-1), and 48% (29 patients) are novel; 13% possesses mutations in *OCRL1* (Dent-2) (Tables 1–3). In the remaining 16%, no mutations in *CLCN5* or *OCRL1* were identified (Dent-NI). The second critical finding is that there are large clinical differences, such as the incidence of hypercalciuria, nephrocalcinosis, urolithiasis and rickets, between patients in Japanese Dent disease and those in Europe/USA, despite the similarity of genetic background. For example, hypercalciuria, which is usually more prominent in children, was observed only in 51% of Japanese Dent disease patients, whereas in other countries it is nearly 90% (Table 5) [22]. Only two patients, both with Dent-2, developed rickets. Furthermore, apparent Fanconi syndrome was not identified in this Japanese cohort. The third is that several Japanese Dent disease patients developed renal impairment. This is somehow unexpected because most of Japanese patients are young, and did not necessarily have the complication of nephrocalcinosis or urolithiasis.

Regarding the *CLCN5* mutations, many novel missense, nonsense and frameshift mutations were identified in this study. As reported previously, mutations in *CLCN5* exist throughout the gene and are specific to each pedigree or individual. One of the critical concerns for these mutations, especially missense mutation, is the mechanism(s) by which each mutation disrupts the function of CLC-5. Recently, Smith *et al.* classified CLC-5 mutants into three classes [23]. Class 1 mutation, including p.Ser270Arg, p.Gly513Glu, p.Arg516Trp and p.Ile524Lys, results in the retention of CLC-5 in the endoplasmic reticulum; Class 2 comprises the functionally defective mutations, such as p.Glu527Asp and Class 3 mutations, p.Gly57Val and p.Arg280Pro, result in altered subcellular distribution of CLC-5. Grand *et al.* also examined several CLC-5 missense mutations identified in the patients, and divided the mutations into two types. Type I mutations depress chloride current, while they are actually expressed in the plasma membrane. Type II includes mutants that could not traffic to the membrane. At the moment, CLC-5 is considered to function as an chloride/proton antiporter and its role in endosomal acidification is unclear [24, 25]. Besides *in vitro* expression study, analyses using bioinformatics methods are useful in speculating the significance of each residue and the impact of its mutation. Wu *et al.* [26] reported that most missense mutations of CLC-5 are located in helices forming the subunit interface using a three-dimensional homology model of human CLC-5. Smith *et al.* [23] also reported that all of the examined mutants are located in the dimer interface.

In order to examine the impact of the missense mutation identified in the present study, we first made the alignment of CLC-5 protein among different mammalian species (see supplementary data S1). As expected, all of the residues, where missense mutations are identified, are conserved among all species tested. In parallel, we are examining several mutants by an *in vitro* expression system, and started to perform a bioinformatics analysis. Preliminary data indicate that novel mutations identified in this study are not necessarily located in the dimer interface of CLC-5. One characteristic mutation identified exclusively in this study is located in a narrow region at the C-terminus of CLC-5. This type of mutation includes p.Phe703Ser, p.Leu706Pro, p.Cys711Trp, p.Lys725Glu, p.Arg704X and p.Thr694Thrfs*48. The C-terminus is

