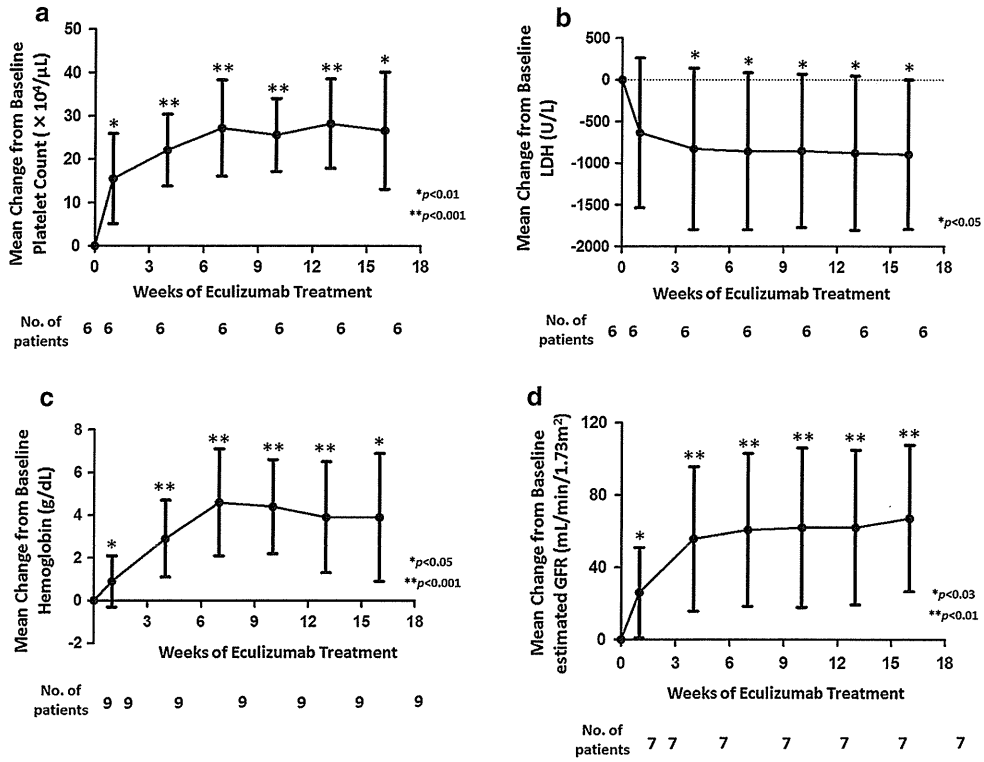

Fig. 1 The improvement of blood and renal parameters in aHUS after the administration of eculizumab therapy. **a** Platelet count ($n = 6$), **b** lactate dehydrogenase concentration ($n = 6$), **c** hemoglobin concentration ($n = 9$) and **d** estimated glomerular filtration rate ($n = 7$)

complement proteins and removing mutant abnormal proteins or auto-antibodies [7–9]. According to the guideline published by the European Paediatric Study Group for HUS in 2009, plasma therapy was recommended to be started as soon as possible (within 24 h) after the diagnosis of aHUS [22]. Nevertheless, it was reported that 29 % of children with aHUS still died or reached ESRD within one year [6]. It was also described that plasma therapy induced complete or partial remission of 63, 25, 57, 88 and 75 % in aHUS patients with CFH, CFI, C3, THBD mutations or anti-CFH autoantibodies, respectively [4]. The analysis of European pediatric aHUS patients mainly treated with plasma therapy in 2009 demonstrated that 11 % of the patients failed to achieve hematological remission and 17 % remained dialysis-dependent at day 33 [23]. These data indicate that the efficacy of plasma therapy in aHUS is limited. Moreover, the incidence of complications in plasma therapy related to vascular access in children is higher than in adults [9].

Eculizumab has become the alternative therapeutic option for aHUS since 2009. This new drug received approval for the indication of aHUS in the United States and Europe in 2012, and in Japan in 2013. The previous case series and the results of clinical trials demonstrated that eculizumab was significantly more effective than conventional plasma therapy for aHUS patients [9–16]. Zuber et al. [9] reviewed the case series of aHUS patients who were treated with eculizumab as a first-line therapy or a rescue therapy. In these cases, all the patients could achieve hematological remission, and 80 % of children and 31 % of adults showed a full recovery to baseline renal function. In a prospective phase 2 clinical trial of eculizumab for patient with aHUS over 12 years of age, who were resistant to or dependent on plasma therapy, eculizumab therapy resulted in improvement of both thrombocytopenia and renal impairment [10].

In our study, after the administration of eculizumab, hematological remission was quickly achieved and plasma therapy was also immediately discontinued in all patients. Notably, platelet count was normalized within several days after the first infusion of eculizumab in most patients. This is one of the most distinctive features of the efficacy of eculizumab [10, 11]. This quick response in the resolution of hematological abnormality strongly suggests that all patients were affected by complement-related aHUS, and it can be the appropriate indicator for therapeutic efficacy of eculizumab. It may also indicate that this therapy can rapidly stop further intravascular thrombus formation by direct blockade of downstream of abnormally activated complement cascade.

On renal outcome, renal impairment was also improved in most patients, although one patient (Patient 7) could not discontinue dialysis and two patients (Patient 1, 7) had developed ESRD at the last observation. Previous literature

suggested that earlier initiation of eculizumab therapy was associated with better improvement of renal function [9–11], although some cases experienced a significant improvement in renal function after RRT of several months [9, 24, 25]. In our study, two of the eight patients who required RRT during the course of the disease (Patients 1, 3) started eculizumab more than one month after the onset of aHUS. Patient 3 could successfully withdraw from RRT 69 days after the administration of eculizumab. Although there is not enough evidence to elucidate the mechanism of such late improvement in renal function as several months after chronic RRT, the extent of renal damage in capillary endothelial cells and interstitium before eculizumab therapy might influence the outcome of renal function. In addition, pediatric patients may have a greater potential to recover renal function than adult patients because each nephron is still in developing during the early stages of life.

There are also other advantages to this new therapy. As eculizumab blocks the downstream complement cascade at the late component C5, it preserves the early components of the cascade which play important roles in phagocytosis and opsonization of microorganisms [9]. In addition, eculizumab is a fully humanized recombinant monoclonal antibody with minimal immunogenicity. Therefore, serious infusion reactions and the development of neutralizing antibodies against the drug are rare [26]. Eculizumab has changed the strategy of management of aHUS dramatically, and it has become not only the substitute for plasma therapy but also the frontline therapy.

However, eculizumab therapy also has some serious adverse events and critical disadvantages. One of the serious adverse events is an increased susceptibility to meningococcal infection. As eculizumab causes late complement pathway deficiencies and inhibits the membrane attack complex formation, patients are at risk of severe encapsulated bacterial infections, especially by *Neisseria meningitidis* [2, 7, 9]. It is well known that individuals deficient in components of the terminal complement pathway are highly predisposed to invasive, often recurrent meningococcal infections [27]. In patients treated with eculizumab for paroxysmal nocturnal hemoglobinuria, the meningococcal infection rate was 0.42 per 100 patients per year [26]. Although annual incidence of this infection, such as bacterial meningitis or sepsis, is only approximately ten patients per year in Japan [28], vaccination against *Neisseria meningococcus* is mandatory for all the patients treated with eculizumab at least two weeks prior to the initiation of the therapy. In this study, all patients were provided with meningococcal vaccine, but they were unapproved and imported because there were no approved vaccines in Japan at that time [29].

If the meningococcal vaccine cannot be given, patients should receive antibiotic prophylaxis until at least two weeks after the vaccination [21]. However, limited-time antibiotic prophylaxis cannot be sufficient to prevent from this critical infection. Tetravalent vaccines against serotypes A, C, Y and W135 are commonly available, but they are not effective for serotype B which is major type found in Japan [30]. Moreover, serum antibody titer acquired by the vaccination can be decreased in several years. It means that we have to keep in mind the risk of meningococcal infection for all the patients treated with eculizumab even after the vaccination.

Furthermore, as the optimal duration of eculizumab treatment has not yet been established, life-long therapy is recommended to prevent relapses at this time in spite of very high-cost therapy [9]. Zuber et al. [9] reviewed five patients treated with a single infusion of eculizumab who experienced relapse mostly within one year and progressed to ESRD. Legendre et al. [10] described that five of the 18 patients who received inappropriate eculizumab doses had subsequent severe complications of TMA. However, it remains unclear whether all patients with aHUS require chronic life-long eculizumab therapy. Ardissino et al. [31] reported that seven of the ten patients could discontinue eculizumab under home monitoring of urine hemoglobin, although three patients with CFH-related aHUS relapsed after discontinuation. Therefore, the risk of relapse in aHUS patients after discontinuation of eculizumab therapy could depend on the genetic background. Better understanding of the genetic correlation and establishing useful biomarkers for monitoring aHUS disease activity are needed to address the optimal duration of this extremely expensive therapy.

Unfortunately, a portion of aHUS patients are resistant to eculizumab therapy. The lack of efficacy of eculizumab may come from a delay in starting eculizumab therapy [9–11], the pathogenesis of aHUS with non-complement-dependent mechanisms such as DGKE mutations [32], or host genetic C5 variants which inhibit the binding of eculizumab to the target epitope [33]. However, there are still some patients in whom the reason of the ineffectiveness is not clear. In this study, Patient 7 could achieve hematological remission but did not have any improvement of renal impairment even though she was administered eculizumab as early as 17 days after aHUS onset. In such cases, unknown factors or mechanisms other than complement activation may be involved in severe renal injury.

An increasing number of children with aHUS will receive this revolutionary treatment in the future. The French Study Group for aHUS/C3G suggested that eculizumab may be considered as a first-line therapy in children with a first episode of aHUS [9]. We should give careful consideration to the indication for eculizumab because some critical issues remain with this new therapy.

However, delayed initiation of eculizumab could also lead to not only irreversible renal impairment but also severe systemic multiple organ damage caused by TMA. As complement investigations take a long time and complement-related mutations have been found only in 50–70 % of patients with aHUS [1–7], identification of a genetic mutation is not necessary for clinical diagnosis and treatment initiation [9, 10]. Therefore, empirical eculizumab therapy for aHUS may be a choice in the early phase of disease after excluding STEC infection and TTP. If patients achieve hematological remission promptly, they could quite likely have aHUS related to abnormalities of complement regulatory factors.

