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Genetic background and clinicopathologic features of adult-onset nephronophthisis

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Title Page

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Conflict of Interest Statement

T.M. reports personal fees from honoraria for lectures, personal fees from Employment, outside the submitted work. J.H. has received a research grant from Otsuka Pharmaceutical Co. The other authors declare that they have no relevant financial interests.

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Running head: Genotype-phenotype of adult-onset nephronophthisis

Keywords: nephronophthisis; renal cystic disease; renal pathology; adult-onset kidney disease; chronic kidney disease; human genetics

Abstract

Introduction

Recently, nephronophthisis is considered to be a monogenic cause of end stage renal disease in adults. However, adult-onset nephronophthisis is difficult to accurately diagnose and has not been reported in a cohort study. In this study, we assessed the genetic background and clinicopathologic features of adult nephronophthisis.

Methods

We investigated 18 sporadic adult patients who were suspected as nephronophthisis by renal biopsy. We analyzed 69 genes that cause hereditary cystic kidney disease, and compared clinicopathologic findings between patients with and without pathogenic mutations in nephronophthisis-causing genes.

Results

Seven of 18 patients had pathogenic nephronophthisis-causing mutations in *NPHP1*, *NPHP3*, *NPHP4*, or *CEP164*. Compared with patients without pathogenic mutations, those with pathogenic mutations were significantly younger but did not significantly differ in the classic nephronophthisis pathologic findings, such as tubular cyst. On the

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other hand, the number of tubules with thick tubular basement membrane (TBM) duplication, which was defined as >10-μm thickness, was significantly higher in patients with genetically proven adult nephronophthisis than in those without pathogenic mutations. Alpha smooth muscle actin positive myofibroblasts were detected inside thick TBM duplication.

Conclusions

In adult patients with nephronophthisis, thick TBM duplication was the specific finding. Our analysis also suggested that older patients tended to have no pathogenic mutations, even when they were suspected to have nephronophthisis by renal biopsy. These findings could be the novel clinical clue for the diagnosis of nephronophthisis in adult patients.

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Main Text

Introduction

Nephronophthisis (NPH) is an autosomal recessive kidney disease and is the most frequent genetic cause of pediatric end stage renal disease (ESRD) [1–3]. In principle, molecular genetic analysis is currently the only method for the accurate clinical diagnosis of NPH [3,4]. To date, more than 25 different genes have been found to be associated with NPH [3]. Because of the increasing number of NPH genes identified, Sanger sequencing has become more tedious and costly, and comprehensive mutation analysis using next generation sequencing (NGS) is now required [5]. Recently, we have developed a target panel of genes that are related with inherited renal cystic diseases, including NPH [6]. This system enables us to perform comprehensive mutation analysis of NPH. However, in the real clinical setting, genetic testing is not performed routinely because of the limited availability of sequencing platforms and the associated high cost.

Recently, advances in genetics have revealed the importance of adult-onset NPH. In 2018, a Dutch study on five international cohorts reported that 26 of 5606 patients (0.5%) with adult-onset ESRD showed homozygous *NPHP1* deletions [7], suggesting that

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NPH is a relatively frequent monogenic cause of adult-onset ESRD. Although the importance of NPH in adult patients has been increasing, only few case reports are available and there had been no clinical cohort studies on adult NPH, probably because of difficulty in the diagnosis.

In a real clinical setting around adult-onset NPH patients, a major clinical problem is difficulty in the accurate diagnosis. In the Dutch study mentioned above, only three (12%) patients were correctly diagnosed as NPH and the other 88% were misdiagnosed as other kidney diseases or were defined as having chronic kidney disease (CKD) with unknown etiology. Several factors make the diagnosis of NPH very difficult in adult patients. First, the extrarenal abnormalities in NPH are fewer in adult patients than in pediatric cases [7–11]. In addition, the clinical and radiologic symptoms of NPH are unspecific, compared with those in the more common causes of CKD [9–11]. Therefore, most cases of adult-onset NPH are suspected only after renal biopsy [11,12]. However, although the renal histology exhibits a characteristic triad of corticomedullary cysts, tubular basement membrane (TBM) disruption, and tubulointerstitial nephropathy[1,13], it is not disease-specific and is commonly seen in any chronic

tubulointerstitial disorder [4,14]. This absence of specific histologic findings makes the correct diagnosis of adult NPH more difficult.

Even in adult patients, accurate diagnosis of NPH is important because management options, such as kidney transplantation and appropriate genetic counseling, are available [12]. Therefore, knowledge on the specific clinical and histologic findings in adult NPH is highly required. Furthermore, the Dutch study only investigated complete *NPHP1* deletion in adult NPH using GWAS data [7]. Although complete *NPHP1* deletion is responsible for 20% of pediatric NPH cases [2–4], more than 25 genes have been reported to cause NPH [3]. Therefore, adult NPH cases may be caused by other kinds of mutations in different genes. However, because only few case reports are available, the genetic background of adult patients with NPH remains unknown.

