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ORIGINAL ARTICLE

Growth impairment in children with pre-dialysis chronic kidney disease in Japan

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Abstract

Background Growth impairment is a major complication of chronic kidney disease (CKD) in children. However, no cohort studies have examined the growth of Asian children with pre-dialysis CKD.

Methods We sent cross-sectional surveys to 113 Japanese medical institutions that were treating 447 children with CKD stages 3–5 in 2010 and 2011. Of 447 children included in our survey conducted in 2010, height and CKD stage were evaluable for 297 children in 2011, and height standard deviation score (height SDS) was calculated in these children.

Results Height SDS decreased with increasing CKD stage ($P < 0.001$) in boys and girls. Height SDS also decreased significantly with increasing CKD stage among patients with congenital anomalies of the kidney and urinary tract ($P < 0.001$). Risk factors for growth impairment included CKD stages 4 and 5 (relative to stage 3), being small-for-date, and asphyxia at birth. Among children with a height SDS ≤ -2.0, growth hormone was used in 19.5, 31.0, and 25.0 % of children with CKD stages 3, 4, and 5, respectively.

Conclusions This prospective cohort study revealed marked growth impairment in Japanese children with CKD stages 3–5 relative to healthy children. CKD-related risk factors for growth impairment included advanced CKD (stages 4 and 5), being small-for-date, and asphyxia at

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birth. Growth hormone was infrequently used in this cohort of children with pre-dialysis CKD.

Keywords Child · Chronic kidney disease · Growth · Growth hormone · Japan

Introduction

Chronic kidney disease (CKD) is relatively rare in children, but it frequently progresses to end-stage kidney disease (ESKD), which requires dialysis or kidney transplantation [1–7]. CKD is associated with several clinical disorders, including growth impairment, CKD-mineral bone disorder, cardiovascular disease, metabolic acidosis, and anemia. Impaired growth, in particular, is a major complication of CKD in children treated with dialysis or kidney transplantation [2, 8–13]. Impaired growth is caused in part by defects in the growth factor (GH)—insulin-like growth factor I axis, and is associated with a variety of medical and psychological problems, together with an increased risk of death [14].

In an effort to prevent or minimize growth impairment and associated disorders, children on dialysis or after kidney transplantation are often treated with growth hormone (GH) or nutritional interventions [8, 14–18]. GH is recommended to treat growth impairments in pediatric patients with CKD [14], and was reported to have good outcomes in a Cochrane review [17], in which treatment with GH was associated with significant increases in height standard deviation score (SDS) at 1 year compared with placebo. However, because patient management begins long before dialysis is started, it is essential to diagnose and treat possible growth impairments at this time. Furthermore, the current status of GH use in Asian children with pre-dialysis CKD is unknown.

Using a prospective cohort of Japanese children with pre-dialysis stage 3–5 CKD, we reported that the prevalence of stage 3–5 CKD in Japan was 29.8 cases/million, and that most of these children had non-glomerular diseases, particularly congenital anomalies of the kidney and urinary tract (CAKUT) [19]. Since that study, we have conducted other surveys to obtain further insights into the characteristics of Asian children with pre-dialysis CKD, including related disorders and treatments. In the present study, we sent additional surveys to the clinicians who participated in the original study with the following aims: (1) to determine the association between CKD and growth status; (2) to identify possible risk factors for growth impairment; and (3) to determine the frequency of GH use in Japanese children with pre-dialysis CKD.

Subjects and methods

Study design and population

The study design and patient population are described in more detail in our original report [19]. In 2010, we sent two surveys to 1190 institutions in Japan to collect data on children with CKD treated as of April 1, 2010. The institutions included all members of the Japanese Society for Pediatric Nephrology, all university hospitals, all children's hospitals, and all general hospitals with >200 beds in Japan, as these were deemed more likely to be treating children with CKD than other medical centers in Japan. The first survey documented the number of children with CKD stages 3–5 in each institution. The respondents were asked to search their medical records to determine the numbers of patients with a confirmed diagnosis of CKD or patients with abnormal serum creatinine (SCr) values. In the second survey, the respondents were asked to record the clinical characteristics of each patient. A total of 925/1190 institutions (77.7 %) responded to the first questionnaire. In the second questionnaire, the participating institutions provided data for 479 children. Of these, 447 children were evaluable based on the following criteria: (1) aged 3 months to 15 years as of April 1, 2010; (2) presence of CKD stages 3–5 lasting >3 months; and (3) no history of dialysis or kidney transplantation. Cases with transient increases in SCr were excluded.

In September 2011, we sent a third questionnaire to the 113 medical institutions that provided data for the 447 children included in the original report [19]. This survey asked clinicians to provide further details for each case, including height, age, sex, CKD stage, primary disease, treatments received (including the use of GH), serum creatinine levels, and the presence of other diseases likely to cause growth impairment. All surveys were to be returned using provided envelopes, and data entry was conducted by the data center. The participating institutions provided data for 429 children in the third questionnaire, of which data on 297 could be evaluated in this study.

Patients were excluded from the present analyses if any of the following criteria were met: (1) CKD returned to stage 2 between 2010 and 2011; (2) progression to ESKD; (3) death; (4) the patient had syndromes associated with short stature; or (5) no response.

CKD stages 3, 4, and 5 were defined as SCr levels more than twice, four times, and eight times, respectively, the median normal levels in age- and sex-matched Japanese children, as previously described [19]. Short stature was defined as a height ≤2 SD below the mean, and growth impairment was defined non-specifically as a disruption of normal growth. The estimated glomerular filtration rate

(eGFR) was calculated using the revised Schwartz formula [20] and a creatinine-based equation for Japanese children and adolescents aged 2–18 years [21]: eGFR = 110.2 × [− 1.259 (height)^5 + 7.815 (height)^4 − 18.57 (height)^3 + 21.39 (height)^2 − 11.71 (height) + 2.628]/(serum creatinine) + 2.93 for boys and eGFR = 110.2 × [− 4.536 (height)^5 + 27.16 (height)^4 − 63.47 (height)^3 + 72.43 (height)^2 − 40.06 (height) + 8.778]/(serum creatinine) + 2.93 for girls.

The study was conducted in accordance with the principles of the Declaration of Helsinki and the ethical guidelines issued by the Ministry of Health, Labour and Welfare, Japan. The study was approved by a central ethics board at Tokyo Metropolitan Children's Medical Center, the principal investigator's institution (approval number: 23–49). Because data were reported using patient medical records, informed consent was not obtained in accordance with the above guidelines.

Statistical analysis

For analyses of growth impairment, only patients with valid data for serum creatinine (to calculate CKD stage) and height were included (n = 297). Characteristics of patients are presented as mean ± standard deviation or n (%). The height SDS was calculated for all children with available data, and is presented graphically as box and whisker plots or histograms according to patient factors (age, sex, CKD stage, and primary disease). The Jonckheere–Terpstra trend test was used to confirm that height SDS decreased with increasing CKD stage. Risk factors for growth impairment were determined using multiple linear regression analysis with height SDS as the continuous dependent variable and patient- and disease-related factors as independent variables to calculate the regression coefficient (β) with standard error and the corresponding P value.