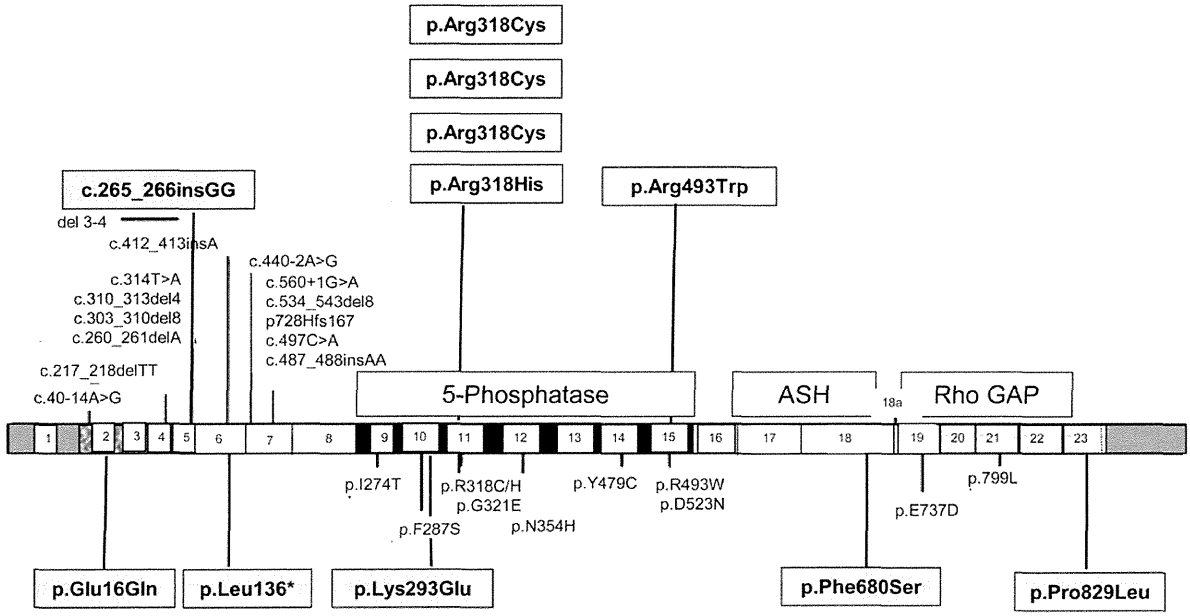

FIGURE 1: *OCRL1* mutations manifesting Dent-2 phenotype. Mutations in closed boxes are those identified in the present study. Mutations in white boxes are those previously reported, and those in gray boxes are novel mutations. The original figure in the report by Hichri [21] was modified and newly identified in this study mutations are added.

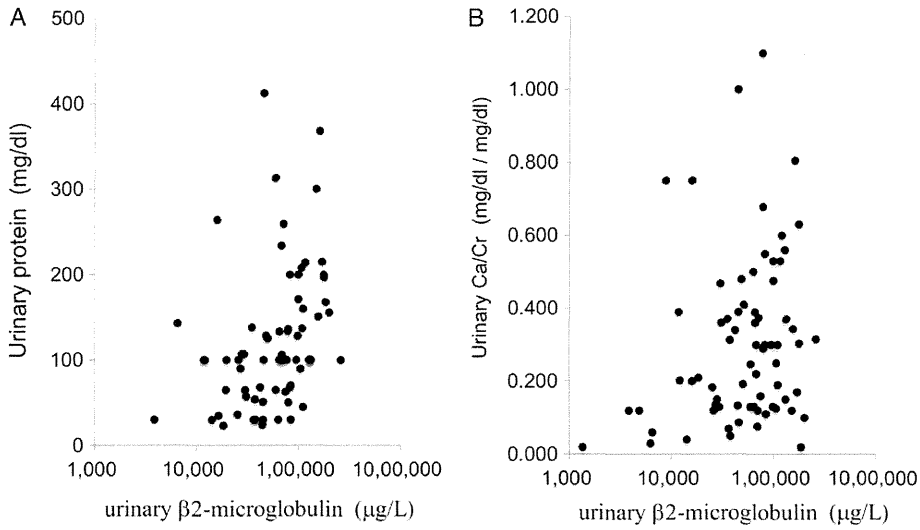

FIGURE 2: (A) Relationship between urinary β2-MG and urinary excretion of total protein. (B) Relationship between urinary β2-MG and urinary Ca/Cr ratio.

considered to bind ATP; as a result, conformational change in CLC-5 protein occurs and functions as proton/chloride channel. Thus, these mutations in the C-terminus would result in disruption of ATP binding, and abolish CLC-5 function. In the present study, we identified 15 novel missense mutations including C-terminus in *CLCN5*. In the next study, we would obtain further clues to elucidate the structure–functional relationship of CLC-5.

Regarding *OCRL1* mutations, six, i.e. p.Asp89Glyfs*18, p.Glu16Gln, p.Lys293Glu, p.Phe680Ser, p.Pro829Leu and p.Leu136*, are novel mutations in Dent-2. The reason why mutations in *OCRL1* lead to Dent disease or Lowe syndrome still remains unclear. Two hypotheses have been proposed. The first is that all of the frameshift mutations or nonsense mutations identified in Dent-2 are clustered in the 5′ region of *OCRL1* (from exons 1 to 7); missense mutation identified in Dent-2 are all found in exons 9–23, mostly between 9 and 15, which comprise a catalytic phosphatase domain [27]. Hichri *et al.* [27] also indicate the same hypothesis [21]. Using bioinformatics analysis, Shrimpton *et al.* proposed an alternative