There were some limitations to this study. Firstly, it was a retrospective study and the number of patients enrolled was small. Secondly, most patients were observed for very short periods after the administration of eculizumab. Therefore, we need to confirm the long-term efficacy and outcome of eculizumab therapy in pediatric aHUS. Finally, although immediate and definite efficacy of eculizumab was confirmed in all the patients, genetic mutations of complement-related proteins could not be detected in half of them. Even for the detected mutations, novel ones were not evaluated whether they were definitely causative or not. In fact, Patient 5 carried a novel MCP mutation, but also revealed mildly enhanced hemolytic assay suggesting aberrant CFH function. Patient 10 demonstrated enhanced hemolytic assay, but no CFH-related mutation was detected. In these patients, interacting with some other mutations or SNPs of the complement-related proteins may contribute to the onset of aHUS. We need further investigation to elucidate these discrepancies.

In conclusion, eculizumab is an efficacious and well-tolerated therapy in Japanese pediatric aHUS patients. The appropriate indication for use of this breakthrough therapy and the proper period of the treatment have yet to be determined.

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Autoimmune-type atypical hemolytic uremic syndrome treated with eculizumab as first-line therapy

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Abstract We report a case of atypical hemolytic uremic syndrome (aHUS) in a 4-year-old boy. Although the patient had the typical triad of aHUS (microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury), urgent dialysis was not indicated because he had neither oliguria nor severe electrolyte abnormality. He was given eculizumab as first-line therapy, which led to significant clinical improvement, thus avoiding any risk of complications associated with plasma exchange and central venous catheterization. Retrograde functional analysis of the patient's plasma using sheep erythrocytes indicated an increase in hemolysis, suggesting impairment of host cell protection by complement factor H. The use of eculizumab as first-line therapy in place of plasma exchange might be reasonable for pediatric patients with aHUS.

Key words atypical hemolytic uremic syndrome, eculizumab, plasma exchange.

Hemolytic uremic syndrome (HUS) is defined by the typical triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal injury. More than 90% of cases in children are secondary to infection with enterohemorrhagic *Escherichia coli* (EHEC) which produces Shiga toxin. The remaining 10% of cases, however, are classified as atypical hemolytic uremic syndrome (aHUS). aHUS has a poor prognosis with a high mortality rate and a high rate of progression to end-stage renal failure.[1] Plasma exchange (PE) has been recommended as first-line rescue therapy for such aHUS episodes, and for prevention of relapse.[2,3] This treatment, however, has some problems in terms of long-term acceptance, and its efficacy is controversial. Also, vascular access carries risk of complications, including bleeding and vascular injury. Eculizumab (Soliris®; Alexion Pharmaceuticals, Cheshire, CT, USA) is a humanized monoclonal anti-C5 antibody that inhibits the terminal complement pathway and hinders the generation of pro-inflammatory C5a and C5b-9 (membrane attack complex: MAC). Recent reports have indicated the efficacy and safety of eculizumab in patients with aHUS.[4,5] In Japan, it was approved for the treatment of aHUS in September 2013.

Here we describe the clinical features of a child with aHUS due to autoantibody against complement factor H (CFH), who was treated successfully with eculizumab as first-line therapy.

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Case report

The patient was a 4-year-old Japanese boy who was the second child of non-consanguineous parents. He had an elder brother and a younger sister, both of whom were healthy. He had been brought to his family physician with a 2 day history of headache,

nausea, appetite loss, and low-grade fever. Given that anemia, thrombocytopenia, and acute kidney injury were evident, he was tentatively diagnosed as having HUS, and referred to hospital for intensive care. The clinical course is summarized in Figure 1. On admission his complexion was pale and slightly icteric. Other physical data included bodyweight, 14.9 kg; body temperature, 37.5°C; pulse rate, 144 beats/min; and blood pressure, 106/60 mmHg. Neither hepatosplenomegaly nor enlargement of superficial lymph nodes was found. The laboratory findings on admission are summarized in Table 1. Among them, severe anemia, thrombocytopenia, hyperbilirubinemia, elevated lactate dehydrogenase, elevation of serum renal function markers including creatinine, and low C3, were remarkable. Both the direct and indirect Coomb's tests were negative. Hemostatic tests showed that prothrombin time and activated partial thromboplastin time were both within the normal range, but that fibrin/fibrinogen degradation products were elevated. Furthermore, red blood cell (RBC) fragmentation was found in a peripheral blood smear. Stool culture failed to identify Shiga toxin-producing EHEC, or both Shiga toxins 1 and 2. Although the patient had macrohematuria, moderate proteinuria, and elevation of serum renal function markers, he did not fall into the category of oliguria or severe electrolyte abnormality. For this reason, urgent dialysis was not initiated. On the following day (hospital day [HD] 2), fresh frozen plasma (FFP; 23 mL/kg) was infused in order to supply normal complement regulatory factors under a tentative diagnosis of aHUS, given that diarrhea was absent. On the third day (HD 3), however, the patient's clinical symptoms worsened, and RBC concentrates were therefore transfused. Given that plasma a disintegrin-like and metalloproteinase with thrombospondin type I motifs, number 13 (ADAMTS13) activity was 120% on the night of HD 3, the patient was definitively diagnosed as having aHUS, and given eculizumab at a dose of 600 mg. On the second day after eculizumab treatment (HD 5), the macrohematuria dramatically resolved, and thereafter hematology showed gradual improvement. On HD 10, the patient started to receive eculizumab at the maintenance dose (300 mg) by injection every 2 weeks. The patient was vaccinated against *Neisseria meningococcus* and *Streptococcus pneumonia* on HD 29 and HD37, respectively, and received prophylactic antibiotic therapy with cefditoren pivoxil until 2 weeks after vaccination for meningococcus. He was discharged with no sequelae on HD32, and thereafter received an injection of eculizumab at the maintenance dose (300 mg) every 2 weeks. There were no adverse events associated with eculizumab treatment, including infusion reaction or infection, in the whole period of observation, or any further recurrence of aHUS.

Retrograde analysis including hemolytic assay, and Western blotting for detection of anti-CFH antibody and complement factor H-related protein 1/3 (CFHR 1/3) were performed using the patient's plasma, which had been obtained before plasma infusion using the method reported previously.[6,7] Comprehensive gene mutation analysis of CFH, complement factor I (CFI), complement factor B (CFB), C3, membrane cofactor protein (MCP), and thrombomodulin, was also performed as described previously.[6] The patient's plasma enhanced the hemolysis of sheep erythrocytes and this effect was suppressed by addition of purified CFH, indicating impairment of host cell protection by CFH (Fig. 2). Anti-CFH antibody was detected in the patient's plasma, but no deficiency of the protein encoded by CFHR 1/3 was observed (Fig. 2).[8] Additionally, there were no mutations of CFH, CFI, CFB, C3, MCP, or thrombomodulin. Therefore, the patient was diagnosed as having aHUS due to autoantibody against CFH without CFHR 1/3 protein deficiency.

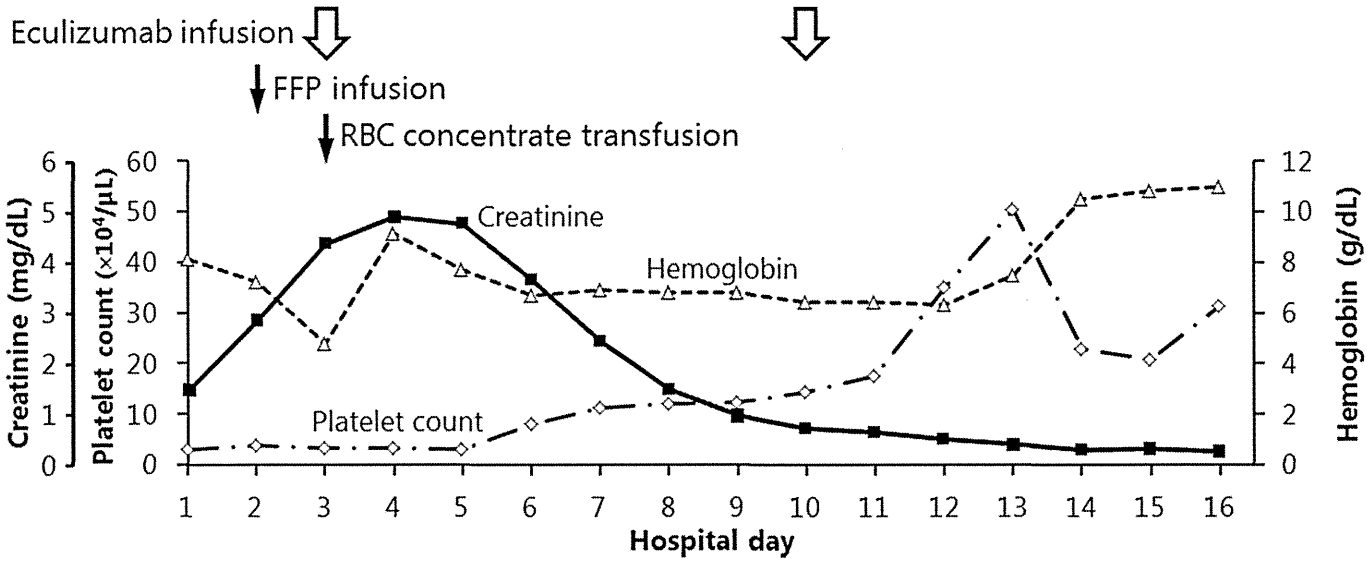

Fig. 1 Clinical course of treatment for atypical hemolytic uremic syndrome. Although fresh frozen plasma (FFP; 23 mL/kg) and red blood cell (RBC) concentrate were transfused on hospital days 2 and 3, laboratory findings including creatinine and platelet count worsened. After initiation of eculizumab, however, all of the laboratory parameters improved.