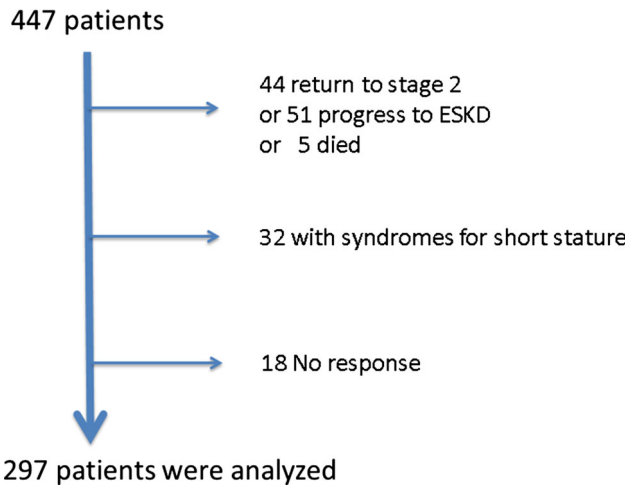

Fig. 1 Patient disposition

Results

Patient disposition and characteristics

Case-report forms were received for the present survey for 429/447 patients (96.0 %) included in our original report, of which data on 297 were analyzed in the present study (Fig. 1). Characteristics of all patients included in this study are presented in Table 1 according to their CKD stage. Primary diseases included CAKUT (n = 186, 62.6 %), cortical necrosis (n = 31, 10.4 %), polycystic kidney disease (n = 16, 5.4 %), drug-induced kidney disease (n = 12, 4.0 %), nephronophthisis (n = 11, 3.7 %), and Alport's syndrome (n = 8, 2.7 %).

Association between CKD stage and height

Overall, 297 patients (188 boys and 109 girls) were included in the analyses examining the association between CKD stage and height, after excluding patients for the reasons presented in Fig. 1.

As illustrated in Fig. 2a, the median height SDS decreased significantly as CKD stage increased ($P < 0.001$ trend test). Figure 2b shows that the height SDS was −2 or lower in many patients, irrespective of CKD stage or gender. Among boys, height SDS was ≤ −2 in 24/122, 17/58, and 5/8 patients with CKD stages 3, 4, and 5, respectively. The numbers of boys using GH and with a height SDS > −2 were 2, 8, and 2 with CKD stages 3, 4, and 5, respectively. The numbers of girls with a height SDS ≤ −2 were 18/72, 11/32, and 3/5 with CKD stages 3, 4, and 5, respectively, while the numbers of girls using GH and with a height SDS > −2 were 2, 4, and 0 with CKD stages 3, 4, and 5, respectively. The age distribution of patients is shown in Fig. 3.

Because other factors may influence height SDS, including the type of disease, we calculated the distributions of height SDS according to primary disease (CAKUT vs. non-CAKUT diseases; Fig. 4). As shown in Fig. 4, the distribution of height SDS was significantly different among the three CKD stages in patients with CAKUT ($P < 0.001$; trend test), but not in patients without CAKUT ($P = 0.140$; trend test). However, the distributions of height SDS showed similar patterns within each subgroup of primary disease.

Risk factors for growth impairment were determined using multiple linear regression analysis with height SDS as the continuous dependent variable; the results of this analysis are presented in Table 2. Of note, CKD stage 4, CKD stage 5, being small-for-date, and asphyxia at birth were significantly associated with growth impairment, with β values of −0.498 ($P = 0.034$), −1.732 ($P = 0.004$), −1.324 ($P < 0.0001$), and −0.986 ($P = 0.0005$),

Table 1 Patient characteristics according to CKD stage

	All patients	Stage 3	Stage 4	Stage 5
n	297	194	90	13
Boys/girls (*n*)	188/109	122/72	58/32	8/5
Age (years)	10.1 ± 4.5	9.8 ± 4.7	10.6 ± 4.1	11.4 ± 3.7
Height SDS	−1.3 ± 1.6	−1.1 ± 1.4	−1.7 ± 1.7	−2.7 ± 2.0
CAKUT/non-CAKUT	186/111	122/72	58/32	6/7
Serum Cr (mg/dl)	1.82 ± 1.22	1.25 ± 0.46	2.52 ± 0.74	5.60 ± 2.21
eGFR (ml/min/1.73 m^2)*	36.9 ± 15.1	45.5 ± 10.7	22.2 ± 4.8	10.2 ± 2.2
eGFR for Japanese children (ml/min/1.73 m^2)**	34.3 ± 12.6	41.7 ± 8.7	22.1 ± 4.2	11.8 ± 1.8

CKD chronic kidney disease, *SDS* standard deviation score, *CAKUT* congenital anomalies of the kidney and urinary tract, *Cr* creatinine, *eGFR* estimated glomerular filtration rate

* Calculated using the revised Schwartz formula [20]

** Calculated using the creatinine-based equation for Japanese children and adolescents aged 2–18 years [21]

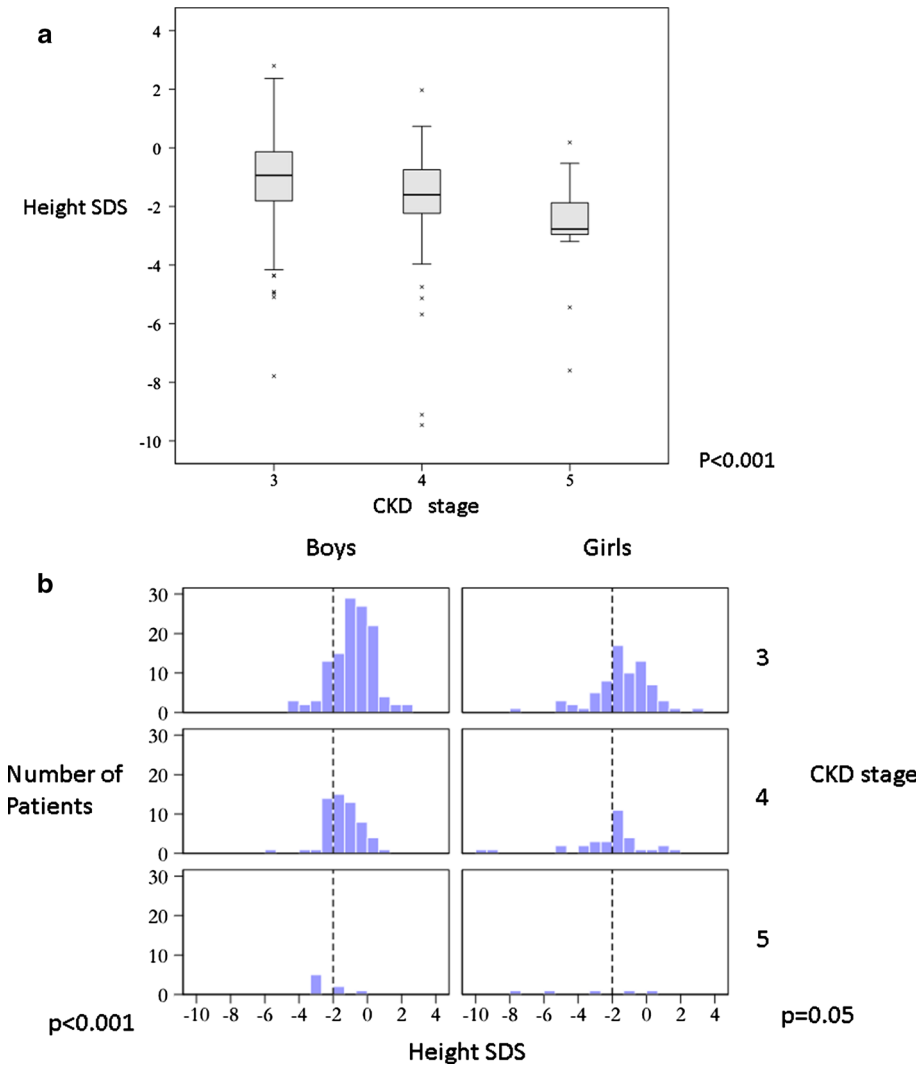

Fig. 2 **a** Height SDS according to CKD stage. The *bottom*, *middle*, and *top lines* of each box represent the 25th, 50th (median), and 75th percentiles of height SDS, respectively. The ends of the whiskers represent the range from 1.5 times the interquartile range (IQR) added to the 75th percentile to 1.5 times the IQR subtracted from the 25th percentile. Outliers (values beyond the whiskers) are indicated with crosses. **b** Distribution of height SDS according to CKD stage and sex. *SDS* standard deviation score, *CKD* chronic kidney disease

respectively. Sex, age in 2011, gestational week <37, and the presence of CAKUT/non-CAKUT disease were not associated with growth impairment. When children who were small-for-date were excluded from the analysis, CKD stage 5 and asphyxia at birth remained significant factors (data not shown).