The present study aimed to assess the genetic background and clinicopathologic features of adult NPH, wherein adult patients who were suspected to have NPH on renal biopsy were analyzed.

Methods

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Patients

We investigated 18 adult patients who were suspected to have NPH by renal biopsy. After the pathologists in each institution suspected NPH based on the presence of tubular dilatation or TBM thickening and lamellation, the clinicians consulted us for genetic testing of NPH. All patients had no extrarenal findings, such as retinitis pigmentosa and liver function disorder, and no family history of autosomal dominant CKD. Patients younger than 17 years were excluded. The patients were recruited at 16 institutions in Japan between 2015 and 2019. This study was approved by the research ethics committee of each institution.

The clinical data of the patients at the time of renal biopsy were collected from the medical records. Estimated glomerular filtration rate (eGFR) was calculated using the Japanese glomerular filtration rate equation [15]. Liver or renal cyst was defined as the presence of at least one cyst detected by computed tomography (CT) or magnetic resonance imaging (MRI).

Genetic analysis

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Comprehensive genetic testing was performed using capture-based NGS of 69 genes that cause nine types of hereditary cystic kidney disease, including NPH; NPH-related ciliopathies (Joubert syndrome, Meckel syndrome, Senior–Løken syndrome, Bardet–Biedl syndrome, and skeletal ciliopathies); autosomal dominant polycystic kidney disease; autosomal recessive polycystic kidney disease; and autosomal dominant tubulointerstitial kidney disease [6] (Supplementary Table S1). The detailed methods are described in Supplementary Methods, as well as in our previous reports [6,16].

To detect large genomic rearrangements, such as gross deletions or duplications, copy number variation (CNV) analysis was conducted using Copy Number Analysis for Targeted Resequencing (CONTRA; http://contra-cnv. sourceforge.net/) [17]. If homozygous entire deletion of *NPHP1* was detected by CNV analysis, we performed polymerase chain reaction (PCR) for exons 1, 10, and 20 of *NPHP1*. The primer sequences are shown in Supplementary Table S2.

Pathologic assessment

For each patient, the tissue slides that were stained with hematoxylin & eosin, periodic

acid-methenamine silver, and periodic acid-methenamine silver (PAM) stain were digitized using the NanoZoomer HT Scan system (Hamamatsu Photonics, Hamamatsu, Japan). Whole standard glass slides were scanned at 40× magnification (0.23 μm/pixel).

We defined three types of pathologic findings that were classically known to be specific for NPH; these included tubular diverticulum, tubular floret, and tubular cyst (Supplementary Figures 1 a–c) [13,14]. For tubular diverticulum, tubular lumens that extended through the long axis of the tubule were excluded. Tubular floret was defined as branching in at least four directions (Supplementary Figure 1a) [14]. Tubular cyst was defined as having >200 μm in diameter (Supplementary Figure 1c) [14]. Atrophic tubules, which were defined according to the Banff working classification [18], were excluded. In addition, we defined thick TBM duplication as thickness of >10 μm. Tubules with >50% fibrosis within the thick TBM duplication were excluded.

A nephrologist and a pathologist blindly assessed the pathologic findings using the tissue slides with PAM stain. First, they counted the number of the three types of tubules (i.e., tubular diverticulum, tubular floret, and tubular cyst) in the specimen of

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each patient. Thereafter, they assessed and calculated the number of tubules that had thick TBM duplication, as follows:

number of tubules with thick TBM duplication = (number of tubules with thick TBM duplication/ total number of counted tubules) $\times$ 10

The cumulative number of the three types of counted tubules was noted.

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Low-vacuum scanning electron microscope and immunofluorescence analysis

To investigate what the essence of thick TBM duplication was, we performed low-vacuum scanning electron microscope (LVSEM) (Hitachi, Tokyo, Japan) analysis and immunofluorescence (IF) analysis in the representative cases with or without pathogenic mutations. For LVSEM analysis, 5-μm formalin-fixed paraffin-embedded (FFPE) sections were stained with periodic acid-methenamine silver to evaluate the tubular basement membrane as described previously [19,20]. For IF analysis, monoclonal antibody against alpha smooth muscle actin (α-SMA) (Merck KGaA, Darmstadt,

Germany) for myofibroblast and *Phaseolus vulgaris* (PHA-E) (Vector Laboratories, Inc., CA, USA) for proximal tubules, respectively, were used in FFPE sections. Streptavidin, Alexa Fluor 488 conjugate and goat anti-mouse IgG (H+L), Alexa Fluor 568 (Thermo Fisher Scientific Inc, USA) were used and sections were counterstained with DAPI. IF images were captured using BZ-X800 (KEYENCE, Osaka, Japan).