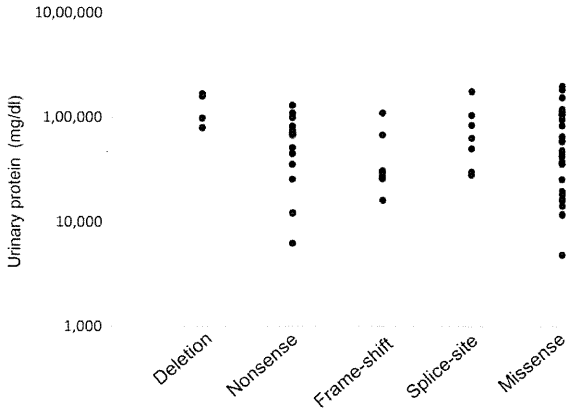

FIGURE 3: The level of urinary β2-MG among the mutational types of *CLCN5*. There are no statistical difference among the mutational types (ANOVA, P = 0.212).

splicing variant of OCRL1 whose translation methionine is Met189. All the six novel *OCRL1* mutations identified in this study follow this rule, and reinforce this hypothesis. In particular, p.Phe680Ser and p.Pro829Leu, which are located outside the catalytic domain (exons 8–15), might be useful for solving this question. The second hypothesis is that Dent-2 is a variant and a mild phenotype of Lowe syndrome, and there are some modifying molecules (e.g. compensatory phosphatase and interacting proteins) whose expression and function depend on the genetic background of other molecules, such as INPP5B [28]. Indeed, Hichri *et al.* reported that one patient with a p.Ile274Thr missense mutation developed bilateral congenital cataract, while his elder brother with the same mutation showed no ocular abnormalities [21]. Pasternack *et al.* also reported a 13-year-old patient with a nonsense mutation (p.Gln199X) in exon 8 of OCRL1, who developed a typical cerebro-renal phenotype of Lowe syndrome, but without any ocular involvement [29]. These results indicate selective organ involvement among *OCRL1* mutations [30]. Except for Dent-2 and Lowe phenotype, there are large clinical variabilities in clinical manifestations of patients with *OCRL1* mutations [28]. We recently experienced several patients with congenital cataract and mental retardation, whereas there is no or only extremely mild renal manifestation. In these patients, we did not identify mutations in *OCRL1*. Thus, as Dent-2 and renal manifestation in Lowe syndrome, other molecules might participate in the proximal function.

In Dent-2, the characteristic laboratory findings are elevated levels of serum CK and LDH. Bökenkamp *et al.* reported that the levels of serum CK and/or LDH are elevated in 31 patients with Dent-2 and Lowe syndrome. However, D-88, D-105, D-21 and D-118 showed normal levels of serum CK and LDH when considering their ages [27]. Thus, the elevation of serum CK and/or LDH levels does not necessarily occur in patients with Dent-2.

Whether Japanese Dent disease patients would develop renal dysfunction is one of the important issues. Igarashi *et al.* [14] reported a case of a 51-year-old Japanese Dent disease patient with a splice donor site mutation in *CLCN5* who

Table 4. Clinical data of the patients who develop renal dysfunction

Dent No.	Dent type	CLCN5 mutation	Patients' age (years)	Serum Cr	BUN	Ca	IP	Urinary Ca/Cr (mg/mg)	Renal calcification	Urolithiasis	Urinary-B2MG (<270 μg/L)
D-38	Dent-1	p.Trp279*	24.3	1.58	13	9.7	3.4	0.12	Presence	None	70 036
D-34	Dent-1	p.Ile524Lys	20.7	1.63	14	10	3.8	0.39	None	None	11 791
D-37	Dent-1	p.Thr694Thrfs*48	19.4	5.33	44.1	9.7	3.8	0.12	Presence		25 800
D-16	Dent-1	p.Arg704*	20.2	1.61	16	9.8	1.4	0.183	Presence	None	25 347
D-115	Dent-NI		24.0	12.8	101.7			0.087	Presence	None	45 800
D-79	Dent-NI		59.0	2.36	14			0.34	None	Presence	α1-MG: 165

Table 5. Comparison of main clinical data of Japanese Dent patients and those reported so far