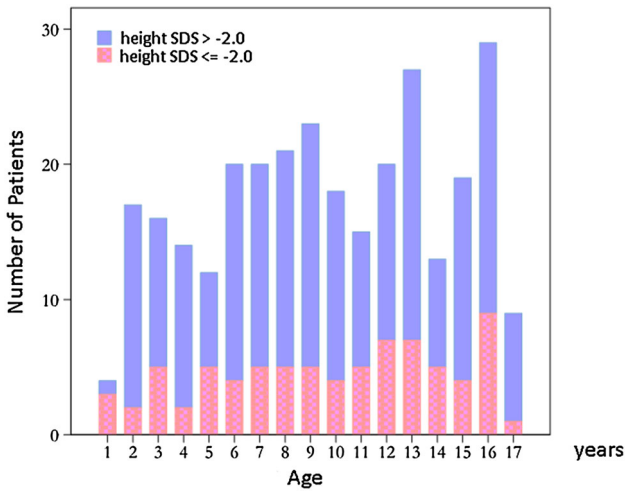

Fig. 3 Age distribution of patients by gender. The numbers of patients are plotted against age in years. *Blue bars* height SDS > -2, *Pink bars* height SDS ≤ -2

Use of GH in Japanese children with CKD

In this survey, we asked clinicians to report on the use of GH and we calculated the percentages of patients who were being treated with GH according to CKD stage and height SDS (Fig. 5). Among children with a height SDS > -2, growth hormone was used in 3.3, 19.7, and 40.0 % of patients with stages 3, 4, and 5 CKD, respectively. Among children with a height SDS ≤ -2.0, growth hormone was used in 19.5, 31.0, and 25.0 % of those with CKD stage 3, 4, and 5, respectively.

Discussion

Growth impairment is a well-known complication of CKD in children and is itself associated with severe conditions, including medical and psychological problems, together with an increased risk of death. To date, however, very few studies have focused on growth impairment in children with pre-dialysis CKD, particularly in Asia. Here, we found that the height SDS was -2 or lower in the majority of Japanese children with CKD stages 3–5, and that height SDS decreased significantly with increasing CKD stage. Risk factors for growth impairment included CKD stage, SFD, and asphyxia at birth. These data indicate that this cohort of children with pre-dialysis CKD exhibited marked growth impairment, the extent of which worsened with CKD stage.

The results of our study are consistent with those of earlier studies performed in Western countries [8–12]. Of note, the North American Pediatric Renal Transplant Cooperative Study revealed that the use of steroids, cyclosporine, and transplantation contributed to growth impairments. However, it must be acknowledged that these earlier studies generally involved post-transplant patients rather than pre-dialysis patients, except for the CKiD study, which also included pre-dialysis patients [2]. To our knowledge, our study is the first in Asia to show an association between CKD and growth impairment in pre-dialysis pediatric patients.

Our study also identified possible risk factors for growth impairment in this cohort of patients. In particular, the β value for CKD stage 5 relative to stage 3 ($\beta = -1.732$; $P = 0.004$) indicates that children with CKD stage 5 are

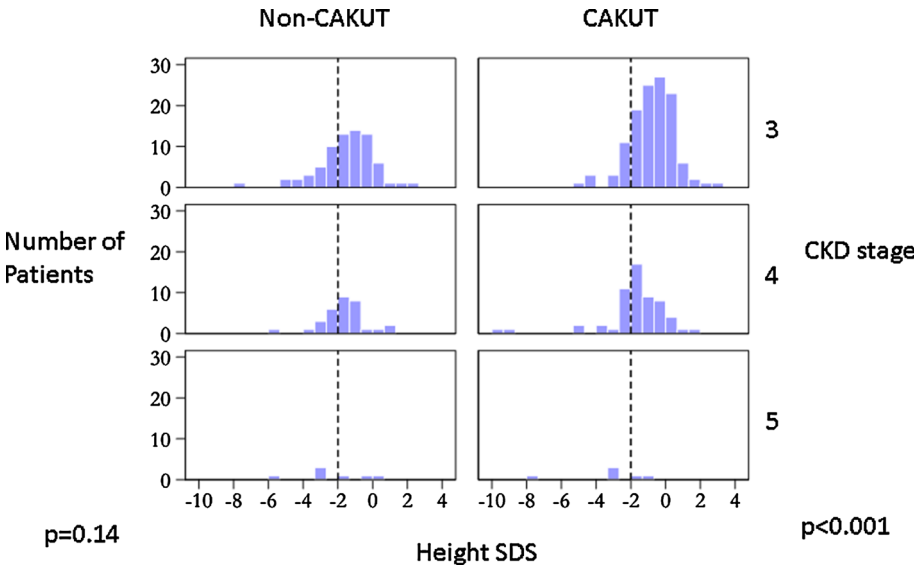

Fig. 4 Distribution of height SDS according to CKD stage and primary disease (CAKUT/non-CAKUT). *SDS* standard deviation score, *CKD* chronic kidney disease, *CAKUT* congenital anomalies of the kidney and urinary tract

Table 2 Risk factors for growth impairment

	β	SE	*P*
Girl (vs. boy)	−0.158	0.217	0.467
Age in 2011 (continuous)	−0.010	0.025	0.698
CAKUT (vs. non-CAKUT)	0.202	0.221	0.363
CKD stage			
Stage 4 (vs. stage 3)	−0.498	0.233	0.034
Stage 5 (vs. stage 3)	−1.732	0.598	0.004
SFD	−1.324	0.322	<0.0001
Gestational week <37	0.001	0.268	0.998
Asphyxia at birth	−0.986	0.278	0.0005

β β regression coefficient, *SE* standard error, *CAKUT* congenital anomalies of the kidney and urinary tract, *CKD* chronic kidney disease, *SFD* small-for-date

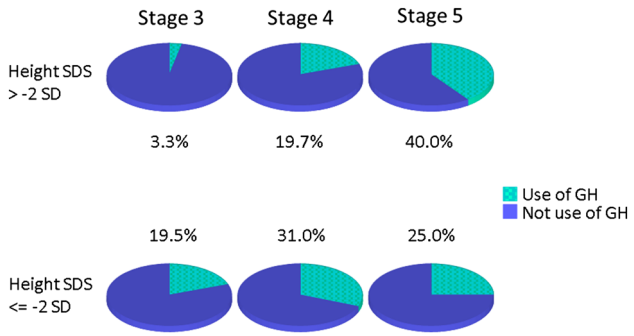

Fig. 5 Use of growth hormone according to CKD stage and height SDS. *SDS* standard deviation score, *CKD* chronic kidney disease

more likely to show growth impairment than are children with CKD stage 3. Perhaps unexpectedly, the presence of CAKUT or non-CAKUT was not significantly associated with growth impairment. We also found that characteristics at birth, including SFD and asphyxia at birth, were risk factors for growth impairment. By reviewing a patient's disease-related characteristics and birth history, it is possible that clinicians could better identify patients with or at risk of growth impairment, allowing timely treatment to facilitate catch-up growth in early childhood. These findings are consistent with those of Greenbaum et al. [22], who showed that abnormal birth history is more common in children with CKD than in the general population, and that low birth weight and being small for gestational age are both associated with short stature and lower weight percentiles in North American children with mild-to-moderate CKD.