Table 1 Laboratory findings on admission

Peripheral blood		Stool culture	
WBC	9500 /μl	Normal flora	
RBC	300×10^4 /μl	EHEC	(−)
Hb	8.1 g/dL	Shiga-toxin	(−)
Ht	23.2%		
Platelet	1×10^4 /μl	Chemistry	
RBC fragmentation	(+)	TP	6.1 g/dL
		Alb	3.1 g/dL
Hemostatic test		Total bilirubin	2.6 mg/dL
PT	11.2 s	Indirect bilirubin	2.2 mg/dL
PT-INR	1.06	AST	101 IU/L
APTT	27.1 s	ALT	27 IU/L
Fibrinogen	278 mg/dL	LDH	3,570 IU/L
FDP	11.8 μg/mL	BUN	48.7 mg/dL
		UA	9.3 mg/dL
Urinalysis		Cr	1.51 mg/dL
Urine color	Light red	CRP	3.55 mg/dL
Occult blood	(4+)	Na	136 mmol/L
Protein	(2+)	K	3.8 mmol/L
Sediment		Cl	102 mmol/L
RBC	10–15/HPF	CK	403 IU/L
WBC	1–4/HPF	Haptoglobin	<10 mg/dL
Epithelium		Coombs test	
Epithelial cast	1+	Direct	(−)
		Indirect	(−)
Complement activity			
CH50	32.2 IU/L	Serological test	
C3	30.8 mg/dL	Total ANA	<40
C4	21.8 mg/dL	PR3-ANCA	<1.0
		MPO-ANCA	<1.0
ADAMTS13 activity	120%	ss-DNA antibody	<1.0
		ds-DNA antibody	<1.0

ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ANCA, anti-neurophil cytoplasmic antibody; APTT, activated partial thromboplastin time; EHEC, enterohemorrhagic *Escherichia coli*; FDP, fibrin/fibrinogen degradation products; INR, international normalized ratio; MPO, myeloperoxidase; PR3, proteinase 3; PT, prothrombin time.

Discussion

Atypical hemolytic uremic syndrome is a rare disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure secondary to thrombotic microangiopathy. In recent years, aHUS has been found to be associated with dysregulation of the complement alternative pathway. In more than half of patients with aHUS, mutations in genes encoding complement-regulating protein including CFH, CFI, and MCP, have been reported.[1] Additionally, functional CFH deficiency due to autoantibodies against CFH has been reported, and this is highly associated with polymorphic homozygous deletion of genes encoding CFHR proteins 1 and 3.[1] The present patient had had no diarrhea, and neither EHEC nor Shiga toxin had been found in his stools. ADAMTS13 activity was 120%, which was within the normal range. aHUS associated with anti-CFH autoantibody was diagnosed on the basis of additional examinations including gene mutation analysis and Western blot analysis for anti-CFH antibody and proteins encoded by CFHR1/3.

Plasma exchange has been recommended as a first-line therapy for aHUS based on expert opinion rather than clinical trials.[2,3] For management of aHUS associated with anti-CFH autoantibodies, PE with FFP has been done for the purpose of removing anti-CFH autoantibodies and simultaneously supplying the circulating CFH pool. Although combination therapy with immunosuppressants has also been used, the rate of remission in response to short-term PE is 70–80%, and the rate of death or end-stage renal disease as a long-term outcome is 30–40% in patients with anti-CFH autoantibodies.[1] Additionally, it is difficult to determine whether the disease activity is stable and leads to remission, because no international standard for determining anti-CFH antibody and the levels of autoantibodies leading to disease relapse or exacerbation has been established.

In contrast, previous case reports have suggested that eculizumab is effective for treatment of aHUS.[4,5] Additionally, Legendre *et al.* noted the efficacy and safety of long-term eculizumab for thrombotic microangiopathy in aHUS patients, via two prospective phase 2 trials lasting 62–64 weeks.[9] Although reduction of the antibody load plays a very important role in aHUS associated with anti-CFH autoantibodies, eculizumab can effectively block the terminal complement cascade and stop further damage in the presence of anti-CFH autoantibodies. Noone *et al.* reported two cases of CFH autoantibody-positive HUS treated with eculizumab and proposed that eculizumab should be used in the acute phase for arresting the complement-mediated damage.[10]

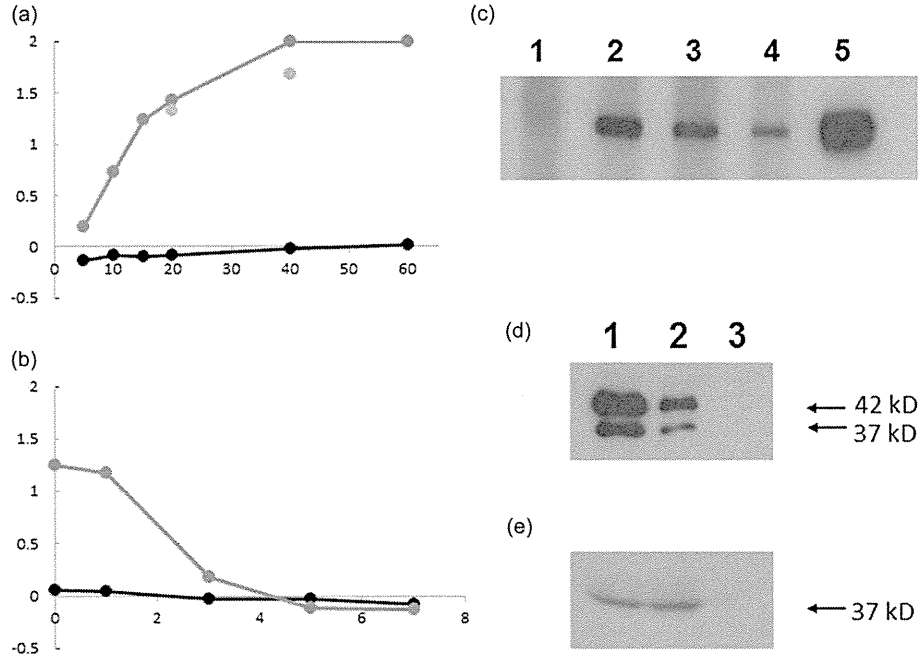

Fig. 2 (a,b) Hemolytic test and (c–e) Western blot analysis for detection of (c) anti-complement factor H (anti-CFH) autoantibodies and (d,e) protein encoded by *complement factor-H related protein CFHR1* and 3. (a) Lysis of sheep erythrocytes by addition of patient plasma. (a) OD_{414} titer sheep erythrocytolysis as a function of patient plasma. Plasma samples ranging from 5 μL to 60 μL were used. (b) Inhibition of enhanced hemolysis with 20 μL of plasma by adding purified CFH in amounts ranging from 0 μg to 7 μg. (—●—) Normal plasma ; (—●—) patient plasma; (●) normal plasma plus purified anti-CFH antibody. (c) Western blot analysis for detection of anti-CFH autoantibody. Lane 1, normal plasma; lane 2, patient plasma in the acute phase (hospital day [HD] 2); lane 3, patient plasma in the chronic phase (HD 31); lane 4, plasma of 3-year-old Japanese boy diagnosed with aHUS associated with anti-CFH autoantibody;[8] lane 5, CFH monoclonal antibody. (d,e) Western blot analysis for (d) CFHR 1 and (e) 3 proteins. (d) Plasma samples from a normal control, the present patient, and a patient who had been previously diagnosed as having deficiency of CFHR plasma proteins and autoantibody-positive HUS (DEAP-HUS)[8] were electrophoresed on a 12.0% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After blocking with 5% dried milk, the membrane was incubated for 1.5 h at room temperature with mouse anti-human CFHR1 monoclonal antibody, the concentration of which was adjusted to 1 μg/mL. Then, 10 000-fold-diluted horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody was used as the secondary antibody, and bound mouse monoclonal antibody was visualized using enhanced chemiluminescence substrate (Western Lightning-ECL; Perkin Elmer, Yokohama, Japan). (e) Western blot analysis for detection of CFHR 3 protein was done using the same method as for CFHR 1, with 1500-diluted rabbit anti-human CFHR 3 polyclonal antibody as the first antibody and 20 000-diluted HRP-labeled goat anti-rabbit IgG antibody as the secondary antibody. Lane 1, normal control; lane 2, present patient; lane 3, DEAP-HUS patient.[8]

Given that the present patient had neither oliguria nor electrolyte abnormalities including hyperkalemia, urgent dialysis was not necessary. Therefore, the patient received eculizumab as first-line therapy and was able to avoid the risk of complications associated with these maneuvers. Therapy with eculizumab was very effective, and no adverse events occurred. Zuber *et al.* proposed the use of eculizumab as first-line therapy for all episodes of aHUS in children because of its efficacy and safety, and for avoiding any potential complications of PE.[4]

Conclusion

The present study has demonstrated the efficacy and short-term safety of eculizumab as first-line therapy in the acute phase for aHUS associated with anti-CFH autoantibodies in a pediatric patient.

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Stenosing ureteritis in Henoch–Schönlein purpura: Report of two cases

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Abstract Stenosing ureteritis (SU), a rare complication of Henoch–Schönlein purpura (HSP), typically presents with severe symptoms. We report the cases of two HSP patients presenting with gross hematuria, blood clotting, and colicky flank pain, followed by purpura on the lower extremities. Early-stage ultrasonography indicated hydronephrosis, thickened renal pelvic mucous membrane, and ureteral dilatation (UD), suggesting HSP complicated with SU. After early SU treatment with prednisolone, kidney function, thickened renal pelvic mucous membrane, and UD progressively normalized and the pain gradually disappeared. Regular ultrasonography of HSP patients from the onset of gross hematuria can be useful to detect early SU and facilitate conservative therapy with prednisolone. Diagnosis of SU can be easily missed by assuming HSP nephritis, particularly owing to the non-specific symptoms. Common characteristics as well as treatment methods and prognosis of SU are given in the literature review.

Key words gross hematuria, Henoch–Schönlein purpura, Henoch–Schönlein purpura nephritis, hydronephrosis, stenosing ureteritis, ultrasonography.