Clinical/biochemical characteristics	Previously reported data: Dent-1*	Dent disease in Japan (incidence/determined)			
		Total	Dent-1	Dent-2	Dent-NI
LWMP	100 (%)	100% (86/86)	100% (61/61)	100% (11/11)	100% (14/14)
Hypercalciuria	89 (%)	51% (37/73)	46% (25/54)	70% (7/10)	56% (5/9)
Nephrocalcinosis	76 (%)	35% (26/75)	38% (20/53)	10% (1/10)	42% (5/12)
Renal failure	42 (%)	8% (6/74)	8% (4/53)	0% (0/10)	18% (2/11)
Rickets/osteomalacia	33 (%)	2% (2/86)	0% (0/61)	9% (1/11)	7% (1/14)

developed end-stage renal failure at 46 years of age. To date, this is the sole case of apparent renal dysfunction in Japanese Dent disease. In the present study, at least 6 (7%) out of 86 patients developed definite renal dysfunction. Among these six cases, four patients possessed mutations in *CLCN5*. Although the remaining two patients, D-79 and D-115, showed no mutations in *CLCN5* or *OCRL1*, clinical findings were compatible with Dent disease. In addition, D-18, D-22 and D-20 might have subclinical levels of renal impairment. Thus, the present findings clearly demonstrate that Japanese Dent disease patients have the risk of developing renal impairment. Except D-79, all of the patients with renal dysfunction were younger than 25 years. In addition, it should be noted that among these six patients with renal impairment, two (D-34 and D-34) showed hypercalciuria (urinary Ca/Cr mg/mg >0.2) and four showed nephrocalcinosis. Thus, hypercalciuria and/or nephrocalcinosis is not necessarily essential for the development of renal insufficiency in Japanese Dent disease patients. Just recently, Frishberg *et al.* reported three unrelated familial cases of Dent-1. Renal biopsy specimens of two unrelated boys revealed focal segmental glomerulosclerosis and/or focal global glomerulosclerosis; tubulo-interstitial changes were minimal [31]. In other reports, Copelovitch *et al.* described precise renal pathological findings in two Dent disease patients with *CLCN5* mutation [32]. In both patients, despite the minimal tubulo-interstitial alterations, FGS was prominent. In one patient, two to three glomeruli out of six showed segmental sclerosis; only one glomerulus was completely normal. Thus, the mechanisms by which patients with Dent disease develop renal dysfunction do not only owe to nephrocalcinosis, but some direct effects of mutations in *CLCN5* or *OCRL1* on renal function might exist. This hypothesis should be challenged in a future study.

The criteria proposed by Hoopes are generally accepted for the clinical diagnosis of Dent disease. Hoopes's criteria include two essential data, i.e. LMW proteinuria and hypercalciuria [20]. In addition, symptoms such as nephrocalcinosis, urolithiasis, hematuria and renal impairment would strengthen the diagnosis [20]. Indeed, accumulated clinical and biochemical data on Dent disease are compatible with Hoopes's criteria. In contrast, hypercalciuria is observed only in 51% of Japanese Dent patients. As has been reported, hypercalciuria is more prominent in young patients than in elderly patients. In Japan, most patients are diagnosed with Dent disease before 15 years of age. Thus, in this population, hypercalciuria should exist in most of the patients. Why did this difference in the clinical spectrum arise? The most probable explanation is the diagnosis of Japanese Dent disease by the annual school urinary screening test. Considering the common molecular basis of Dent disease between patients in Japan and other countries, and the high sensitivity Japanese urinary screening system, 'LMW proteinuria in Japan' has a wider clinical spectrum than Dent disease. In other words, patients with only LMW proteinuria and mutations in *CLCN5* or *OCRL1* might be missed in other countries. In addition, the degree and incidence of hypercalciuria, nephrocalcinosis, urolithiasis and renal impairment in Japanese Dent disease patients are lower than those in Europe/USA and this might be partially owing to the content of the water. In Japan, drinking water contains very low levels of minerals, especially calcium. This is true throughout Japan, except Okinawa prefecture, and the reference data for calciuria are urinary Ca/Cr ratio and normal level, these data are <0.2 in Japan.

In conclusion, the present study demonstrated the genetic and clinical backgrounds of Japanese Dent disease. Japanese Dent disease, in other words 'LMW proteinuria disease', appears to include a wider clinical spectrum than Dent disease in Europe and the USA, although both diseases showed the same genetic background. In addition, many novel mutations in *CLCN5* and *OCRL1* identified in this study would provide clues to the molecular mechanisms underlying Dent disease and the mechanisms of renal impairment.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

On behalf of all authors, T.S. declares no competing interests that should be disclosed.

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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),

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.