GH is widely recommended as part of the treatment for growth impairment in patients with CKD [14] because it is associated with good clinical outcomes in terms of improving growth velocity [17], and reduces the risk of severe complications related to growth impairment [14]. A consensus statement for the use of GH was developed to help nephrologists/urologists determine when GH should be introduced and possible dosing regimens [14]. The authors of that report proposed that GH should be considered in patients with a GFR of <75 ml/min/1.73 m^2 and a height SDS of < -1.88 (corresponding to the 3rd percentile) or < -2. In the present study, however, only 25 % of children with CKD stage 5 and a height SDS of ≤ -2 were being treated with GH, and fewer than one-third of children overall were being treated with GH. These data indicate that GH is underused in Japanese children with CKD, which may reflect the stricter indication for GH in CKD in Japan (eGFR ≤50 ml/min/1.73 m^2 and height SDS ≤ -2), as well as the added expense of its treatment and pain associated with injections. Unfortunately, our survey did not assess why GH was not used in these patients, and the possible reasons will be evaluated in the next survey.

There are several limitations associated with the use of GH, including the risk of adverse events. Additionally, because most of the studies to date have been of limited duration (usually <1–2 years), there is scant data showing that the use of GH allows the patients to reach a normal adult height. Furthermore, children on dialysis may show weaker responses to GH than those treated with GH before dialysis [23]. Therefore, we should consider starting GH therapy at an appropriate stage of the patient's treatment, after introducing nutritional management, and treating kidney-induced anemia and mineral bone disease.

Some limitations of this study warrant discussion. In particular, 30 % of the surveyed patients did not meet our eligibility criteria, and were not included in the current analyses. The patients included in this study had pre-dialysis CKD and had not undergone transplantation. It is clear that CKD disturbs growth rates and growth impairments may become more pronounced when these children start renal replacement therapies. It is also possible that steroid use in some patients might have influenced their growth. We did not obtain data on steroid use in our patients, including four patients with chronic glomerulonephritis and four with focal segmental glomerulosclerosis who might have required steroids. Finally, the current survey was not designed to address the impact of GH therapy on the growth of patients. However, as surveys are planned in future years, it may be possible to examine this issue further.

In conclusion, while recent advances in the treatment of CKD have enabled children to lead normal social lives, this disorder is associated with growth impairment, which may have an impact on quality of life. Here, we showed that Japanese children with pre-dialysis CKD exhibited significant growth impairment relative to normative data.

By identifying patients with or at risk of growth impairment, clinicians can introduce appropriate and timely therapies to improve their growth velocity. Indeed, the

current study suggests that children with pre-dialysis CKD over stage 3 are strong candidates for the treatment of growth impairment. Prospective studies are needed to confirm the efficacy of treatments to improve growth velocity in Asian children, as well as the optimal timing of treatment, and whether the identification of risk factors can help identify candidates for treatment.

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Conflict of interest Kenji Ishikura has received lecture fees and travel expenses from Novartis Pharma and Asahi Kasei Pharma. Osamu Uemura has received lecture fees and travel expenses from Asahi Kasei Pharma and Siemens Group in Japan. Yuko Hamasaki has received research grants from Novartis Pharma, and lecture fees from Novartis Pharma, Astellas Pharma, and Pfizer Japan. Ryojiro Tanaka has received lecture fees from Pfizer Japan. Koichi Nakanishi has received lecture fees from Novartis Pharma, Asahi Kasei Pharma, and Astellas Pharma. Masataka Honda has received lecture fees from Novartis Pharma and Asahi Kasei Pharma.

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EXTENDED REPORT

Genome-wide association study of clinically defined gout identifies multiple risk loci and its association with clinical subtypes

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ABSTRACT

Objective Gout, caused by hyperuricaemia, is a multifactorial disease. Although genome-wide association studies (GWASs) of gout have been reported, they included self-reported gout cases in which clinical information was insufficient. Therefore, the relationship between genetic variation and clinical subtypes of gout remains unclear. Here, we first performed a GWAS of clinically defined gout cases only.

Methods A GWAS was conducted with 945 patients with clinically defined gout and 1213 controls in a Japanese male population, followed by replication study of 1048 clinically defined cases and 1334 controls.

Results Five gout susceptibility loci were identified at the genome-wide significance level ($p<5.0\times10^{-8}$), which contained well-known urate transporter genes (*ABCG2* and *SLC2A9*) and additional genes: rs1260326 ($p=1.9\times10^{-12}$; OR=1.36) of *GCKR* (a gene for glucose and lipid metabolism), rs2188380 ($p=1.6\times10^{-23}$; OR=1.75) of *MYL2-CUX2* (genes associated with cholesterol and diabetes mellitus) and rs4073582 ($p=6.4\times10^{-9}$; OR=1.66) of *CNIH-2* (a gene for regulation of glutamate signalling). The latter two are identified as novel gout loci. Furthermore, among the identified single-nucleotide polymorphisms (SNPs), we demonstrated that the SNPs of *ABCG2* and *SLC2A9* were differentially associated with types of gout and clinical parameters underlying specific subtypes (renal underexcretion type and renal overload type). The effect of the risk allele of each SNP on clinical parameters showed significant linear relationships with the ratio of the case–control ORs for two distinct types of gout (r=0.96 [$p=4.8\times10^{-4}$] for urate clearance and r=0.96 [$p=5.0\times10^{-4}$] for urinary urate excretion).

Conclusions Our findings provide clues to better understand the pathogenesis of gout and will be useful for development of companion diagnostics.

INTRODUCTION

Gout is a common disease caused by deposition of monosodium urate (MSU) crystal due to hyperuricaemia.[1] Humans have long suffered from gout as reported by Hippocrates 2500 years ago.[2] There have been many famous patients with gout such as Sir Isaac Newton[3] in the more recent past, and the numbers are still growing. From the pathophysiological point of view, gout can be classified into the renal underexcretion (RUE) type, the renal overload (ROL) type and the combined type based on clinical parameters[4] (see online supplementary figure S1).

So far the genome-wide association studies (GWASs) of serum uric acid (SUA) level[5–16] have identified a number of genetic loci including *SLC2A9* (also known as *GLUT9*) and *ABCG2* (also known as *BCRP*), and subsequent genetic and functional studies have revealed the biological and pathophysiological significance of *ABCG2*.[4 17 18] Previous GWASs of gout reported a significant association with single-nucleotide polymorphisms (SNPs) of *ABCG2*, *SLC2A9* with European ancestries,[14 15] and of *ALDH16A1* with Icelanders,[14] while another study with African-American and European ancestries reported no significantly associated SNPs of gout.[13] All of these studies were, however, performed with cases including self-reported patients with gout, in which clinical information was insufficient. Therefore, the relation to genetic heterogeneity underlying gout subtypes is also unclear. To better understand its genetic basis, we first performed a GWAS of clinically defined gout cases only. We then investigated the relationship between genetic variation and clinical types of gout.

METHODS

Subjects

In the present study, we avoided use of self-reported gout cases and collected only clinically defined gout

cases. All gout cases were clinically diagnosed as primary gout according to the criteria established by the American College of Rheumatology.[19] All patients were assigned from among the Japanese male outpatients at the gout clinics of Midorigaoka Hospital (Osaka, Japan), Kyoto Industrial Health Association (Kyoto, Japan) or Ryougoku East Gate Clinic (Tokyo, Japan). Patients with inherited metabolism disorders including Lesch–Nyhan syndrome were excluded. Finally, 1994 male gout cases were registered as valid case participants. As controls, 2547 individuals were assigned from among Japanese men with normal SUA level (≤7.0 mg/dL) and no gout history, who were obtained from BioBank Japan[11 20] and Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study).[21]

Genotyping and quality control

Genome-wide genotyping was performed with Illumina HumanOmniExpress v1.0 (Illumina) in 946 cases and 1213 controls. Detailed methods of genotyping and quality control are shown in the online supplementary methods and figure S2. Finally, 570 442 SNPs passed filters for 945 cases and 1213 controls.