Statistical analysis

All statistical analyses were performed using JMP version 15 (SAS Institute, Cary, North Carolina). Non-normally distributed variables were expressed as median with interquartile range. The Mann–Whitney U-test was used to compare the medians of continuous variables, such as age, and the chi-square test was used to compare the percentages of categorical variables, such as sex, between patients with pathogenic mutations and those without pathogenic mutations. A P value of <0.05 was considered statistically significant.

Results

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Patient characteristics

The patient characteristics are presented in Table 1. The median age at renal biopsy was 52 years; 6 patients (33%) were men and 11 patients (61%) had hypertension. For the renal manifestations, 14 patients (78%) had proteinuria. The median eGFR was 18.4 mL/min/1.73 m^2. CKD was stage 3 in three patients (17%), stage 4 in eight patients (44%), and stage 5 in seven patients (39%). No patient received dialysis. Nine patients had at least one renal cyst identified on imaging, such as CT or MRI.

Panel-based genetic diagnosis of NPH

A summary of the results of our comprehensive genetic testing is presented in Figure 1. Of the seven patients (39%) who had pathogenic mutations related with NPH, four patients had mutation in *NPHP1* and the other three patients had compound heterozygous mutations in *NPHP3*, *NPHP4*, and *CEP164*, respectively. Notably, to the best of our knowledge, the patient with *CEP164* mutation was the first case in an adult. Of the patients with *NPHP1* mutation, three patients (patient numbers 883, 896, and 1207) were detected to have homozygous entire deletion of *NPHP1* by CNV analysis.

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The regions that were expected to be identified by CNV analysis are shown in Supplementary Table S3. In all three patients, no PCR products were detected in exons 1, 10, and 20 of *NPHP1*; this confirmed complete gene deletion of *NPHP1*. The other patient (patient number 1107) had homozygous nonsense mutation in *NPHP1* (Supplementary Table S4). The details of the mutations in the patients with compound heterozygous mutations in *NPHP3*, *NPHP4*, and *CEP164* are shown in Supplementary Table S4.

Clinical characteristics of adult NPH patients

As shown in Table 2, the patients with pathogenic mutations were significantly younger, compared with those who had no pathogenic mutations (median age, 26 years vs. 63 years, P = 0.01). Interestingly, no patient who was >50 years old at the time of renal biopsy had pathogenic mutations (Figure 2). In addition, compared with patients who had no pathogenic mutations, those with pathogenic mutations had significantly higher proportion of men (71% vs. 9%, P = 0.01) and significantly lower incidence of hypertension (29% vs. 82%, P = 0.049) but had similar eGFR, level of proteinuria, and

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number of renal or liver cysts (at least one for each).

Pathologic findings specific for adult-onset NPH

The three pathologic findings that are known to be specific for NPH were not significantly different between the adult patients with NPH and those who did not have a mutation causing NPH (Table 2). Therefore, we further searched for novel pathologic findings that were specific to adult NPH cases. We focused on TBM duplication, which was reported to be another specific finding in NPH [13,14]. Although TBM duplication was reported in noninherited renal diseases [14], we noticed that the TBMs were quite thick and reduplicated in genetically proven adult NPH cases. Therefore, we focused on the thickness of TBM duplication; we defined thick TBM duplication as >10 μm (Figure 3). Interestingly, the number of tubules with thick TBM duplication was significantly higher in genetically proven adult NPH than in those who did not have pathogenic mutation (Table 2).

For investigating the ultrastructure and components of thick TBM duplication, we observed renal tissues of representative cases using LVESM and IF. In LVSEM

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observations, thick TBM duplications were detected in the patient with homozygous entire deletion of *NPHP1* (patient number 896) (Figure 4a and 4b). Furthermore, from immunofluorescence results, α-SMA positive myofibroblasts were detected inside thick TBM duplication, in addition to vascular smooth muscle cells (Figure 5a and 5b). On the other hand, in the patient who had no pathogenic mutations related with NPH (patient number 669), thick TBM duplications were not detected in LVSEM analysis (Figure 4c). Additionally, α-SMA positive myofibroblasts were detected only in vascular smooth muscle cells by IF observation (Figure 5c).

In general, IgA nephropathy, diabetic nephropathy, and tubulointerstitial nephritis are known to have tubular interstitial lesions. Therefore, we assessed for the presence of tubules with thick TBM duplication in these noninherited renal diseases (Supplementary Table S5). Among the nine patients, the diagnoses by renal biopsy were IgA nephropathy in four patients, diabetic nephropathy in three patients, and tubulointerstitial nephritis in two patients. Although most patients had >30% of tubulointerstitial fibrosis, we could not find any tubule that had thick TBM duplication in these samples. Therefore, thick TBM duplication could be specific for NPH.