Henoch–Schönlein purpura (HSP) is the most common childhood systemic small-vessel vasculitis and can cause serious complications such as stenosing ureteritis (SU).[1,2] SU symptoms may be masked by gastrointestinal and renal symptoms and, in some cases, it may be diagnosed incidentally.[2–5] Depending on the course, various reports have stated the necessity of surgical treatment for SU, but the benefits of steroid monotherapy have also been noted.[2,6,7] We report the cases of two HSP patients presenting with ureteral stenosis, gross hematuria, and colicky flank pain that improved with prednisolone (PSL) therapy. Although an extensive literature review found >30 cases reports on the clinical spectrum of the complications, only seven English-language case reports published during 1997–2013 have been included (Table 1).[2–10]

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Case reports

Patient 1

A 6-year-old boy, diagnosed with gastroenteritis by a local general practitioner, presented with a 3 day history of colicky flank pain and gross hematuria. The patient had a history of severe sinusitis and bilateral vesicoureteral reflux (stage III at 4 years of age), but no history of urinary tract infection (UTI) after 4 years of age. At presentation, he was afebrile and normotensive; on physical examination, tenderness was observed around the navel, with no evidence of purpura or joint pain.

Blood test results were as follows: white blood cell (WBC) count, 19 570/μL; platelet count, 35.0×10^4/μL; creatinine (Cr), 0.39 mg/dL; C-reactive protein (CRP), 7.6 mg/dL (normal, <0.5 mg/dL); complement component 3 (C3), 118 mg/dL (normal, 86–160 mg/dL); and D-dimer, 5.2 μg/mL (normal, <1.0 μg/dL). Urinary test results were as follows: urine protein, 300 mg/dL; urine sediment, 281/μL (normal, <5/μL) and 4865/μL (normal, <5/μL) for WBC and red blood cells (RBC; non-glomerular), respectively. Urine biochemistry was as follows: calcium/Cr ratio (Ca/Cr), 0.1 (normal, <0.31); and β-2 microglobulin (β2MG), 301 μg/L (normal, <230 μg/L). To exclude urolithiasis and colitis, ultrasonography and abdominal

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Successful Kidney Transplantation in Epstein Syndrome With Antiplatelet Antibodies and Donor-specific Antibodies: A Case Report

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ABSTRACT

An autosomal dominant hereditary disease, Epstein syndrome (ES) is characterized by sensorineural hearing impairment, macrothrombocytopenia, and hereditary nephritis, and can progress to end-stage kidney disease after puberty. Generally, kidney transplantation is difficult to perform in Epstein syndrome owing to the high risk of perioperative bleeding. Additionally, due to previous platelet transfusions, ES patients sometimes have antihuman leukocyte antigen (HLA) antibodies, including antiplatelet antibodies and donor-specific anti-HLA antibodies (DSA), which may result in refractoriness to platelet transfusion and antibody-mediated rejection (AMR). We report a case of successful kidney transplantation in a patient with ES who had DSA and antiplatelet antibodies. To prevent AMR, we used a desensitization protocol (a combination of plasmapheresis, rituximab, and basiliximab induction). Surveillance biopsy performed at 4 months and 1 year after transplantation showed no pathological findings suggesting AMR. To prevent perioperative bleeding complications, we infused the patient with HLA-matched platelets, thereby maintaining the platelet count at $>10.0 \times 10^4/\mu L$, and no postoperative episodes of bleeding occurred.

EPSTEIN SYNDROME (ES) is an autosomal dominant hereditary disease characterized by sensorineural hearing impairment, macrothrombocytopenia, and hereditary nephritis [1]. This disease is also known as myosin heavy chain 9 (MYH9)-related disorder; the *MYH9* gene encodes the nonmuscle myosin heavy chain IIA, a cytoskeletal contractile protein [2].

In ES, nephritis tends to progress to renal failure after puberty [3,4]. In patients with end-stage renal disease due to ES, kidney transplantation (KT) is the optimal treatment choice. However, the main difficulty in the postoperative management of these patients is prevention of perioperative bleeding complications.

Although macrothrombocytopenia is present in all affected individuals, bleeding tendency is generally believed to be moderate. Alving et al emphasized that macrothrombocytopenia in these patients is not a contraindication to KT under careful bleeding control [5]. Nevertheless, several recent case reports suggest that ES patients are at great risk of perioperative bleeding complications requiring surgical intervention after KT [6,7]. This inconsistency in patients with ES may be due not only to thrombocytopenia but also bleeding tendency resulting from chronic renal failure [8]. Moreover, ES patients frequently receive platelet transfusions upon surgical intervention [9]. These blood transfusions may induce antihuman leukocyte antigen (HLA) antibodies, including antiplatelet antibodies and donor-specific anti-HLA antibodies (DSA), which can result in refractoriness to platelet transfusion therapy and antibody-mediated rejection (AMR), respectively [10,11].

We report here a case of successful KT in a patient with ES who had DSA and antiplatelet antibodies.

CASE REPORT

The patient was a 35-year-old Japanese man with ES. He was misdiagnosed with refractory chronic idiopathic thrombocytopenia purpura from early childhood. Proteinuria, which was found during his elementary school years, had progressed gradually to renal

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failure, and a renal biopsy performed at the age of 16 years showed pathological findings compatible with focal segmental glomerulosclerosis. Sensorineural hearing impairment was also noted at that time. He started peritoneal dialysis (PD) when he was 17 years old, but his dialysis modality was changed to hemodialysis because of the frequent obstruction of his PD catheter. At the age of 33 years, he was diagnosed as having ES by both clinical manifestations and identification of the S96L mutation of the *MYH9* gene. Additionally, the same gene mutation was found in his first son.

He received a live-donor kidney allograft from his 63-year-old mother when he was 35 years old. Preoperatively, he had a low platelet count ($0.7 \times 10^4/\mu L$), macrothrombocytopenia, and antiplatelet antibodies, which might have resulted from his history of multiple platelet transfusions during previous blood access- and PD-related surgeries. Furthermore, pretransplant complement-dependent cytotoxicity (CDC) crossmatch was negative, but flow cytometry crossmatches to donor T and B cells were positive, with low-level DSA to HLA-A24 and HLA-B51. Therefore, he underwent desensitization using plasmapheresis (PP) with rituximab before KT. For desensitization, mycophenolate mofetil (MMF) was given 6 weeks prior to transplantation. In addition, tacrolimus (TAC) was administered about a month before transplantation. Rituximab (200 mg/body) was given twice on −10 day and −1 day before KT. He underwent PP 3 times before KT; double-filtration PP was performed on day −5 and day −3, and PP was performed on day −1 before KT. This protocol decreased the mean fluorescence intensity (MFI) of DSA remarkably from 13,664 to 1778 (for anti-HLA A24) and from 13,103 to 4175 (for anti-HLA B51).

Post-transplant immunosuppression was standard triple therapy including TAC, MMF, and steroids with basiliximab induction. Moreover, daily transfusion of HLA-matched platelets was given to keep platelet counts over $10.0 \times 10^4/\mu L$ from day −1 until day 7 after the transplantation. Perioperative course of platelet count and serum creatinine (SCr) are shown in Fig 1.

At surgery, the platelet number increased to $13.3 \times 10^4/\mu L$. Immediate function of the kidney allograft and smooth decrease in SCr level without bleeding complications were observed.

After HLA-matched platelet transfusion was stopped on day 7, the platelet count decreased slowly to the pretransplant level ($<1.0 \times 10^4/\mu L$) on day 14 after surgery. No AMR and no bleeding complications were observed after KT.

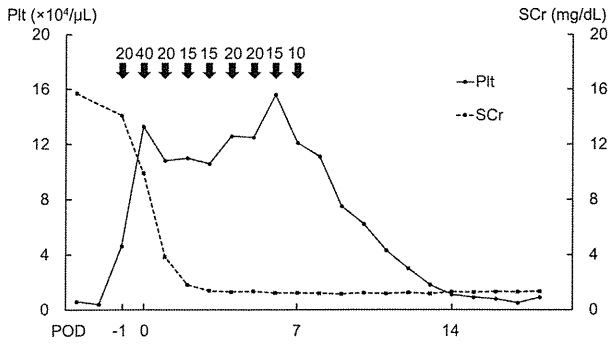

Fig 1. Perioperative time course of platelet count and serum creatinine in this case. Solid line: platelet count; dashes: serum creatinine. *Black arrows* indicate HLA-matched platelet transfusion. *Numbers with black arrows* indicate the amount of platelet units transfused.

At 1 year after transplantation, his SCr was 1.2 to 1.3 mg/dL. The surveillance biopsy performed at 4 months and 1 year after transplantation showed no pathological findings suggesting AMR.

DISCUSSION

ES, also known as MYH9 RD, is a rare disorder characterized by hearing impairment, macrothrombocytopenia, and renal failure, and the English literature contains only a few reports of successful KT in patients with ES.

The main difficulty in the operative management of patients with ES is perioperative bleeding. One report insists that macrothrombocytopenia has little relation to bleeding complications of surgical interventions for MYH9 RD and is not a contraindication for surgery [12]. On the other hand, Saposnik et al, who evaluated the risk for bleeding complications in a cohort of 109 cases with MYH9 RD during an 8-year follow-up period [13], demonstrated that maintaining platelet counts at or above $5.0 \times 10^4/\mu L$ decreases bleeding complications in such cases. They showed that the risk for bleeding was related more to platelet count than to platelet size.

Ogura et al reported a case of ABO-incompatible KT in a patient with ES [6]. In this case, they recovered platelet count of the recipient to $10.8 \times 10^4/\mu L$ by transfusion before transplantation, and maintained it above $5.0 \times 10^4/\mu L$ after transplantation. However, intraperitoneal bleeding and hemorrhagic duodenal ulcer developed within a month after transplantation, leading the authors to suggest that platelets count greater than $10.0 \times 10^4/\mu L$ might be necessary for the safe and successful postoperative management in KT for ES patients. Min et al also reported 2 cases of KT in patients with Fechtner syndrome (a condition similar to ES) [7]. Based on the observation of intraperitoneal bleeding on postoperative day 1 and intracranial hemorrhage on postoperative day 4 in the first patient (whose platelet count was $5.0 \times 10^4/\mu L$) and no complication in the second patient (whose platelet count was above $10.0 \times 10^4/\mu L$), they concluded that the platelet counts of kidney transplant recipients should be kept above $10.0 \times 10^4/\mu L$ perioperatively. However, the second and biggest problem was that this patient had antiplatelet antibodies as well as anti-HLA antibodies, probably due to previous platelet transfusions. Therefore, maintaining platelet count $>10.0 \times 10^4/\mu L$ by the usual platelet transfusions is quite difficult. Several previous studies have demonstrated that HLA-matched platelet transfusions would be effective in this situation. Recently, successful cochlear implantation using HLA-matched platelet infusion was reported in 2 patients with ES [9,14]. Also, daily HLA-matched platelet infusion seemed to prevent perioperative bleeding in our case.