In total, 123 SNPs passing the significance threshold at $p<1.0\times10^{-5}$ in the GWAS stage were used for subsequent analyses. Among these SNPs, we examined their linkage disequilibrium (LD) and selected 16 SNPs for replication study (see online supplementary methods). These 16 SNPs were then genotyped by an allelic discrimination assay (Custom TaqMan Assay and By-Design, Applied Biosystems) with a LightCycler 480 (Roche Diagnostics).[18] After quality control, subsequent statistical analysis was performed with 1048 cases and 1334 controls.

Statistical analyses for GWAS

We conducted an association analysis using a 2×2 contingency table based on the allele frequency, and p value of association was assessed by χ^2 test. The quantile–quantile plot and the genomic inflation factor were used to assess the presence of systematic bias in the test statistics due to potential population stratification (see online supplementary methods and figure S3).

We then combined results from the GWAS and replication stages by meta-analysis.[22] The inverse-variance fixed-effects model meta-analysis was used for estimating summary OR. Cochran's Q test[23] and I^2 statistic[24 25] were examined to assess heterogeneity in ORs between GWAS and replication study. If heterogeneity was present by the statistical test ($p_{het}<0.05$) or measurement ($I^2>50\%$), we implemented DerSimonian and Laird random-effects model meta-analysis.[26] All the meta-analyses were performed using the STATA V.11.0. Genome-wide significance threshold was set to be $\alpha=5.0\times10^{-8}$ to claim evidence of a significant association. Detailed methods of imputation and per cent variance are shown in the online supplementary methods.

Subtype analyses

Gout contains two distinct types, 'ROL' type and 'RUE' type. The ROL type was defined when urinary urate excretion (UUE) was over 25.0 mg/h/1.73 m^2 (600 mg/day/1.73 m^2)[4 27–29] and their urate clearance (urate clearance/creatinine clearance ratio, FE_{UA}) was 5.5% or over. Also, the RUE type was determined when UUE was 25.0 mg/h/1.73 m^2 or under and FE_{UA} was under 5.5%.[4 30 31] Detailed methods of subtype analyses are described in the online supplementary methods.

RESULTS

Genome-wide association study

Clinical characteristics of participants in this study are shown in online supplementary tables S1–S3. GWAS with 945 clinically defined gout cases and 1213 controls identified SNPs in three loci showing evidence of associations at the genome-wide significance level ($p<5.0\times10^{-8}$): rs2728125 of *ABCG2* ($p=1.5\times10^{-27}$; OR=2.05), rs3775948 of *SLC2A9* ($p=6.7\times10^{-15}$; OR=1.64) and rs2188380 of *MYL2-CUX2* ($p=5.7\times10^{-13}$; OR=1.78, figures 1 and 2, table 1 and online supplementary figure S4).

Replication study was conducted with 1048 cases and 1334 controls. As a result, the three SNPs surpassing the genome-wide significance threshold in the GWAS stage were successfully replicated; rs2728125 ($p=8.3\times10^{-29}$; OR=2.03), rs3775948 ($p=7.6\times10^{-14}$; OR=1.57) and rs2188380 ($p=2.0\times10^{-12}$;

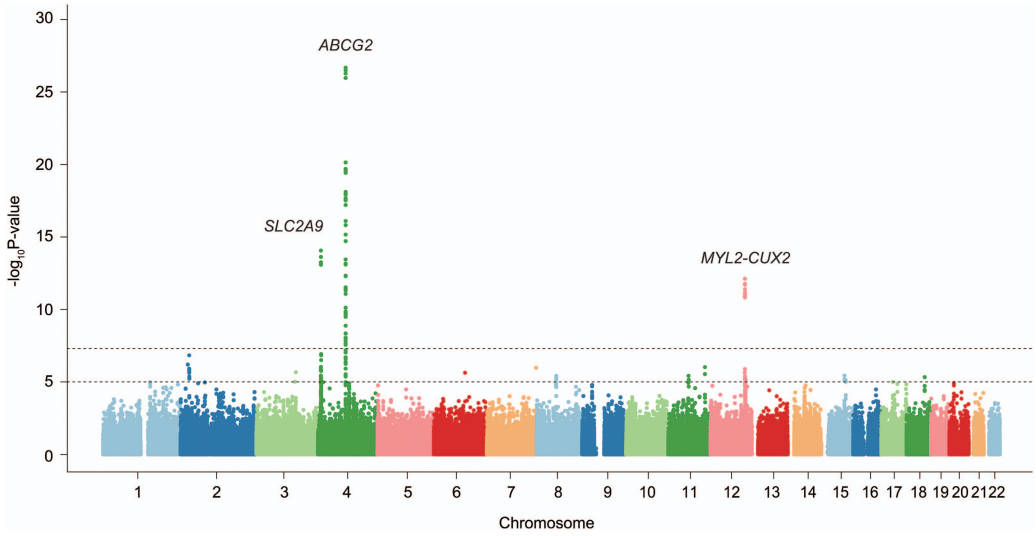

Figure 1 Manhattan plot of a genome-wide association analysis of gout. X-axis shows chromosomal positions. Y-axis shows -$\log_{10}$ p values. The upper and lower dotted lines indicate the genome-wide significance threshold ($p=5.0\times10^{-8}$) and the cut-off level for selecting single-nucleotide polymorphisms for replication study ($p=1.0\times10^{-5}$), respectively.