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Discussion

To the best of our knowledge, this was the first cohort study to investigate the clinicopathologic findings and genetic background of adult patients with NPH. Through genetic analysis of 18 adult patients who were suspected to have NPH by renal biopsy, we found pathogenic mutations related with NPH in 7 patients. Compared with patients who had no pathogenic mutations, adult patients who had genetically proven NPH were significantly younger and had significantly higher proportion of men, significantly lower incidence of hypertension, and significantly higher number of tubules with thick TBM duplication.

In this study, the pathogenic mutations in the seven patients with NPH were in the genes *NPHP1*, *NPHP3*, *NPHP4*, and *CEP164*. In a genome-wide association study on adult-onset ESRD patients, Snoek et al analyzed only homozygous *NPHP1* full gene deletions using generated genomic data [7]. However, based on our results, four of seven genetically confirmed NPH cases had pathogenic mutations other than homozygous *NPHP1* full gene deletions. Moreover, mutations in the *CEP164* were reported to cause

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Senior–Løken syndrome or Joubert syndrome [21,22]. In this study, patient number 930, who was a 26-year-old man, had compound heterozygous mutations in *CEP164*. To the best of our knowledge, this was the first reported case of adult NPH with *CEP164* mutation. These findings strongly indicated that comprehensive genetic testing may be useful for adult patients suspected to have NPH.

In our study, we detected αSMA positive cells in the thick TBM duplication. αSMA is commonly used as a marker for myofibroblasts and the resulting fibrosis [23]. Interestingly, through the study of primary cilia in cultured epithelial cells, primary cilia undergo a dynamic biphasic change during epithelial-myofibroblast transition as well as fibroblast-to-myofibroblast transition induced by transforming growth factor-β [24]. Furthermore, the study of inhibition of ciliogenesis demonstrated that deficiency of primary cilia induces epithelial to mesenchymal transition [25]. Therefore, as it is well known that products of genes causing NPH are localized at segments associated with primary cilia, it could be possible that abnormal primary cilia in NPH patients result in increased alpha-SMA positive myofibroblasts and fibrosis, resulting in thick TBM duplication.

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In this study, adult patients with genetically proven NPH were significantly younger, compared with those who had no pathogenic mutations, at the time of renal biopsy. Snoek et al analyzed 5606 patients with adult-onset ESRD and revealed that 26 (0.5%) of the patients had homozygous *NPHP1* full gene deletions [7]. Of the patients who were >50 years old at the time of initiation of renal replacement therapy, only two had homozygous *NPHP1* full gene deletions. Similar to Snoek's report, our study showed that no patient who was >50 years old at the time of renal biopsy had pathogenic NPH mutations. These finding suggested that older patients tended to have no pathogenic mutations, even if they were suspected to have NPH by renal biopsy.

In this study, the incidence of hypertension was significantly higher in patients without pathogenic mutations than in those who had pathogenic mutations. Hypertensive nephrosclerosis is a disorder that is usually associated with disease chronicity. The renal pathologic features of arteriolar nephrosclerosis are characterized by the involvement of arteries, arterioles, glomeruli, and the tubulointerstitium [26]; the presence of chronic tubular and interstitial lesions in the form of tubular atrophy and interstitial fibrosis [26,27]; and lamellated TBMs in atrophic tubules [28]. In this study, the

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patients who had no pathogenic mutation tended to have a relatively high number of tubular cysts. In aging kidneys, tubular diverticulum is often observed and is a probable source of renal cysts [29–31]. Therefore, in patients without pathogenic mutations, tubular disorders can be caused by secondary factors, such as hypertension and aging. Moreover, we found that patients with pathogenic mutations included a relatively high proportion of men. However, in the Dutch study about adult-onset ESRD, 12 of 26 patients who had homozygous *NPHP1* deletion were men [7], demonstrating no sex difference. Further study will be required to confirm this observation.

This study had several limitations. First, sample size of our study was small. Nevertheless, only few adult cases of suspected NPH by renal biopsy have been reported [7–9,11], and no cohort studies on adult NPH cases have been available. Considering that there were only 26 patients with homozygous *NPHP1* deletions even in the study using nationwide data from five countries [7], number of patients in our study could be reasonable. Therefore, although we collected samples from all over Japan, this number of study patients was inevitable. Furthermore, to the best of our knowledge, this was the first study to investigate the clinicopathologic findings and genetic background

of adult patients who were suspected to have NPH. We expect an increase in the number of cases in the future. Second, in our study, 2 of 7 patients with mutations in the genes related with nephronophthisis had only novel missense mutations. Therefore, we could not exclude the possibility that these missense mutations were not disease causing mutations. Third, we could not analyzed other genes that had been identified very recently as rare causal genes for NPH-related ciliopathies, such as *C8orf37*[32], *KIAA0586*[33], and *MAPKBP1*[34]. However, the phenotypes of the patients with mutations in these genes were Bardet–Biedl syndrome, Joubert syndrome, or NPH with extrarenal findings. Therefore, considering that all patients in this study do not exhibit any extrarenal findings, it is unlikely that our 11 patients without identified pathogenic mutations have mutations in these genes.