The presence of DSA in the recipient before KT is a strong risk factor for the development of AMR. Furthermore, application of the solid-phase antibody detection systems, such as Flow PRA (One Lamda, Canoga Park, Calif., United States) and Luminex (Luminex Corp., Austin, Texas, United States) (single-antigen beads), have allowed

for a more exact determination of anti-HLA antibody specificity. An increasing number of studies showed an association between level of DSA and risk of AMR. Fidler et al revealed that the risk of AMR was significantly greater in patients with high pretransplantation levels of DSA (MFI >8000) than in those with low DSA levels (MFI <8000) [10]. Current desensitization protocols using a combination of PP, rituximab, and antithymocyte globulin or basiliximab induction may be successful in decreasing the AMR rate in CDC crossmatch negative, flow cytometry crossmatch positive patients with low DSA levels. MIF of DSA was significantly decreased in our case using desensitization.

In conclusion, this is first report of successful KT in an ES patient with both DSA and antiplatelet antibodies. Our desensitization protocol prevented AMR, and HLA-matched platelet infusion prevented perioperative bleeding by maintaining the platelet count at $>10.0\times10^4/\mu L$.

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ORIGINAL ARTICLE

Clinical and genetic characteristics of Japanese nephronophthisis patients

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Abstract
Background Nephronophthisis (NPH) accounts for 4–5 % of end-stage renal disease occurring in childhood.
Method We investigated the clinical context and characteristics of renal and extrarenal symptoms, as well as the *NPHP* genes, in 35 Japanese patients with clinical and histologic features suggesting NPH.
Results NPH occurred fairly uniformly throughout Japan irrespective of region or gender. In three families, NPH affected siblings. The median age of patients was 12.5 years. Renal abnormalities attributable to NPH discovered through mass screening, such as urine tests in school. However, NPH accounted for less than 50 % of children with abnormal findings, including incidentally discovered renal dysfunction during evaluation of extrarenal symptoms or during routine check-ups. Typical extrarenal manifestations leaded to discovery including anemia and delayed physical development. The urine often showed low gravity specific density and low molecular weight proteinuria. Frequent renal histologic findings included cystic dilation of tubules, mainly in the medulla, and irregularity of tubular basement membranes. Genetically abnormalities of *NPHP1* were not common, with large deletions frequently noted. Compound heterozygotes showing single abnormalities in each of *NPHP1*, *NPHP3*, and *NPHP4* were observed.
Conclusions Our findings resemble those reported in Western populations.

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Keywords End-stage renal disease · Renal cysts · *NPHP* genes · Children · Renal tubules

Introduction

Nephronophthisis (NPH) is a disease characterized by renal medullary cyst formation. Additional histologic findings include tubulointerstitial nephritis accompanied by progressive sclerosis and hyaline glomeruli. Although NPH characteristically shows autosomal recessive inheritance, it may occur sporadically [1]. NPH accounts for approximately 4–5 % of end-stage renal disease (ESRD) in childhood. Disease subtypes include: infantile NPH (NPH2), which progresses to ESRD around the age of 5 years; juvenile NPH (NPH1), which develops from early childhood to school age and usually progresses to ESRD by an age of about 13 or 14 years; and adolescent NPH (NPH3), with development of ESRD at an average age of 19 years. Juvenile NPH is reported to be the most common subtype [1].

NPHP1, the gene most often responsible for juvenile nephronophthisis, encodes the nephrocystin-1 molecule. This gene has an extent of approximately 11 kbp, and is located on chromosome 2q12-13 [2]. The nephrocystin-1 protein consists of 677 amino acids and includes three coiled domains; two highly acidic negatively charged glutamic acid-rich domains; and an Src-homology 3 domain. Nephrocystin-1 has a molecular weight of 83 kD. As this protein is located in the transition zone of primary cilia of renal tubular epithelial cells, its abnormalities typically cause dysfunction of these primary cilia (ciliopathy) [1, 2].

NPHP4, whose abnormalities cause a second form of NPH1, is located on chromosome 1p36 and encodes the nephrocystin-4 (nephroretinin) molecule. Nephrocystin-4

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Primers for *NPHP1*

exon	F primer	5′-nucleotide sequence -3′	R primer	5′-nucleotide sequence -3′	Amprified fragment length (bp)
exon1	NPHP1E01F010	GACCACCGCAAGAGAACATTT	NPHP1E01R010	AAGCTCCCAGGATTAGGTGGG	319
exon2	NPHP1E02F010	GGTATATGGGTTTTCACTGTA	NPHP1E02R010	TTCCATTTGATTCCAAAGGAC	319
exon3	NPHP1E03F010	TAATTGCCTTGCCTGCTCAAC	NPHP1E03R010	CAGACTTAGCAAGCCTGTTCG	320
exon4	NPHP1E04F010	GATAGGTGTAATGTCACACTG	NPHP1E04R010	CATGGGATCTAACACCTTCTA	418
exon5	NPHP1E05F010	CCAGCTCCAAATATGGGATAT	NPHP1E05R010	CAGGTGTACAGGCAGAGTTTTC	380
exon6	NPHP1E06F010	GGGAAGCTTTTGATAAACCTT	NPHP1E06R010	GTCATTCACTAGTCAACTGAC	349
exon7	NPHP1E07F010	GTTTTTGTTTTTACTGGAGGG	NPHP1E07R010	GTTGTCTCCATTTCAAGAAAG	306
exon8	NPHP1E08F010	CTCGTTTTCATCTGGAAACTG	NPHP1E08R010	GGAAAGCAGGATCAATGAGAA	443
exon9	NPHP1E09F010	CTTCCACTAAAGTCTGTATGT	NPHP1E09R010	GTGAGATTCAACATCTTCTTC	322
exon10	NPHP1E10F010	TTTGGAAGTGCCTGTACTCTA	NPHP1E10R010	GTCCAAATTCTGCCTTAGTTA	360
exon11	NPHP1E11F010	GCCTGCCAATATTTATTGTTCC	NPHP1E11R010	TACTCTCTTGGGAATTGGGGA	494
exon12	NPHP1E12F010	TCCTCACTTAGTGTAGCCACT	NPHP1E12R010	GTCCTCAAAGAACACCAAAGA	302
exon13	NPHP1E13F010	CACCTTCAACATTGGGATTAC	NPHP1E13R010	CATTCTCATTCCTCAAGGGAT	365
exon14	NPHP1E14F010	GCAAAATGAGATTCTACTGTG	NPHP1E14R010	AGTTATTGGCATGCTCATAGA	342
exon15	NPHP1E15F010	GGCATAAATGAAATGTCTGAG	NPHP1E15R010	GTCTCATATGTGTTACCAAGA	374
exon16	NPHP1E16F010	GCACTACTGGGTGGTATATTT	NPHP1E16R010	GGGAAGAATTAAAGAGGACAA	330
exon17	NPHP1E17F010	GAAGCAAAATTTGGGACTGTT	NPHP1E17R010	AAAGTCACAACCAGAAACAGA	316
exon18	NPHP1E18F010	CCTAGAAGTCAAAGTGTGTAG	NPHP1E18R010	GGAGACATCATCCTAGTAACA	326
exon19	NPHP1E19F010	CAGCATTTTTAACCCTGTCCA	NPHP1E19R010	GGGATTATGACTATGGCTACT	261
exon20	NPHP1E20F010	CCCTCCATCCTACCTCTTAGG	NPHP1E20R010	CTAAGTTGTAAAGTGACAGTG	478

Primers for *NPHP2*

exon	F primer	5′-nucleotide sequence -3′	R primer	5′-nucleotide sequence -3′	Amprified fragment length (bp)
5UTR	I5Uf1	TTTCCCATTGGGCTCTCGGCC	I5Ur1	TGAGTCTGCAGCAGGGGCCAA	366
exon1	IEx1F	CCCACTTGGAACTGATGAGAC	IEx1R	AACAAACTTCCTCAGGACAAAC	265
exon2	IEx2F	ATAATAAACAGCGAATATAGTTCCTAC	IEx2R	TGTCCATTGCATAGTTCCAC	327
exon3	IEx3F	GTGGAATTACAAGCATTTTTCC	IEx3R	AATTCAGGCCTTCTTCCTTG	411
exon4	IEx4F	TTGTTACTGTTGTTATTTCGAGAACC	IEx4R	ACTTCTTGGGGGATGAGTCC	356
exon5	IEx5F	CACCAAATGTAATTTATTGAGGATTC	IEx5R	AGTGGAAGGGAAGGCACAG	317
exon6	IEx6F	CTGCTGTTCAGAAACCGTTG	IEx6R	GGTGTAGGAGTGCAAAAAGC	421
exon7	IEx7F	AGGGGAAAATGCTTTGCTTC	IEx7R	AATTTTATAGCAACATCTACACTTTGG	351
exon8	IEx8F	GATGGGGAAATCAAGAGAGG	IEx8R	TGTGCAGCTTTCTGCTAAGG	348
exon9	IEx9F	CCATAAGAATAAAGCATTAAAGGAAC	IEx9R	TGTGGGTGATCTCTTCATCTTG	494
exon10	IEx10F	CCACATATCCAAAAATACTTACTCC	IEx10R	AGAAAGGATGTATGATAAAGAGCAC	528
exon11	IEx11F	TTCCACATCTTAGAATGAAGTTTCC	IEx11R	CTCATCTGTTTCCCTCTCCTG	427
exon12	IEx12F	CACACAGAGACTTGAGGAGGTG	IEx12R	CGGCAGAAGATGACAAAGG	382
exon13	IEx13F	TGTAACTGCCACTATTATGGTGATG	IEx13R	CACCACATGGAACTCACTGG	939
exon14	IEx14F	AATGGGAGCTTGAATGAACC	IEx14R	TGGTACTCTGGGGGTACTTG	410
exon15	IEx15F	CACACACCTGCAAGCTCAAG	IEx15R	TCTTTGGGGATGAAACAAAGG	255
exon16	IEx16F	CCAATGAACTATTCCCTTCAGC	IEx16R	GCAGAAAATCTGAACTCTGCAC	242

Fig. 1 Genomic DNA extraction, PCR, and determination of *NPHP1, 2, 3* and *4* gene sequence. PCR primers were prepared to amplify approximately 200–300 bp fragments based on *NPHP 1–4* gene sequences registered in GenBank, the following primers were used as shown

has been shown to carry out signal transmission between renal tubular epithelial cells, in cooperation with nephrocystin-1 [3].

