Genetic Background and Clinicopathologic Features of Adult-onset Nephronophthisis

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Introduction: Recently, nephronophthisis (NPH) has been considered a monogenic cause of end-stage renal disease (ESRD) in adults. However, adult-onset NPH 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 NPH.

Methods: We investigated 18 sporadic adult patients who were suspected as having NPH 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 NPH-causing genes.

Results: Seven of 18 patients had pathogenic NPH-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 NPH pathologic findings, such as tubular cysts. On the 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 NPH than in those without pathogenic mutations. ß-Smooth muscle actin (ß-SMA)-positive myofibroblasts were detected inside thick TBM duplication.

Conclusions: In adult patients with NPH, 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 NPH by renal biopsy. These findings could be the novel clinical clue for the diagnosis of NPH in adult patients.

Kidney Int Rep (2021) 6, 1346–1354; https://doi.org/10.1016/j.ekir.2021.02.005
PH is an autosomal recessive kidney disease and is the most frequent genetic cause of pediatric ESRD. In principle, molecular genetic analysis is currently the only method for the accurate clinical diagnosis of NPH. To date, more than 25 different genes have been found to be associated with NPH. 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 is now required. Recently, we have developed a target panel of genes that are related with inherited renal cystic diseases, including NPH. 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 5 international cohorts reported that 26 of 5606 patients (0.5%) with adult-onset ESRD showed homozygous NPHP1 deletions, suggesting that NPH is a relatively frequent monogenic cause of adult-onset ESRD. Although the importance of NPH in adult patients has been increasing, only a few case reports are available, and there had been no clinical cohort studies on adult NPH, probably because of the difficulty in the diagnosis.

In a real clinical setting around adult-onset NPH patients, a major clinical problem is the difficulty in an accurate diagnosis. In the Dutch study mentioned previously, only 3 (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 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. In addition, the clinical and radiologic symptoms of NPH are unspecific compared with those in the more common causes of chronic kidney disease. Therefore, most cases of adult-onset NPH are suspected only after renal biopsy. However, although the renal histology exhibits a characteristic triad of corticomedullary cysts, TBM disruption, and tubulointerstitial nephropathy, it is not disease specific and is commonly seen in any chronic tubulointerstitial disorder. 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. 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 genome wide association study data. Although complete NPHP1 deletion is responsible for 20% of pediatric NPH cases, more than 25 genes have been reported to cause NPH. Therefore, adult NPH cases may be caused by other kinds of mutations in different genes. However, because only a 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

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 or liver function disorder, and no family history of autosomal dominant chronic kidney disease. 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. The estimated glomerular filtration rate was calculated using the Japanese glomerular filtration rate equation. A liver or renal cyst was defined as the presence of at least 1 cyst detected by computed tomography or magnetic resonance imaging.

Genetic Analysis

Comprehensive genetic testing was performed using capture-based next-generation sequencing of 69 genes that cause 9 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 (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 analysis was conducted using Copy Number Analysis for Targeted Resequencing (http://contra-cnvs.sourceforge.net/).17 If homozygous entire deletion of NPHP1 was detected by copy number variation analysis, we performed polymerase chain reaction 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-Schiff, and periodic acid–methenamine silver 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 3 types of pathologic findings that were classically known to be specific for NPH; these included tubular diverticulum, tubular floret, and tubular cyst (Supplementary Figure S1A–C).13,14 For tubular diverticulum, tubular lumens that extended through the long axis of the tubule were excluded. A tubular floret was defined as branching in at least 4 directions (Supplementary Figure S1A).14 A tubular cyst was defined as having >200 μm in diameter (Supplementary Figure S1C).14 Atrophic tubules, which were defined according to the Banff working classification,18 were excluded. In addition, we defined thick TBM duplication as thickness >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 periodic acid–methenamine silver stain. First, they counted the number of the 3 types of tubules (i.e., tubular diverticulum, tubular floret, and tubular cyst) in the specimen of 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) × 10. The cumulative number of the 3 types of counted tubules was noted.