In conclusion, our analysis showed that older patients tended to have no pathogenic mutations, even if they were suspected to have NPH by renal biopsy. On pathology, the number of tubules with thick TBM duplication could be an effective measure to diagnose NPH in adult patients. In addition, comprehensive genetic testing with a panel system could be useful for adult patients suspected to have NPH.

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Disclosure

T.M. reports personal fees from honoraria for lectures, personal fees from Employment, outside the submitted work. J.H. has received a research grant from Otsuka Pharmaceutical Co. The other authors declare that they have no relevant financial interests.

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Supplementary Material (PDF)

Supplementary Methods.

Supplementary Methods References.

Supplementary Table S1. Disease categories and the targeted genes included in this panel.

Supplementary Table S2. Primer sequences of *NPHP1*.

Supplementary Table S3. Detected copy number variants in *NPHP1*.

Supplementary Table S4. Mutations in the genes related with nephronophthisis.

Supplementary Table S5. Clinical characteristics of the pathologic control group.

Supplementary Figure 1. Representative pathologic findings.

Supplementary information is available at KI Report's website.

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Tables

Table 1. Characteristics of patients at the time of renal biopsy

Characteristics	N = 18
Age, years	52 (25.8–74.5)
Male	6 (33)
Hypertension	11 (61)
Proteinuria	14 (78)
Serum Cr, mg/dL	2.4 (1.60–3.66)
eGFR, mL/min/1.73 m^2	18.4 (10.4–29.0)
CKD stage	
3	3 (17)
4	8 (44)
5	7 (39)
Liver cyst	2 (11)
Renal cyst	9 (50)

Values are reported as medians (25th–75th percentile) or numbers (%).

Cr, creatine; eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease

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Table 2. Phenotypic characterization of the patients, according to the presence of pathogenic mutation

Phenotype	Pathogenic mutation N = 7	No pathogenic mutation N = 11	*P*-value
Clinical findings at renal biopsy			
Age, years	26 (22–35)	63 (55–77)	0.01
Male	5 (71)	1 (9)	0.01
Hypertension	2 (29)	9 (82)	0.049
Proteinuria	5 (71)	9 (82)	1.00
eGFR, mL/min/1.73 m^2	28.8 (5.2–39.5)	15.2 (10.4–25.9)	0.53
Liver cyst	1 (14)	1 (9)	1.00
Renal cyst	3 (43)	6 (55)	1.00
Pathologic findings			
Tubule with thick TBM duplication, /10 counted tubules [d]	4.5 (1.5–5.4)	0 (0–0.4)	<0.001
Tubular diverticulum	8 (5–9)	5.5 (5–7)	0.22
Tubular floret [a]	1 (0.5–2.5)	2.5 (1–3.5)	0.12
Cyst [b]	0 (0–0)	1 (0–1.5)	0.15
Total counted tubules [c]	9 (6–11.5)	9.5 (8.5–11)	0.56

Values are reported as median (25th–75th percentile) or numbers (%).

eGFR, estimated glomerular filtration rate; TBM, tubular basement membrane

[a]Branching in at least four directions

[b]Diameter ≥200 μm

[c]Sum of the number of tubular diverticula, tubular florets, and cysts

[d]Number of tubules with thick TBM duplication divided by the total number of counted tubules then multiplied by 10

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Legends to figures and Figures

Figure 1. Disease-causing mutations in adults with suspected nephronophthisis

Of the seven patients (39%) who had pathogenic mutations related with NPH, four patients had mutation in *NPHP1* and the other three patients had compound heterozygous mutations in *NPHP3*, *NPHP4*, and *CEP164*, respectively.

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Figure 2. Age distribution of patients according to the presence of pathogenic mutations

No pathogenic mutations in the known genes are detected in patients >50 years old.

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Figure 3. Thick tubular basement membrane duplication

Thick tubular basement membrane duplication was defined as thickness of >10 µm (yellow arrows) a,b) patient number 883, PAM stain, 20× and 40× magnification, respectively. c,d) patient number 478, PAM stain, 20× and 40× magnification, respectively. e,f) patient number 896, PAM stain, 20× and 40× magnification, respectively. All slides were scanned on a NanoZoomer NDP system with 40× resolution (0.23 µm/pixel) (Hama-matsu Photonics, Hamamatsu-City, Japan).

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Figure 4. Low-vacuum scanning electron microscope imaging of the patient with pathogenic mutation in *NPHP1* (patient number 896) and without pathogenic mutations (patient number 669)

Low-vacuum scanning electron microscope images for periodic acid-methenamine silver using formalin-fixed paraffin-embedded samples (5 µm section). a, b) imaging of the patient with pathogenic mutation in *NPHP1*. b presents higher magnification images of a. White arrow shows thick TBM duplication. c) imaging of the patient without pathogenic mutations. White bars were 20 µm.