NPHP2, the gene responsible for infantile NPH (NPH2), is located on 9q22-31 [4]. *NPHP2* encodes a protein termed inversin (INVS). An abnormality in INVS can cause situs inversus, pancreatic islet-cell dysplasia, cardiovascular abnormalities, and hepato-biliary disorders. In addition, INVS abnormalities can cause cyst formation resembling that in juvenile nephronophthisis. However, the renal prognosis is worse progression to ESRD in early childhood.

The gene responsible for adolescent NPH (NPH3), *NPHP3* is located on chromosome 3q21-22 [5]. *NPHP3* is believed to encode a protein involved in signal

Primers for *NPHP3*

exon	F primer	5′-nucleotide sequence -3′	R primer	5′-nucleotide sequence -3′	Amprified fragment length (bp)
exon1	NPHP4E01F010	ATGCAAATCAGGATGGGCCCG	NPHP4E01R010	AACCCACGTAGCCAAACGGCA	598
exon2	NPHP4E02F010	AGGTTCCTCTGGGATTTAGTG	NPHP4E02R010	AATCAAAGCATCGTAAGCCAG	373
exon3	NPHP4E03F010	TGATATCTGAGCGAGGTGGCC	NPHP4E03R010	AAGTCTGAGACGCGCTGTGAG	368
exon4	NPHP4E04F010	TGCTGTGGCACGTGTAGGAAG	NPHP4E04R010	ACTGCACTCTAGCCTGGTTGA	379
exon5	NPHP4E05F010	AAAGCTCTAGTGGGCGTGGTG	NPHP4E05R010	CAGATAGCAGTTTACACTGAG	273
exon6	NPHP4E06F010	CCTGTTTGTGGTGTTTCTAAC	NPHP4E06R010	TTCCATCCTCCTCCACTGTCC	426
exon7	NPHP4E07F030	TGGAGGAGGTTTGGGGTAGAT	NPHP4E07R020	AGGGGAAAAGACAGAACTACA	569
exon8	NPHP4E08F010	CTGCTCCAGTTTCTTCTCTCT	NPHP4E08R010	TCCCACGTGGGTGAGTCAACA	383
exon9	NPHP4E09F010	ACTTGCTGTTGCAGCAGCACC	NPHP4E09R010	CCATCTCATCTGTATCCTTTG	446
exon10	NPHP4E10F010	CACTGAGCTCTCGTTGAATTT	NPHP4E10R010	GGCATACCCATGACATGAAAA	420
exon11	NPHP4E11F010	GACTTTGTTTTAGGGCAGAGC	NPHP4E11R010	ATGTGGTGATTACCGTACTAG	339
exon12	NPHP4E12F010	AGACAAGGTGGTGAGGCCTGT	NPHP4E12R010	AAGCACGCAGGGATCCACTGT	274
exon13	NPHP4E13F010	TTGAGAAGCGGTCCCAGGTTC	NPHP4E13R010	TGCCACCTAACTAAGGACAGG	384
exon14	NPHP4E14F010	CCAGAGGCAATTAATCGATGA	NPHP4E14R010	ATTGATGCACCTCCCTGTGGA	354
exon15	NPHP4E15F010	CAGACTGTTGGACCTTGTGAA	NPHP4E15R010	TCAGCACAGACAGTTCTGCCA	392
exon16	NPHP4E16F010	GACTAAGGTGCCTGGACCATC	NPHP4E16R010	GGTCACCGTATGATTCTAATG	419
exon17	NPHP4E17F010	GTAGCTATGACAGAAGCAGAA	NPHP4E17R010	ACAAGTCCTGTGCGGGATAGC	392
exon18	NPHP4E18F010	AGGGTCTTATCCTGCGCACAC	NPHP4E18R010	ATTCTCCCGGTTTCCTCCTGG	441
exon19	NPHP4E19F010	AGGCCATTTGAAAGCCACAGC	NPHP4E19R010	CACATGCACACACGCATGCAC	326
exon20	NPHP4E20F010	CCCTCCCTATATAGGTGGTCC	NPHP4E20R010	AGGTAAGAGAGAATCATGTGG	404
exon21	NPHP4E21F010	AATGTCCTCTTCTGAGATCGC	NPHP4E21R010	AGAGAAGTCAAATCGCCCCGG	444
exon22	NPHP4E22F010	TCTCTCCCACTCCTCTGAGCA	NPHP4E22R010	TGCACAGGTAAGGGAGGGACA	391
exon23	NPHP4E23F010	TCAGTGTGAGAGGAGGCTGGT	NPHP4E23R010	AAAAGGCCATTCCCAGGCCCA	346
exon24	NPHP4E24F010	GTCTTGGCAACAGTGGAGATA	NPHP4E24R010	ACCAGGGCATGAAGCCATGAG	360
exon25	NPHP4E25F010	TGACGAGCCTGTCCTGTCCTA	NPHP4E25R010	CCTAAAATGAAGAGGATCCCA	286
exon26	NPHP4E26F010	AGATGCGTTCTGGGAGGGACT	NPHP4E26R010	TTTAGGAAGGGGCCAAGCCCA	308
exon27	NPHP4E27F010	TTTCCCTGCACAGCCTCCTGT	NPHP4E27R010	AAAAGCTGCTGTCAGGGCCAC	390
exon28	NPHP4E28F010	AACCACCCATGACCTTGGGCT	NPHP4E28R010	TGTATCCAGTGGTCCGAGTCA	392
exon29	NPHP4E29F010	TCTTATCTCTGTGGGGGTCCC	NPHP4E29R010	GCTGGTGATTTGAGGAACTCG	364
exon30	NPHP4E30F010	CAGCTCCCCTTGGAAATAAAC	NPHP4E30R020	AAACTGCCAAGGGAAGACGTG	768

Primers for *NPHP4*

exon	F primer	5′-nucleotide sequence -3′	R primer	5′-nucleotide sequence -3′	Amprified fragment length (bp)
exon1	NPHP3E01F020	TGCTCCGCCCAGTCCTGCTCT	NPHP3E01R020	GAGAATATGGCCTCTCAAATT	694
exon2	NPHP3E02F010	CATGAAGTTCCTGATAATTGG	NPHP3E02R010	GAATCCTACATGACTTACTTC	387
exon3	NPHP3E03F010	GAGGACCAAATGAATATTGGT	NPHP3E03R020	GCAGCTGACAGAGAGAACACA	420
exon4	NPHP3E04F020	CAGTATCTTTGAACTTTGCCA	NPHP3E04R020	GATGGTTTGTCAATGGAAAGC	459
exon5	NPHP3E05F020	GGTATGGCAGTATTAACATGT	NPHP3E05R020	GCTTCCTGTTCTTTAAGACAT	391
exon6	NPHP3E06F020	GTATTTGAGAGAAACTTGCCT	NPHP3E06R020	GCTATATTTGCCAAACTCTGA	595
exon7	NPHP3E07F020	GTTGGACCTTTTCTGGCCACT	NPHP3E07R020	GTTCCAGCCACACTGGTTTCT	401
exon8	NPHP3E08F010	CCTAAGGTTGTTGTGAAGATA	NPHP3E08R010	TTCAAAAAGACAAGCAAGTGG	320
exon9	NPHP3E09F020	AAGGCTGTATGTTTGAACTTG	NPHP3E09R020	CACATCTCAACATGGATAATC	440
exon10	NPHP3E10F010	CAGCTTTTCTCCAGTATTTTC	NPHP3E10R020	GGGCATGAACCTATTGTTTTA	350
exon11	NPHP3E11F020	AGTAACTGACCACCTGATTGC	NPHP3E11R020	GACCCGATTGTATCGAATATT	390
exon12	NPHP3E12F020	ATATTCGATACAATCGGGTCC	NPHP3E12R020	CTGTGGGCATACGATATATTT	458
exon13	NPHP3E13F010	CAGAGTTCAGATTGGTGATAA	NPHP3E13R010	CCTCACTGCAAGTTACATAAA	406
exon14	NPHP3E14F010	GTTGTGATTCATTGCTCAAAG	NPHP3E14R010	CCTTATAACAGATCCCCTATA	410
exon15	NPHP3E15F010	TTTCTGTCGGGGTACTTGTTG	NPHP3E15R010	CAGACTGGTGTAGTGATCAGT	283
exon16	NPHP3E16F020	TGACTCTAGCAGCCCCATAAA	NPHP3E16R020	GGCTATCAGCATTCCTGCATA	435
exon17	NPHP3E17F020	GTTATCTTTGGTGTGCTAGAT	NPHP3E17R010	CTTTGGCAGAAATAATCTTGC	487
exon18	NPHP3E18F010	CATTCCACACTTCTGAGATTT	NPHP3E18R010	GAATAGGGAGAGGATTTAATC	496
exon19	NPHP3E19F020	GGTTCTGCATATCACTGAATT	NPHP3E19R020	GGAAAAGCAGATCTAATAGAG	492
exon20	NPHP3E20F010	CAGTACTCGCCTACTAATAAA	NPHP3E20R020	GCAAGATCTGTCATCTGATTA	440
exon21	NPHP3E21F020	CTCTTCTCTTTTTCCAAGATG	NPHP3E21R020	CCACATGAAGACTAGGCACAG	497
exon22	NPHP3E22F020	CTAGACTTGCTTTGTTTTGTC	NPHP3E22R020	CTTTAAAGAACTGAGGTAGCT	614
exon23	NPHP3E23F010	GTTGCCATGTGGAAATATTTG	NPHP3E23R010	CATACATGAAATTTTGCGTGG	436
exon24	NPHP3E24F010	GGAAAGTAAGATTTTGAGCTG	NPHP3E24R020	GTTCTGCTCAGTTACTTGTTA	536
exon25	NPHP3E25F020	GCTTTTCTATACAGTGTAGCT	NPHP3E25R010	CCTTCATACAAGTCTAACTTC	485
exon26	NPHP3E26F010	CCCATCTTTTAGGAGGATATT	NPHP3E26R010	CCCCACTCTAAGAAAAAAACAT	341
exon27	NPHP3E27F010	AGGGGAAATGGGCAAATATTT	NPHP3E27R020	CCTTGGATACCATATAATAGG	512