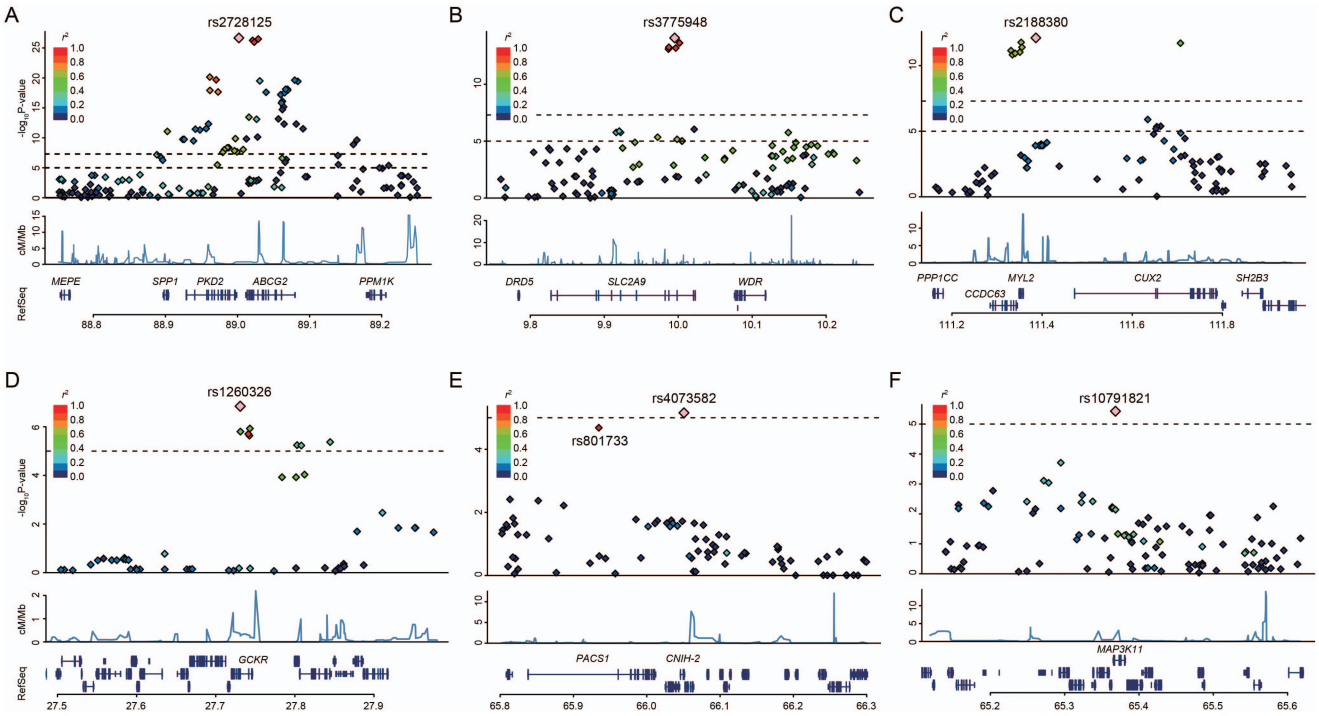

Figure 2 Regional association plots for six discovered loci of gout. Five regions exceeding the genome-wide significance level (A–E) and one region showing a suggestive association (F). The highest association signal in each panel is located on *ABCG2* (A), *SLC2A9* (B), *MYL2-CUX2* (C), *GCKR* (D), *CNIH-2* (E) and *MAP3K11* (F). Region within 250 kb from single-nucleotide polymorphism (SNP) showing lowest p value is displayed. (Top panel) Plots of -$\log_{10}$ p values for the test of SNP association with gout in the genome-wide association study stage. SNP showing the lowest p value is depicted as a pink diamond. Other SNPs are colour-coded according to the extent of linkage disequilibrium (measured in r^2) with SNP showing the lowest p value. (Middle panel) Recombination rates (centimorgans per Mb) estimated from HapMap Phase II data are plotted. (Bottom panel) RefSeq genes. Genomic coordinates are based on Genomic Reference Consortium GRCh37.

OR=1.73). Additionally, two SNPs showed significant associations at $p<3.1\times10^{-3}$ (=0.05/16) with Bonferroni correction; rs1260326 of *GCKR* ($p=2.8\times10^{-6}$; OR=1.32) and rs4073582 of *CNIH-2* ($p=1.6\times10^{-4}$; OR=1.55) as shown in table 1 and online supplementary table S4.

All five SNPs that showed significant associations in the replication study achieved genome-wide significance in the meta-analysis of GWAS and replication study (table 1): rs2728125 ($p_{meta}=7.2\times10^{-54}$; OR=2.04), rs3775948 ($p_{meta}=5.5\times10^{-27}$; OR=1.61), rs2188380 ($p_{meta}=1.6\times10^{-23}$; OR=1.75), rs1260326 ($p_{meta}=1.9\times10^{-12}$; OR=1.36) and rs4073582 ($p_{meta}=6.4\times10^{-9}$; OR=1.66). In addition, an intronic SNP of *MAP3K11* (rs10791821) showed a suggestive level of association ($p_{meta}=1.0\times10^{-7}$; OR=1.57). There was >80% power to detect a risk variant for OR=1.6 at the genome-wide significance level ($p<5.0\times10^{-8}$) for an SNP with a minor allele frequency of 0.35 (see online supplementary table S5). Imputation was also performed with the GWAS genotyping data for 1 Mb across the identified SNPs of novel loci (rs2188380 of *MYL2-CUX2*, rs1260326 of *GCKR*, rs4073582 of *CNIH-2* and rs10791821 of *MAP3K11*). SNPs that passed the significant threshold of GWAS stage ($p<1.0\times10^{-5}$) in this imputation are shown in online supplementary table S6A–D.

Two dysfunctional SNPs of *ABCG2*

We previously demonstrated that two dysfunctional SNPs of *ABCG2*, rs72552713 (Gln126Ter) and rs2231142 (Gln141Lys), were located on different haplotypes[4 18] and strongly associated with hyperuricaemia and gout.[4 18 32] Therefore, we additionally performed genotyping of these two SNPs by an allelic discrimination assay because SNPs are not on Illumina HumanOmniExpress V.1.0 (Illumina). SNP showing the highest significance in the present GWAS (rs2728125) was in strong LD with rs2231142 ($r^2=0.76$) but not in LD with rs72552713 ($r^2=0.03$). A multivariate logistic regression analysis including these three SNPs of *ABCG2* showed that rs2728125 no longer had a significant association (p=0.19), but rs72552713 and rs2231142, that is, two non-synonymous SNPs, remained highly significant (see online supplementary table S7A, B), indicating that rs2728125 was merely a surrogate marker for rs2231142. Therefore, we used these two non-synonymous variants for subsequent analyses.

Cumulative effect of risk alleles for gout

Accumulation of the number of risk alleles of the gout-associated SNPs (rs3775948, rs2188380, rs1260326, rs4073582, rs72552713 and rs2231142) increased the probability of gout logarithmically. When setting the reference category as having four or fewer risk alleles, ORs for having 5, 6, 7, 8 and 9 or more risk alleles were 1.79 ($p=3.5\times10^{-3}$), 3.16 ($p=2.3\times10^{-10}$), 5.10 ($p=9.7\times10^{-21}$), 10.1 ($p=5.3\times10^{-39}$) and 18.6 ($p=3.6\times10^{-45}$), respectively (see online supplementary figure S5 and table S8).

Subtype analysis of gout

We examined type-specific ORs and the case–subtype heterogeneity test.[33] The subgroup analysis (table 2) showed that the associations of two non-synonymous SNPs of *ABCG2* (rs72552713 and rs2231142) were stronger for the ROL type (ORs=4.35 and 3.37, respectively) than for the RUE type (ORs=1.28 and

Table 1 Five SNPs showing significant association at genome-wide significance level and one suggestive SNP

SNP¶	Chromosome	Position (bp)††	Gene	A1/A2‡‡	GWAS†				Replication study‡				Meta-analysis§	
					Freq.				Freq.					
					Cases	Controls	OR (95% CI)	p Value	Cases	Controls	OR (95% CI)	p Value	OR (95% CI)	p Value
rs2728125	4	89 001 893	*ABCG2*	C/T	0.40	0.25	2.05 (1.80 to 2.34)	1.5×10^{-27}	0.40	0.24	2.03 (1.79 to 2.30)	8.3×10^{-29}	2.04 (1.86 to 2.23)	7.2×10^{-54}
rs3775948	4	9 995 182	*SLC2A9*	G/C	0.68	0.56	1.64 (1.45 to 1.86)	6.7×10^{-15}	0.67	0.56	1.57 (1.40 to 1.77)	7.6×10^{-14}	1.61 (1.47 to 1.75)	5.5×10^{-27}
rs2188380	12	111 386 127	*MYL2-CUX2*	T/C	0.85	0.76	1.78 (1.52 to 2.08)	5.7×10^{-13}	0.86	0.78	1.73 (1.48 to 2.02)	2.0×10^{-12}	1.75 (1.57 to 1.96)	1.6×10^{-23}
rs1260326	2	27 730 940	*GCKR*	T/C	0.62	0.54	1.39 (1.23 to 1.57)	1.2×10^{-7}	0.61	0.55	1.32 (1.18 to 1.49)	2.8×10^{-6}	1.36 (1.25 to 1.48)	1.9×10^{-12}
rs4073582	11	66 050 712	*CNIH-2*	G/A	0.95	0.91	1.78 (1.39 to 2.29)	5.3×10^{-6}	0.94	0.91	1.55 (1.23 to 1.96)	1.6×10^{-4}	1.66 (1.40 to 1.96)	6.4×10^{-9}
rs10791821**	11	65 368 323	*MAP3K11*	G/A	0.94	0.90	1.75 (1.38 to 2.22)	2.8×10^{-6}	0.94	0.92	1.41 (1.12 to 1.77)	3.4×10^{-3}	1.57 (1.33 to 1.85)	1.0×10^{-7}

†945 gout cases and 1213 controls.
‡1048 gout cases and 1334 controls.
§Meta-analyses of the combined GWAS and replication samples (1993 gout cases and 2547 controls).
¶dbSNP rs number. A suggestive SNP is marked with '**'.
††SNP positions are based on the National Center for Biotechnology Information human genome reference sequence Build 37.4.
‡‡A1 is a risk-associated allele and A2 is a non-risk-associated allele.
Freq., frequency of A1; GWAS, genome-wide association study; SNP, single-nucleotide polymorphism.