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Figure 5. Immunofluorescence imaging of the patient with pathogenic mutations in *NPHP1* (patient number 896) and without pathogenic mutations (patient number 669)

a, b) imaging of the patient with pathogenic mutation in *NPHP1*. Alpha smooth muscle action (α-SMA) (red) positive myofibroblasts were detected inside thick tubular basement membrane duplication (yellow arrow head) in addition to vascular smooth muscle cells in the artery (white arrow). b presents higher magnification images of a. c) imaging of the patient without pathogenic mutations. α-SMA (red) was detected only in vascular smooth muscle cells (white arrow). *Phaseolus vulgaris* (PHA-E) (green) was used to identify proximal tubules. Nuclear counterstain with DAPI (blue). White bars are 100 μm.

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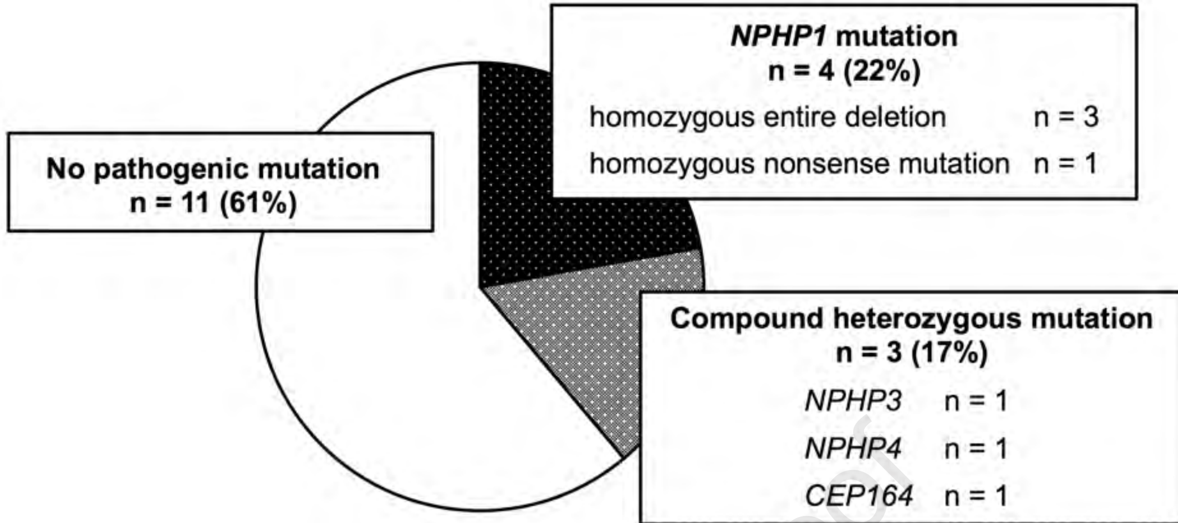

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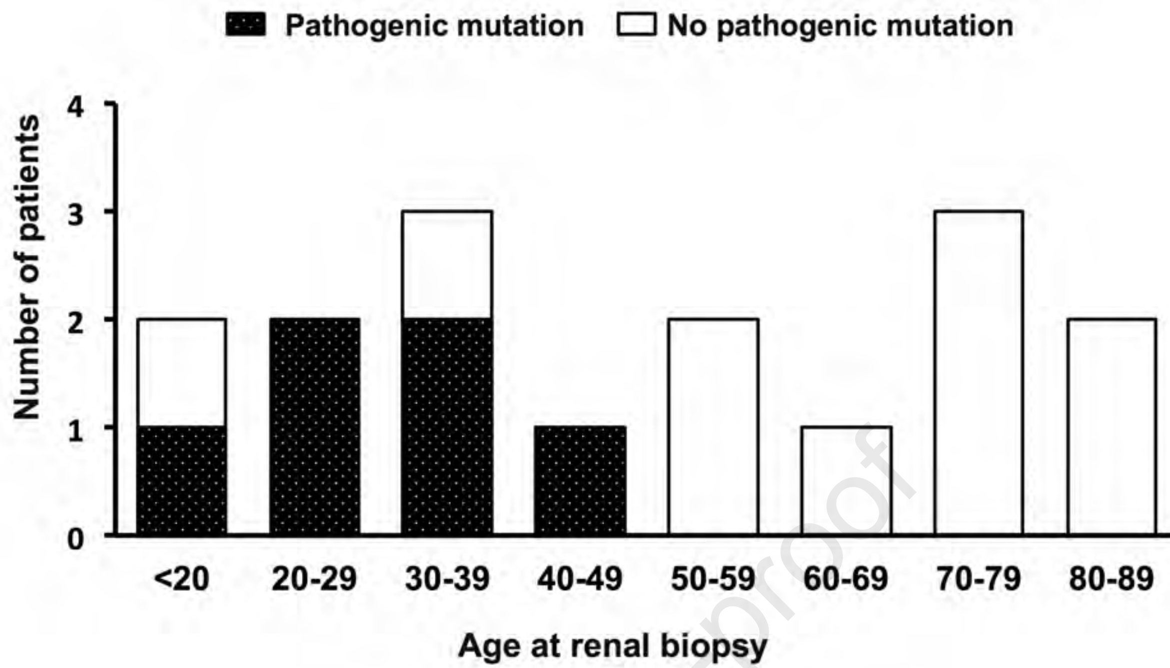