### 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 (1.80–3.66) |
| eGFR, ml/min per 1.73 m² | 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) |

CKD, chronic kidney disease; Cr, creatine; eGFR, estimated glomerular filtration rate. Values are reported as medians (25th–75th percentile) or numbers (%).

### Low-vacuum Scanning Electron Microscopic and Immunofluorescence Analysis

To investigate what the essence of thick TBM duplication was, we performed low-vacuum scanning electron microscopic (Hitachi, Tokyo, Japan) analysis and immunofluorescence (IF) analysis in the representative cases with or without pathogenic mutations. For low-vacuum scanning electron microscopic analysis, 5-μm formalin-fixed paraffin-embedded sections were stained with periodic acid–methenamine silver to evaluate the TBM as described previously.19,20 For IF analysis, monoclonal antibody against α-SMA (Merck KGaA, Darmstadt, Germany) for myofibroblast and Phaseolus vulgaris (Vector Laboratories, Inc., Burlingame, CA) for proximal tubules, respectively, were used in formalin-fixed paraffin-embedded sections. Streptavidin, Alexa Fluor 488 conjugate and goat anti-mouse IgG (H+L; Thermo Fisher Scientific Inc, Waltham, MA), and Alexa Fluor 568 (Thermo Fisher Scientific Inc) were used, and sections were counterstained with 4′,6-diamidino-2-phenylindole. IF images were captured using BZ-X800 (Keyence, Osaka, Japan).

### Statistical Analysis

All statistical analyses were performed using JMP version 15 (SAS Institute, Cary, NC). Nonnormally distributed variables were expressed as the 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 categoric variables, such as sex, between patients with pathogenic mutations and those without pathogenic mutations. A P value < 0.05 was considered statistically significant.

### RESULTS

### 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 estimated glomerular filtration rate was 18.4 ml/min per 1.73 m². Chronic kidney disease was stage 3 in 3 patients (17%), stage 4 in 8 patients (44%), and stage 5 in 7 patients (39%). No patient received dialysis. Nine patients had at least 1 renal cyst identified on imaging, such as computed tomography or magnetic resonance imaging.

Panel-based Genetic Diagnosis of NPH
A summary of the results of our comprehensive genetic testing is presented in Figure 1. Of the 7 patients (39%) who had pathogenic mutations related with NPH, 4 patients had a mutation in NPHP1, and the other 3 patients had compound heterozygous mutations in NPHP3, NPHP4, and CEP164, respectively. Notably, the patient with the CEP164 mutation was the first case in an adult. Of the patients with an NPHP1 mutation, 3 patients (patient numbers 883, 896, and 1207) were detected to have homozygous entire deletion of NPHP1 by copy number variation analysis. The regions that were expected to be identified by copy number variation analysis are shown in Supplementary Table S3. In all 3 patients, no polymerase chain reaction 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 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, there was a significantly higher proportion of men for those with pathogenic mutations (71% vs. 9%, P = 0.01) and a significantly lower incidence of hypertension (29% vs. 82%, P = 0.049) but a similar estimated glomerular filtration rate, level of proteinuria, and number of renal or liver cysts (at least 1 for each).

Pathologic Findings Specific for Adult-onset NPH
The 3 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. Although TBM duplication was reported in noninherited renal diseases, we noticed that the TBM 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 low-vacuum scanning electron microscopy and IF. In low-vacuum scanning electron microscopic 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 b). 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 low-vacuum scanning electron microscopic 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 9 patients, the diagnoses by renal biopsy were IgA nephropathy in 4 patients, diabetic nephropathy in 3 patients, and tubulointerstitial nephritis in 2 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.

**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 a significantly higher proportion of men, a significantly lower incidence of hypertension, and a significantly higher number of tubules with thick TBM duplication.