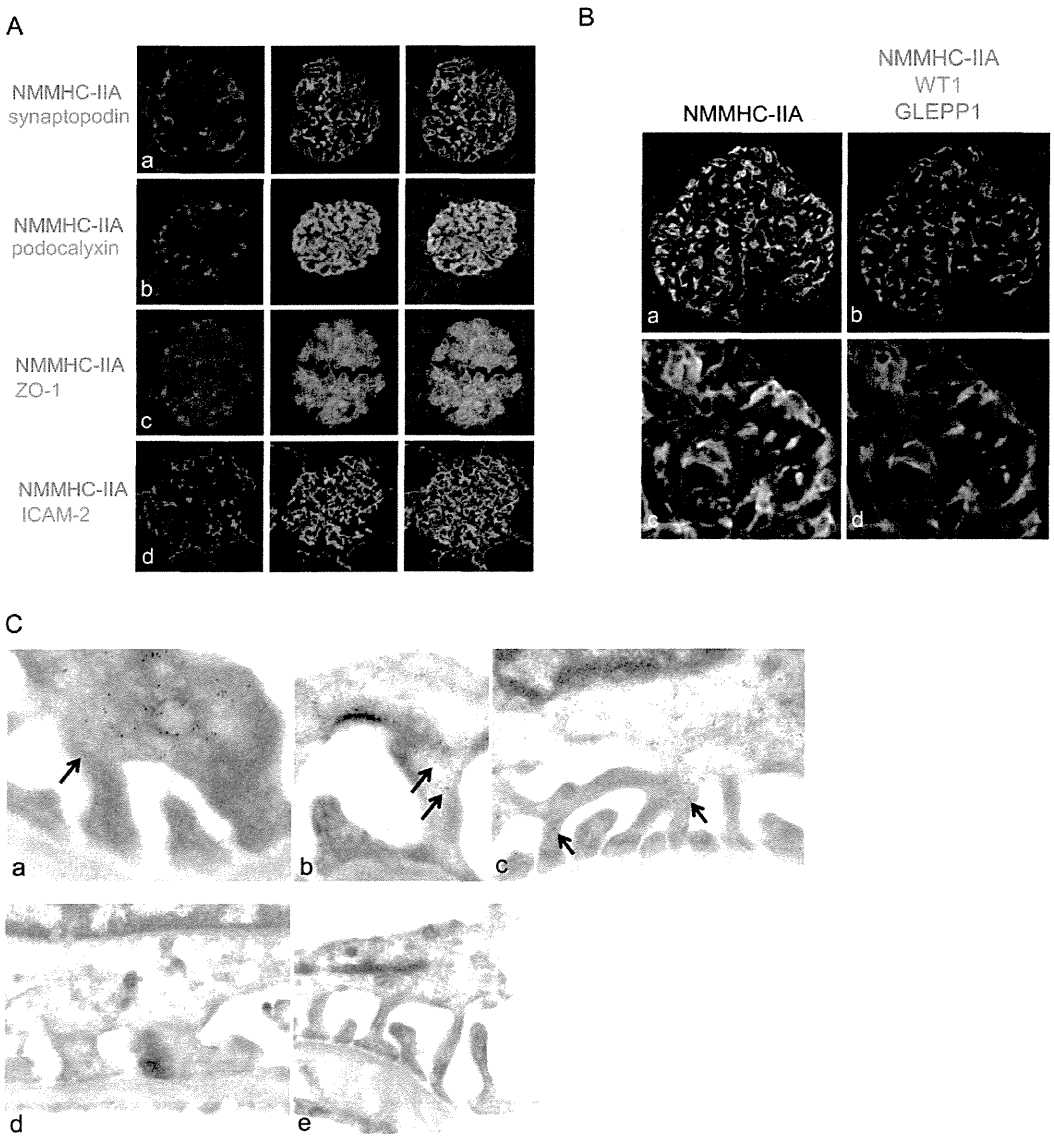

FIGURE 1: Localization of NMMHC-IIA. (**A**). Cryostat sections (5 μm thick) of the normal rat kidney were co-stained with an antibody specific for NMMHC-IIA (red) and other podocyte-associated molecules (green), synaptopodin (*a*), podocalyxin (*b*) and zonula occludens (ZO)-1 (*c*). Intercellular adhesion 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