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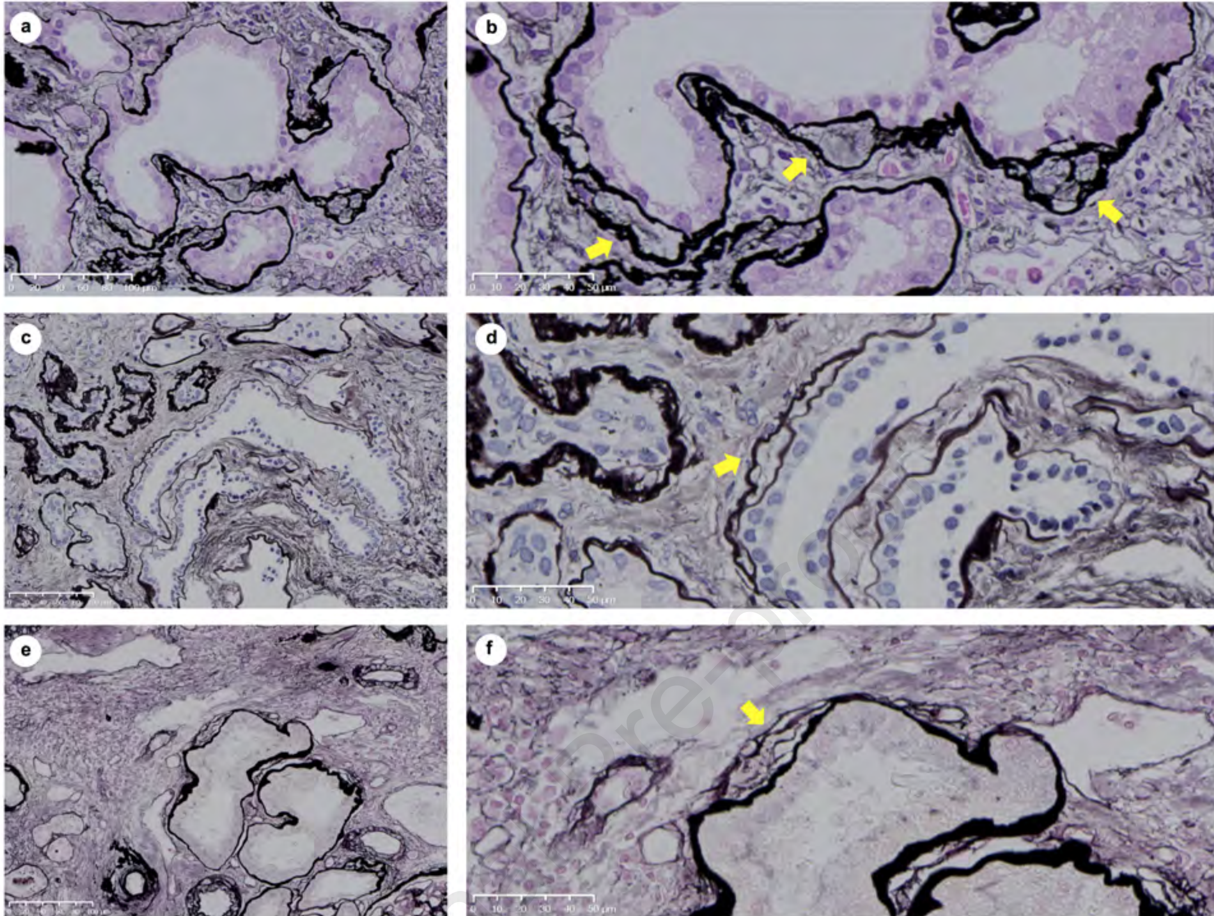

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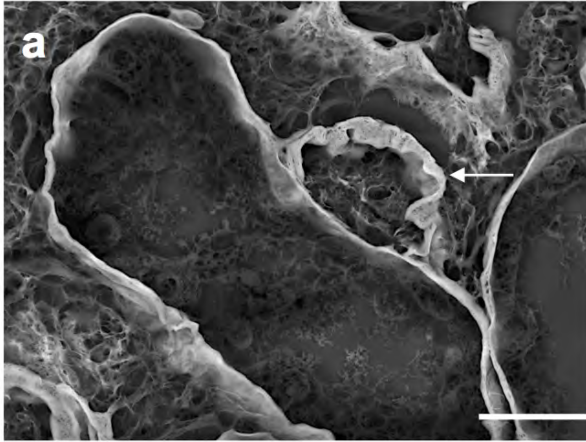