1.88, respectively). The differences in ORs between the gout types were highly significant ($p=2.4\times10^{-5}$ and 1.0×10^{-7}, respectively). The association of rs3775948 of *SLC2A9* was stronger for the RUE type (OR=1.94) than for the ROL type (OR=1.38). The case–subtype heterogeneity test showed a significant difference in ORs ($p=2.7\times10^{-4}$). The other SNPs evidenced no significant differences. Then, associations between SNPs and clinical parameters (FE_{UA} and UUE) were assessed. Only SNPs that showed a significant difference in ORs between different gout types were significantly associated with FE_{UA} and UUE (table 2 and online supplementary figure S6, table S9); the gout risk alleles of *ABCG2* and *SLC2A9* were associated with increased and decreased levels of these parameters, respectively. The effect of the risk allele of each SNP on clinical parameters showed significant linear relationships with OR in the case–subtype heterogeneity test, which was an estimate of the ratio of the case–control ORs for the gout types ($r=0.96$ [$p=4.8\times10^{-4}$] for FE_{UA} and $r=0.96$ [$p=5.0\times10^{-4}$] for UUE) (figure 3).

DISCUSSION

Through the GWAS with clinically defined cases, we identified five gout-associated loci that showed different association patterns in subtype analysis. Previous GWASs of SUA[5–16] showed genome-wide significant associations with *ABCG2*, *SLC2A9* and *GCKR*. These genes were also reported to have significant associations with gout as a consequence of hyperuricaemia.[13–15] The present study revealed for the first time that three loci (*GCKR*, *MYL2-CUX2* and *CNIH-2*) were associated with gout at the genome-wide significance level. In particular, *MYL2-CUX2* and *CNIH-2* are novel loci for gout.

The total variance explained by the seven SNPs was estimated to be 9.0% (see online supplementary methods): three SNPs of well-known urate transporter genes (*SLC2A9* and *ABCG2*) with large effects accounted for 6.9%, and the four SNPs identified in this GWAS with modest effects explained 2.1%. Additional discoveries of unidentified genetic variants by performing a meta-analysis of GWAS data sets will improve the explained genetic variation of gout.

ABCG2 and *SLC2A9* are well-known urate transporter genes for urate excretion[17 18] and renal urate reabsorption,[34 35] respectively. *ABCG2* is identified to have an association with SUA levels by recent GWASs.[9–16] Subsequent genetic and functional analysis[17 18] revealed that ABCG2 is a high-capacity urate exporter and shows the reduced transport of urate by a common half-functional variant, rs2231142 (Gln141Lys). We also demonstrated that common dysfunctional genotype combinations of *ABCG2* gene (non-functional rs72552713 [Gln126Ter] and rs2231142) are a major cause of hyperuricaemia and gout,[18] especially for early-onset gout.[32] We earlier found that the risk alleles of these two SNPs reside on different haplotypes,[4 18] indicating independent risks of gout. Recently, these dysfunctional SNPs were revealed to decrease extrarenal (intestinal) urate excretion and to cause ROL hyperuricaemia,[4] through studies with hyperuricaemic patients[4] and *Abcg2*-knockout mice.[4 36] This is consistent with the fact that ABCG2 exporter is expressed on the apical membrane in several tissues, including intestine[37] and kidney,[38] which have urate-excreting functions in humans.

SLC2A9 is a member of the glucose transporter (GLUT) family. SLC2A9 was found to transport urate,[7 34] and several GWAS have demonstrated an association of *SLC2A9* with SUA levels.[5–16] SLC2A9 has two isoforms, GLUT9L (long isoform) and GLUT9S (short isoform),[34] and is highly expressed in the kidney proximal tubules in humans.[39] Genetic and functional

Table 2 Associations of seven SNPs with gout types

SNP†	Gene	Freq.		ROL type vs controls*		RUE type vs controls*		Case–subtype heterogeneity test	
		ROL type	RUE type	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value‡
rs3775948	*SLC2A9*	0.62	0.70	1.38 (1.14 to 1.68)	1.0×10^{-3}	1.94 (1.63 to 2.31)	1.0×10^{-13}	0.66 (0.53 to 0.83)	**2.7×10^{-4}**
rs2188380	*MYL2-CUX2*	0.84	0.85	1.45 (1.11 to 1.89)	6.5×10^{-3}	1.47 (1.16 to 1.86)	1.2×10^{-3}	0.92 (0.68 to 1.25)	0.60
rs1260326§	*GCKR*	0.60	0.62	1.25 (1.04 to 1.50)	0.016	1.35 (1.15 to 1.58)	3.0×10^{-4}	0.94 (0.77 to 1.14)	0.51
rs4073582	*CNIH-2*	0.95	0.94	1.96 (1.30 to 2.95)	1.2×10^{-3}	1.51 (1.09 to 2.08)	0.013	1.26 (0.80 to 1.99)	0.32
rs10791821	*MAP3K11*	0.93	0.95	1.37 (0.96 to 1.96)	0.084	1.79 (1.26 to 2.54)	1.2×10^{-3}	0.79 (0.51 to 1.23)	0.30
rs72552713§	*ABCG2*	0.067	0.029	4.35 (2.82 to 6.72)	3.0×10^{-11}	1.28 (0.78 to 2.12)	0.32	2.90 (1.77 to 4.75)	**2.4×10^{-5}**
rs2231142§	*ABCG2*	0.50	0.38	3.37 (2.76 to 4.12)	2.8×10^{-32}	1.88 (1.58 to 2.24)	2.5×10^{-12}	1.76 (1.43 to 2.17)	**1.0×10^{-7}**

*We performed multivariate logistic regression analyses, in which all seven SNPs, alcohol drinking and body mass index were included in the model. In total, 1613 patients with gout and 1334 controls with genotypes for rs72552713 and rs2231142 of *ABCG2*, which were not on the Illumina OmniExpress platform, were used. Also, 375 and 509 patients with gout were grouped into ROL type and RUE type, respectively.
†dbSNP rs number.
‡p Values <0.05 are shown in bold.
§Non-synonymous SNPs (rs1260326, Leu446Pro; rs72552713, Gln126Ter; and rs2231142, Gln141Lys).
Freq., frequency of risk-associated allele; ROL, renal overload; RUE, renal underexcretion; SNP, single-nucleotide polymorphism.

analysis[34 35] with patients with renal hypouricaemia (RHUC) revealed that RHUC is caused by dysfunctional mutations in *SLC2A9*, which decrease urate reabsorption in the renal proximal tubules. For example, non-functional mutations of either GLUT9L (Arg198Cys and Arg380Trp) or GLUT9S (Arg169Cys and Arg351Trp, corresponding to Arg198Cys and Arg380Trp in GLUT9L), which were found from patients with RHUC, dramatically reduced the urate transport activity.[34] Therefore, SLC2A9 plays an important role in renal urate reabsorption.[34] Thus, *SLC2A9* is a causative gene for RHUC type 2,[34 40] which was confirmed by the report of homozygous mutations in patients with RHUC type 2.[35] In our subtype analysis, OR of RUE type was higher than that of ROL type (OR=1.94 and 1.38, respectively, table 2), which is compatible with the fact that SLC2A9 is a transporter for urate reabsorption in human kidney.