In this study, the pathogenic mutations in the 7 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. However, based on our results, 4 of 7 genetically confirmed NPH cases had pathogenic mutations other than homozygous NPHP1 full gene deletions. Moreover, mutations in CEP164 were reported to cause Senior-Løken syndrome or Joubert syndrome. In this study, patient
number 930, who was a 26-year-old man, had compound heterozygous mutations in \textit{CEP164}. To the best of our knowledge, this was the first reported case of adult NPH with a \textit{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 \textit{\alpha}-SMA–positive cells in the thick TBM duplication. \textit{\alpha}-SMA is commonly used as a marker for myofibroblasts and the resulting fibrosis.\textsuperscript{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-\textit{\beta}.\textsuperscript{24} Furthermore, the study of inhibition of ciliogenesis demonstrated that deficiency of primary cilia induces epithelial-to-mesenchymal transition.\textsuperscript{25} Therefore, because 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 \textit{\alpha}-SMA–positive myofibroblasts and fibrosis, resulting in thick TBM duplication.

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 \textit{et al.}\textsuperscript{7} analyzed 5606 patients with adult-onset ESRD and revealed that 26 (0.5\%) of the patients had homozygous \textit{NPHP1} full gene deletions. Of the patients who were \textit{\textgreater}50 years old at the time of the initiation of renal replacement therapy, only 2 had homozygous \textit{NPHP1} full gene deletions. Similar to the report by Snoek \textit{et al.}, our study showed that no patient who was \textit{\textgreater}50 years old at the time of renal biopsy had pathogenic NPH mutations. These findings

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Thick tubular basement membrane duplication. Thick tubular basement membrane duplication was defined as thickness of \textit{\textgreater}10 \textmu m (yellow arrows). (a and b) Patient number 883, periodic acid–methenamine silver (PAM) stain, 20× and 40× magnification, respectively. (c and d) Patient number 478, PAM stain, 20× and 40× magnification, respectively. (e and f) Patient number 896, PAM stain, 20× and 40× magnification, respectively. All slides were scanned on the NanoZoomer NDP system with 40× resolution (0.23 \textmu m/pixel) (Hamamatsu Photonics, Hamamatsu-City, Japan).}
\end{figure}
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; the presence of chronic tubular and interstitial lesions in the form of tubular atrophy and interstitial fibrosis; and lamellated TBM in atrophic tubules. In this study, the 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. 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 deletions were men, demonstrating no sex difference. Further study will be required to confirm this observation.

This study had several limitations. First, the sample size of our study was small. Nevertheless, only a few adult cases of suspected NPH by renal biopsy have been reported, 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 5 countries, the number of patients in our study could be reasonable. 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 analyze other genes that had been identified very recently as rare causal genes for NPH-related ciliopathies, such as C8orf37, KIAA0586, and MAPKBP1. 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

Figure 4. Low-vacuum scanning electron microscopic 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 and b) Imaging of the patient with pathogenic mutation in NPHP1; b presents higher magnification images of a. The white arrow shows thick tubular basement membrane duplication. (c) Imaging of the patient without pathogenic mutations. White bars = 20 μm.
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.

DISCLOSURE

TM reports personal fees from honoraria for lectures and personal fees from employment outside the submitted work. JH has received a research grant from Otsuka Pharmaceutical Co. All the other authors declared no competing interests.

ACKNOWLEDGMENTS

Supported in part by the Grants-in-Aid for Scientific Research (25221306, 16H05314, 19H01049, and 19H03672), the Challenging Exploratory Research (15K15327, 16K15467, and 18K19534), the Research Activity Start-up (17H06657, 20K22926), the Young Scientists (19K17733), and the Early-Career Scientists (19K17730) from the Japan Society of the Promotion of Science; the Health Labor Science Research Grant from the Ministry of Health Labor and Welfare, AMED under Grant Number JP18ek0109304; grants from the Yukiko Ishibashi Foundation; the Salt Science Research Foundation (1925, 2030); and low-vacuum scanning electron microscope device support from the LVSEM Study Group of Renal Biopsy and Hitachi High-Technologies Corporation in Japan Grant Number 007.

SUPPLEMENTARY MATERIAL

Supplementary Material (PDF)
Supplementary Methods.
Supplementary References.
Table S1. Disease categories and the targeted genes included in this panel
Table S2. Primer sequences of NPHP1
Table S3. Detected copy number variants in NPHP1
Table S4. Mutations in the genes related with nephronophthisis
Table S5. Clinical characteristics of the pathologic control group
Figure S1. Representative pathologic findings.

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