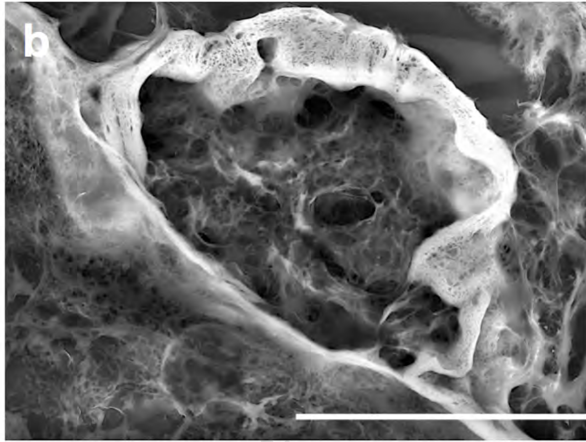

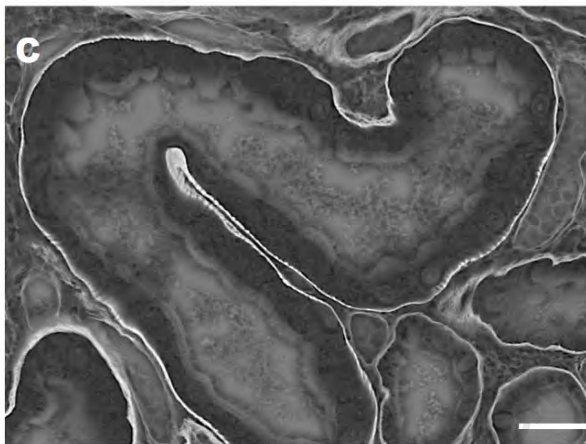

NPHP1 mutation	*NPHP1* mutation
No mutation	

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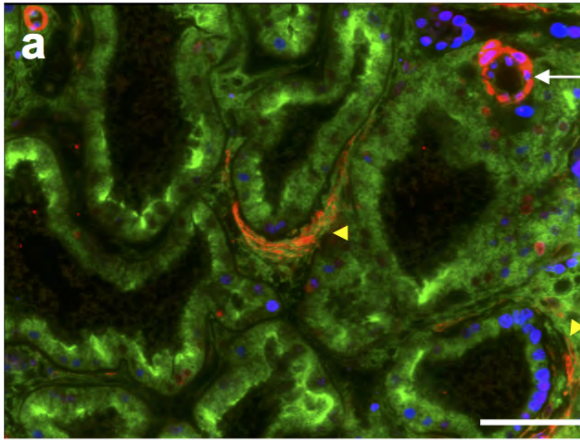

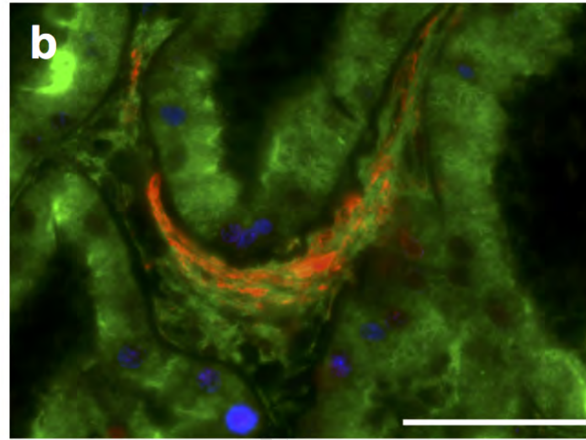

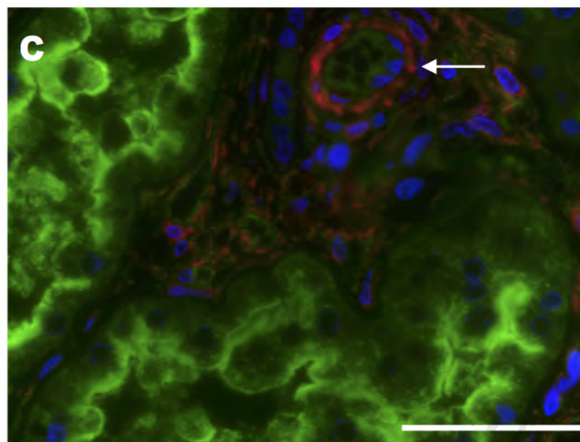

	NPHP1 mutation	*NPHP1* mutation
No mutation		

PHA-E / α-SMA

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