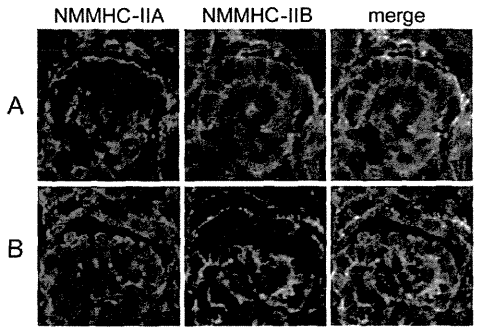

FIGURE 2: Localization of NMMHC-IIA and NMMHC-IIB in neonatal rat glomeruli. Cryostat sections at the S-shaped stage (**A**) and at the capillary loop stage (**B**) were co-stained with an anti-serum specific for NMMHC-IIA and NMMHC-IIB. The merged image showing the extent of co-localization is on the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

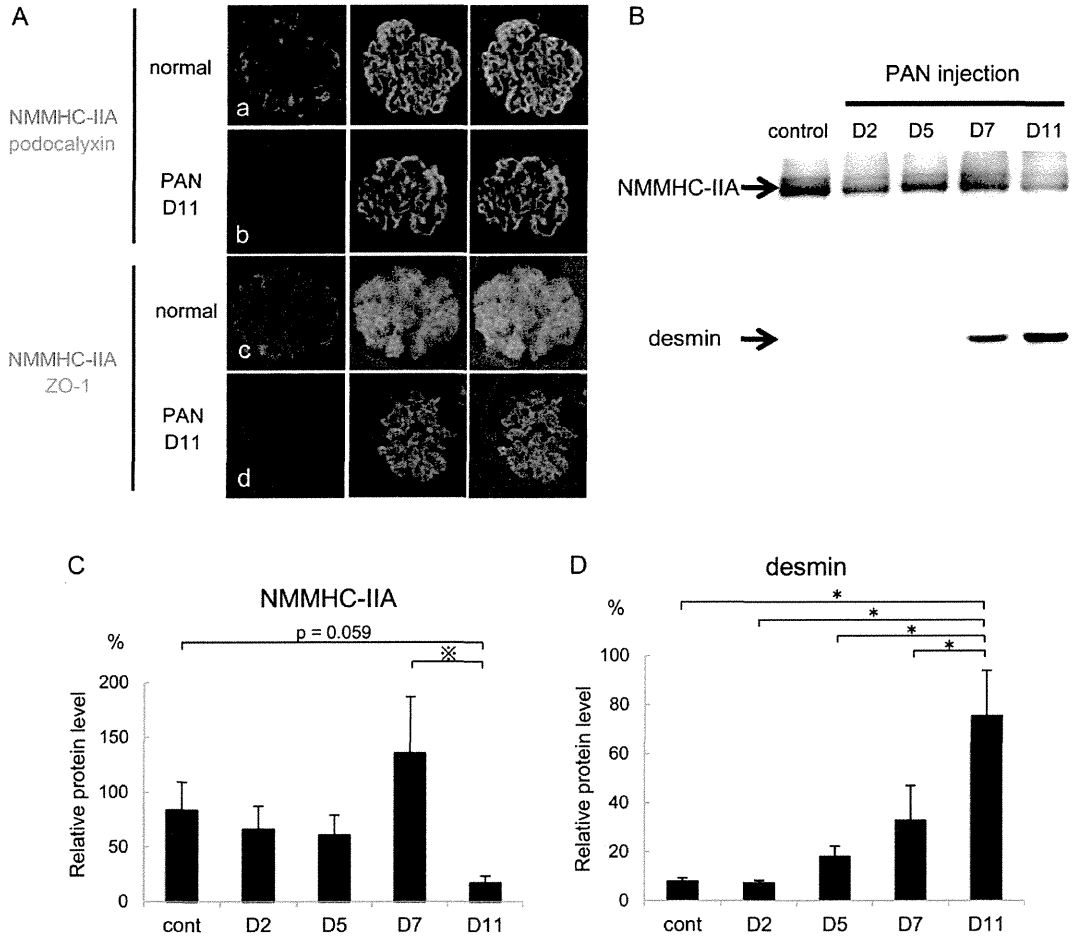

FIGURE 3: Expression of NMMHC-IIA in puromycin aminonucleoside (PAN)-treated rat kidney. (**A**) Immunofluorescent study of NMMHC-IIA in PAN-treated rats. Cryostat sections (45 μm thick) were co-stained with an anti-serum specific for NMMHC-IIA (red) and other podocyte-associated molecules (green), podocalyxin (*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