Fig. 1 continued

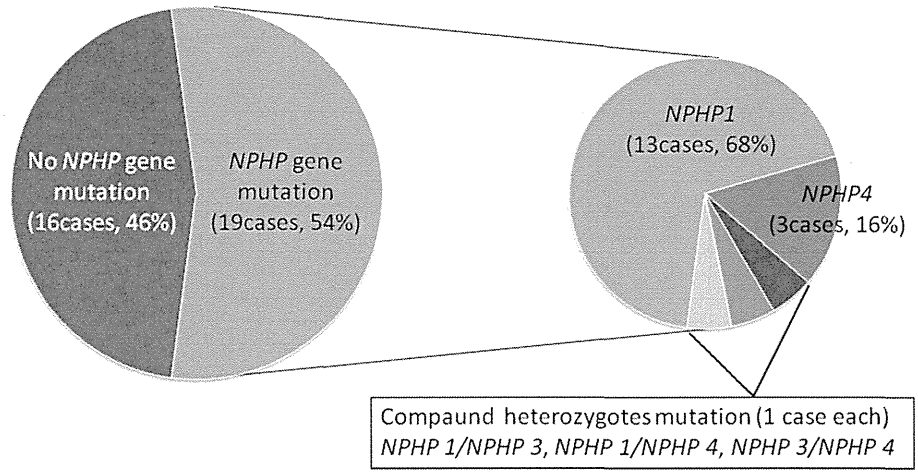

Fig. 2 Percentage of NPH patients with *NPHP* gene mutation. *NPHP* gene mutation was detected in 19 patients. No *NPHP* gene aberration detected within the sequences analyzed in the other 16 patients with suspicion of NPH clinicopathologically

transmission in renal tubular epithelial cells, such as signaling involving diacylglycerol kinase-zeta and receptor-like tyrosine kinase. Abnormalities of the protein disrupt urinary concentrating ability and the structure of cilia of renal tubules, as in the other types of NPH.

Previous reports describe occurrence of *NPHP1* mutations in approximately 30–50 % of juvenile nephronophthisis patients in Western countries [1, 6], where genetic analysis of *NPHP1* is performed initially when juvenile NPH is suspected. If mutation is detected, kidney biopsy usually is deferred [7]. Genetic diagnosis is made less frequently in Japan; so kidney biopsy often is performed to obtain a definitive diagnosis. Not infrequently, NPH is discovered in the advanced or end stage in many Japanese patients, in whom treatment no longer can slow progression. Unfortunately, symptoms typically seen in early stages are incompletely characterized.

In the present study, we investigated clinical, histologic, and genetic features in 35 Japanese patients clinically and histologically suspected to have NPH, aiming to promote early diagnosis. We studied many exons as many as 13 *NPHP* genes. Since such genetic analysis involves significant cost and time, we also screened biopsy specimens by immunohistologic methods employing antibodies against relevant peptides.

Methods

Patient registration and informed consent

Our subjects included 35 patients with clinicopathologic findings suggestive of NPH who were referred to our department from various regions of Japan. The study was performed following approval by the Ethics Committee of Kinki University Faculty of Medicine and acquisition of written informed consent from patients or their parents (Actual state of Japanese juvenile nephronophthisis patients and identification of gene aberrations; approval number 20–99).

Genomic DNA extraction, polymerase-chain reaction (PCR), and determination of *NPHP* gene sequence.

After approximately 5 mL of peripheral blood was collected from patients into tubes containing Na-EDTA, genomic DNA was extracted using NucleoSpin for Blood (TaKaRa Bio Inc, Shiga, Japan). Human genomic DNA (TaKaRa Clontech, code 636401; Shiga, Japan) was used as a control. Patient samples and control genomic DNA were diluted with sterile water to prepare 10 ng/μL solutions. PCR was performed using these as templates and TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa Bio Inc, Shiga, Japan). To determine extent of deletions and identify break points, PCR primers were prepared to amplify approximately 200–300 bp fragments based on *NPHP* gene sequences registered in GenBank (Fig. 1). For PCR, annealing temperatures and times were 63 °C and 15 s for *NPHP1* and *NPHP3*; 60 °C and 15 s for *NPHP2*; and 60 °C and 20 s for *NPHP4*, respectively. For sequence analysis, PCR products were purified by an enzyme reaction, and templates for sequencing were prepared. The sequencing reaction was carried out using the prepared template DNA and a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), employing the dye terminator method. Reaction products were purified by gel filtration, and sequence analysis was performed using a capillary-type sequencer, ABI3730xl (Applied Biosystems, CA, USA). The algorithm established by Salomon et al. [8]. was adapted for use in our analytical procedure. In children with renal dysfunction

Table 1 Characteristics of patients found to have NPHP gene mutations

Age/gender	Motive of discovery	BUN/Orn (mg/dL)[a]	UP	Urinary LMP	Low gravity urine	Extrarenal symptom	Diagnosis at first biopsy	*NPHP*/mutation	The other *NPHP* mutation	Consenguineous marriage	Family history of renal disease
14 years/M	Anemia	49/3.3	(−)	(−)	(−)	n.f	n.d	Large delation (>2.0kbp)		(−)	(−)
13 years/F	Nocturnal enuresis	27/1.3	(−)	(−)	(−)	n.f	n.d	Large delation		(−)	NPH (younger brother)
11 years/M	Sibling with NPH	17/0.6	(−)	(+)	(+)	n.f	n.d	Large delation		(−)	NPH (elder sister)
15 years/F	Protein uria (school urinalysis)	21.3/1.3	(±)	(−)	(−)	n.f	TIN	(−)	D1980G (*NPHP4*, hetero)	(−)	(−)
15 years/F	Protein uria (school urinalysis)	89.6/11.6	(−)	(+)	(+)	RP	NPH	Partial delation (=300bp)		(−)	Acute glomerulonephritis (mother)
14 years/M	Chance discovery of the RD (heatstroke)	17/0.9	(±)	(+)	(+)	n.f	n.d	n.d	L939 (*NPHP4*, hetero)	(−)	(−)
11 years/F	Enuresis, polyuria	74/5.2	(1+)	(+)	(+)	SS(-2.5SD)	NPH	E677Q (hetero)	E642L (*NPHP4*, hetero)	(−)	(−)
18 years/F	Enuresis, polydipsia	82/8.1	(±)	(+)	(+)	SS(-1.8SD)	NPH	Gln547 (hetero)	S80L (*NPHP3*, hetero)	(−)	Protein uria (father)
8 years/M	Glycosuria (school urinalysis)	159/11.1	(2+)	(+)	(+)	n.f	Similar NPH	E677Q (hetero)	*NPHP4*(−)	(−)	(−)
14 years/M	Glycosuria (school urinalysis	48/5.0	(±)	(+)	(+)	RP	NPH	Large delation		(−)	NPH (younger sister)
13 years/F	Sibling with NPH	58/2.7	(1+)	(+)	(+)	RP	NPH	Large delation		(−)	NPH (elder brother)
20 years/F	Chance discovery of the RD (medical examination)	44.6/2.4	(1+)	(+)	(+)	n.f	n.d	(−)	L939Q (*NPHP4*, homo)	(−)	(−)
8 years/F	Chance discovery of the RD	32.2/1.4	(−)	(+)	(+)	n.f	NPH	Large delation		(−)	(−)
15 years/F	Protein uria (school urinalysis)	37/2.6	(−)	(+)	(+)	n.f	Tubular enlargement medullary cysts	Large delation		(−)	(−)
7 years/F	Chance discovery of the RD (urine tract infection)	40/3.0	(1+)	(+)	(+)	Joubert syndrome	n.d	(−)	AA/OO→AG/OT (exon 26/exon 20)	(−)	(−)
19 years/F	Chance discovery of the RD (bronchitis)	90.3/8.4	(1+)	(+)	(+)	n.f	n.d	(−)	A150V (*NPHP3*, hetero)	(−)	(−)

Table 1 continued

Age/gender	Motive of discovery	BUN/Orn (mg/dL)[a]	UP	Urinary LMP	Low gravity urine	Extrarenal symptom	Diagnosis at first biopsy	*NPHP*/mutation	The other *NPHP* mutation	Consenguineous marriage	Family history of renal disease
8 years/M	Visual impairment	46.5/1.9	(−)	(+)	(+)	RP	NPH	Large delation	D1980G (*NPHP4*, hetero)	(−)	(−)
16 years/F	Protein uria (school urinalysis)	43.3/2.6	(1+)	(+)	(+)	n.f	NPH	Large delation		(−)	(−)
13 years/F	Anemia, fatigue	39/2.2	(−)	(+)	(−)	n.f	TIN, tubular enlargement	Large delation		(−)	(−)

RD renal dysfunction, *n.f*, not found, *n.d* not done, *UP* urinary protein, *LMP* low molecule protein, *SS* short stature, *RP* retinitis pigmentosa, *TIN* tubulo interstitial nephritis

[a] Renal function at the time of the discovery

who were 5 years old or younger, the gene responsible for infantile NPHP (*NPHP2)* was analyzed first. In patients older than 5 years, *NPHP1* was analyzed first; if no mutation was detected, *NPHP4* was examined. *NPHP3* analysis was added when no mutation was detected in other genes in patients whose disease progressed to end-stage renal disease at an age of 16 years or older.

Clinical data

Data originally collected at our department as well as data provided by other institutions were surveyed using a questionnaire. Questionnaire consists of personal data including the patient's age, motive of discovery, urinary abnormality and renal dysfunction, detailed clinical data, extrarenal symptom, renal tissue diagnosis at the first biopsy, consanguineous marriage, and family history of renal disease.

Results

NPHP gene analysis

Among 35 patients, an *NPHP* gene mutation was identified in 19 patients. Although NPH was suspected clinicopathologically in the other 16 patients, no *NPHP* gene aberration was detected within the sequences analyzed (Fig. 2). Characteristics of patients with *NPHP1* gene mutations (Table 1) and without *NPHP* gene mutations (Table 2) were shown. A mutation was detected only in *NPHP1* in 13 patients; deletion was extensive in 10 (Fig. 3a) and partial in 1. Two other patients had a point mutation (E677Q and K334 N, both heterozygous). In all, these mutations accounted for 37.1 % (13/35) of patients. In another candidate gene responsible for the juvenile type, *NPHP4*, the mutation L939* was detected in 2 patients (Fig. 3b), while a D1980G mutation was detected in 1, accounting for 8.6 % (3/35) of all patients. Compound heterozygotes containing 1 mutation each in *NPHP1* G547* and *NPHP3* S80L (Fig. 4a), 1 mutation each in *NPHP1* E677Q and *NPHP4* E642L (Fig. 4b), and 1 mutation each in *NPHP3* A150 V and *NPHP4* D1089G (Fig. 4c) also were observed. The disease progressed to ESRD before 20 years of age in these patients, similar to the course of other patients with a single-gene mutation. No *NPHP2* mutation was detected in any patient.

Clinical and demographic features of patients

Patient background

Patients were reported from 46 prefectures without evident selection bias, and with no important regional

Table 2 Characteristics of patients without apparent NPHP gene mutations

Age/gender	Motive of discovery	BUN/Orn (mg/dL)[a]	UP	Urinary LMP	Low gravity urine	Extrarenal symptom	Diagnosis at first biopsy	NPHP1 mutation	NPHP3 mutation	NPHP4 mutation	Consanguineous marriage	Family history of renal disease
6 years/M	Lagging physical development	34/1.2	(−)	(−)	(−)	SS (−1.3SD)	NPH	(−)	n.d	(−)	(−)	(−)
12 years/M	SS, fatigue	48/1.3	(−)	(+)	(−)	Sensory deafness	Chronic interstitial nephritis, glomerulosclerosis	(−)	n.d	(−)	(−)	(−)
26 years/M	Chance discovery of the RD (medical examination)	21/1.6	(−)	(+)	(−)	n.f	Interstitial nephritis	(−)	(−)	(−)	(−)	Renal dysfunction (father, young sister)
11 years/M	Pallor, anemia	27.4/1.5	(−)	(+)	(+)	SS (−2.2SD)	n.d	(−)	n.d	(−)	(−)	(−)
17 years/M	SS	34/1.5	(±)	(+)	(+)	SS (−3.8SD)	n.d	(−)	n.d	(−)	(−)	(−)
22 years/M	Hypertention	32.5/1.5	(1+)	(+)	(−)	RP	TIN	(−)	(−)	(−)	(−)	(−)
11 years/F	polydipsia, polyuria	15.4/0.7	(−)	(+)	(+)	n.f	n.d	(−)	n.d	(−)	(−)	(−)
12 years/F	Chance discovery of the RD (protein uria at 3 years old)	32/1.6	(2+)	(+)	(+)	n.f	TIN, tubular enlargement	(−)	(−)	(−)	(−)	(−)
14 years/M	Protein uria (school urinalysis)	58/4.2	(1+)	(+)	(+)	n.f	TIN, tubular enlargement	(−)	(−)	(−)	(−)	(−)
26 years/M	Hypertention (medical examination)	38.7/1.5	(−)	(+)	(+)	n.f	TIN, glomerulosclerosis	(−)	(−)	(−)	(−)	(−)
10 years/M	Pallor, anemia	53.6/1.0	(1+)	(+)	(+)	n.f	NPH	(−)	n.d	(−)	(−)	(−)
28 years/F	Chance discovery of the RD anemia	47.5/2.9	(1+)	n.d	(+)	n.f	TIN, similar NPH	(−)	(−)	(−)	(−)	(−)
11 years/F	Pallor, polydipsia, polyuria	27.4/1.5	(−)	(+)	(+)	SS(-2.2SD)	n.d	(−)	n.d	(−)	(−)	(−)
18 years/M	Crud, fatigability	49.6/4	(±)	(+)	(+)	Specific complexion	Similar NPH	(−)	(−)	(−)	(−)	NPH (young sister)
16 years/F	Sibling with NPH	38.3/1.8	(−)	(+)	(−)	Specific complexion	Similar NPH	(−)	(−)	(−)	(−)	NPH (elder brother)
46 years/F	Protein uria, hematuria (at 30 years old)	33/1.8	(1+)	(+)	(−)	Sensory deafness	Chronic interstitial nephritis, glomerulosclerosis	(−)	(−)	(−)	(−)	NPH (elder brother)

RD renal dysfunction, *n.f* not found, *n.d* not done, *UP* urinary protein, *LMP* low molecule protein, *SS* short stature, *RP* retinitis pigmentosa, *TIN* tubular interstitial nephritis

[a] Renal function at the time of the discovery

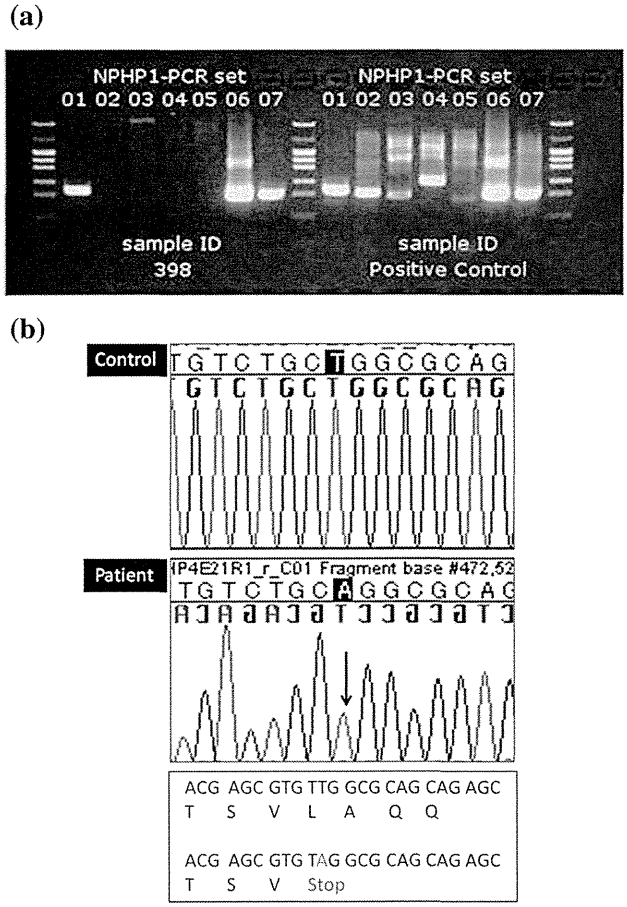

Fig. 3 Analysis of deletion in *NPHP1* (**a**) and analysis of *NPHP4* (**b**). In **a** *lane 1* and *lanes 6 and 7*, contain PCR products of regions within and outside *NPHP1*, respectively; *Lane 2* contains PCR products from the junction between *NPHP1* and the adjacent *MALL* gene. *Lanes 3–5* show the PCR products of *NPHP1* obtained with primers amplifying fragments of approximately 300 bp. *NPHP1* was nearly completely deleted (1.2 kbp deletion). In **b**, substitution of TAG for TTG formed a stop codon, prematurely terminating peptide synthesis

differences (Fig. 5). The male:female ratio was 16:19, with evident gender difference. Ages of patients ranged from 2 to 38 years (median; 12.5). Familial occurrence was noted in 3 families. Other occurrences were solitary, with no family member showing a urinary abnormality, a diagnosis of NPH, or any renal dysfunction of unknown cause.

Initial abnormality deletion

NPH sometimes was discovered following an abnormal urinary finding by mass screening, such as proteinuria detected in a urine test at school (18 %), or renal dysfunction discovered incidentally in working up other medical symptoms, or during medical check-ups (23 %). Approximately 20 % of cases were discovered because of urinary tract symptoms such as polyuria with or without polydipsia, enuresis (often nocturnal), or mellituria. Some 38 % were discovered because of either extrarenal manifestations such as lagging physical development, dwarfism, anemia, pallor, hypertension, or visual disturbance arising from pigmentary retinal degeneration; a prior diagnosis of NPH in a sibling; or both (Fig. 6).

Urinary findings

Urine specific gravity frequently was low (not greater than 1.010); approximately 75 % of cases. Low molecular weight proteinuria, such as β2-microgloburinuria, also was common (85 %), even though inclusion of renal function shown such as between blood urea nitrogen and serum creatinine was relatively mild at that time.

Renal histologic findings

Renal biopsy was performed in 25 patients (71 %). These included 13 patients demonstrated to have an *NPHP* gene mutation and in 12 with no *NPHP* gene mutation identified (suspected cases). Histologic findings included suspected NPH; interstitial nephritis, renal tubular dilation, and glomerulosclerosis. Cystic dilation of renal tubules and irregular contours of tubular basement membranes were observed in most patients, mainly in the renal medulla (Fig. 7a). Sclerotic glomeruli, inflammatory cell infiltration in the renal tubules and interstitium, and fibrosis were frequent, although not seen in all patients (Fig. 7b).

Discussion

Renal tubular epithelial cells are attached to the basement membrane through integrin cross-linking, which transmits extracellular signals to the cell nucleus [2]. Nephrocystin acts importantly in signal transmission between tubular epithelial cells and between these epithelial cells and the extracellular matrix functioning as a docking protein. Nephrocystin also is involved in cell adhesion, together with *N*-cadherin, catenin, and β-catenin [2, 8]. Furthermore, nephrocystin influences actin cytoskeleton structure together with β-tubulin, contributing to maintenance of the cytoskeleton and determination of cell polarity. Nephrocystin forms a complex with Crk-associated substrate, which promotes phosphorylation of Pyk2 and transmits intracellular information through a Pyk2-dependent pathway [2]. Furthermore, nephrocystin is present on primary cilia, where it functions in cooperation with α-tubulin; nephrocystin also is involved in signal transmission in organelles [9]. Accordingly, abnormalities in the nephrocystin molecule disrupt signal transmission between cells