Glucokinase regulatory protein (GCKR) controls the activity of glucokinase, which is a major glucose sensor for insulin secretion. GCKR regulates the first step of glycolysis, the phosphorylation of glucose to glucose-6-phosphate.[41 42] Glucokinase activity is controlled by GCKR, which binds to glucokinase and suppresses its function in the postabsorptive phase. On the other hand, this binding is loosened in the postprandial phase, so that glucokinase could adopt the glycolysis.[43] So far, the gout risk allele of rs1260326 (Leu446Pro) has been reported to be associated with lower fasting glucose levels, and inversely, higher levels of triglyceride[43–45] and SUA.[10 12 15] An association of *GCKR* with dyslipidaemia has also been reported.[46]

MYL2 encodes a regulatory light chain associated with cardiac myosin β (or slow) heavy chain. *MYL2* mutations are associated with mid-left ventricular-type hypertrophic cardiomyopathy. In addition, its association with high-density lipoprotein

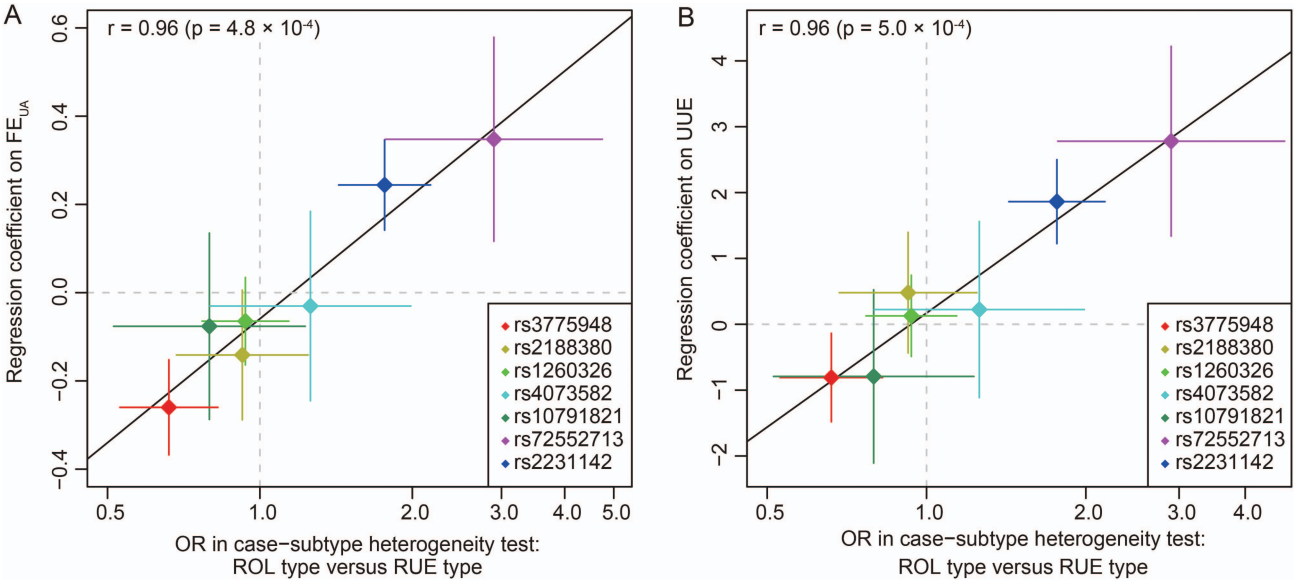

Figure 3 Relationships between effects of risk alleles on clinical parameters and ORs in case–subtype heterogeneity test. (A) FE_{UA} and (B) urinary urate excretion (UUE). The seven single-nucleotide polymorphisms (SNPs) listed in table 2 were examined. OR in case–subtype heterogeneity test is an estimate of the ratio of the case–control OR for the renal overload (ROL) type to that for the renal underexcretion (RUE) type. If an SNP has a stronger effect for the ROL type than for the RUE type, it takes a value >1. Diamonds and lines represent point estimates and their 95% CIs. Pearson's correlation coefficient (r) between the effect on clinical parameters and natural logarithm of OR in case–subtype heterogeneity test and its significance were examined. FE_{UA}, fractional excretion of urate clearance.

cholesterol metabolism was previously reported.[47] CUX2 regulates cell-cycle progression[48] and plays important roles in neural progenitor development in the central nervous system.[48 49] Its association with type 1 diabetes has also been reported.[50] Thus, rs2188380 of *MYL2-CUX2* showed an association with gout because *MYL2* and *CUX2* might influence such metabolic pathways. Rs2188380 locates near rs653178 of *ATXN2* (see online supplementary figure S7), which was reported by Köttgen *et al*[15] to have an association with SUA. Rs653178 is, however, monomorphic in the Japanese population of the HapMap project,[51] and we also confirmed it in our samples by genotyping >250 replication cases. Conversely, rs2188380 of *MYL2-CUX2* is monomorphic in European and African populations,[51] while rs2188380 is a common variant in the Japanese population (table 1). Therefore, this SNP was identified as a novel locus of gout in the present study. The differences in study populations could be one of the reasons why rs2188380 was not found in a large European-driven GWAS on urate and gout.[15] Further analyses including fine mapping and functional analysis are required in this region.

CNIH-2 regulates the function of glutamate receptors of the AMPA-subtype assembly at the cell surface of various neurons and glial cells.[52 53] CNIH-2 modulates AMPA receptor gating by increasing its cell surface expression. The newly identified rs4073582 of *CNIH-2* was in strong LD with rs801733 in *PACS1* (r^2=0.97, figure 2E and see online supplementary figure S4E), which is reported to be associated with severe obesity.[54] Accordingly, *PACS1* can also be a good candidate for a gout susceptibility gene. Additional genetic dissection and functional analysis will be needed to determine whether these genes or others could play roles with true causality at this locus. Since Okada *et al*[16] previously reported the association between SUA and rs504915 of *NRXN2*, which is near *CNIH-2* and *MAP3K11*, we examined their relationships. They are not in strong LD (see online supplementary table S10), and the association of rs4073582 and rs10791821 remained significant after adjustment with rs504915 (see online supplementary table S11). Therefore, rs4073582 of *CNIH-2*, rs10791821 of *MAP3K11* and rs504915 of *NRXN2* are revealed to be independent of each other.

MAP3K11, also known as mixed lineage kinase 3 (MLK3), is a MAP kinase member and plays a significant role in the activation of c-Jun N-terminal kinase (JNK), a stress-activated protein kinase.[55] Signalling from the small GTP-binding proteins Rac1 and Cdc42 induces MLK3 to activate the MEKK-SEK-JNK kinase cascade. Interestingly, the JNK pathway is activated when monocytes/macrophages phagocytose MSU crystals,[56] which cause gouty arthritis. The SNP rs10791821 of *MAP3K11* has been associated with the expression level of *MAP3K11* in monocytes,[57] and therefore, is likely to be a regulatory SNP. However, further study is required to confirm precise involvement of MAP3K11 in the development of gout.

Other genes (*CCDC63*, *C2orf16*, *ZNF512*, *RAB1B*, *EHBP1L1* and *KCNK7*) near each of the novel loci, which are found by imputation analysis (see online supplementary table S6A–D), could also be candidate genes of gout, and further studies including functional analyses are warranted.

Most of the gout-related genes are also associated with SUA.[15] In the present study design, to identify novel gout risk loci, clinically defined gout and normouricaemic controls were recruited. Therefore, further investigations with different study designs will be needed to identify gout loci associated with crystal deposition and inflammation.

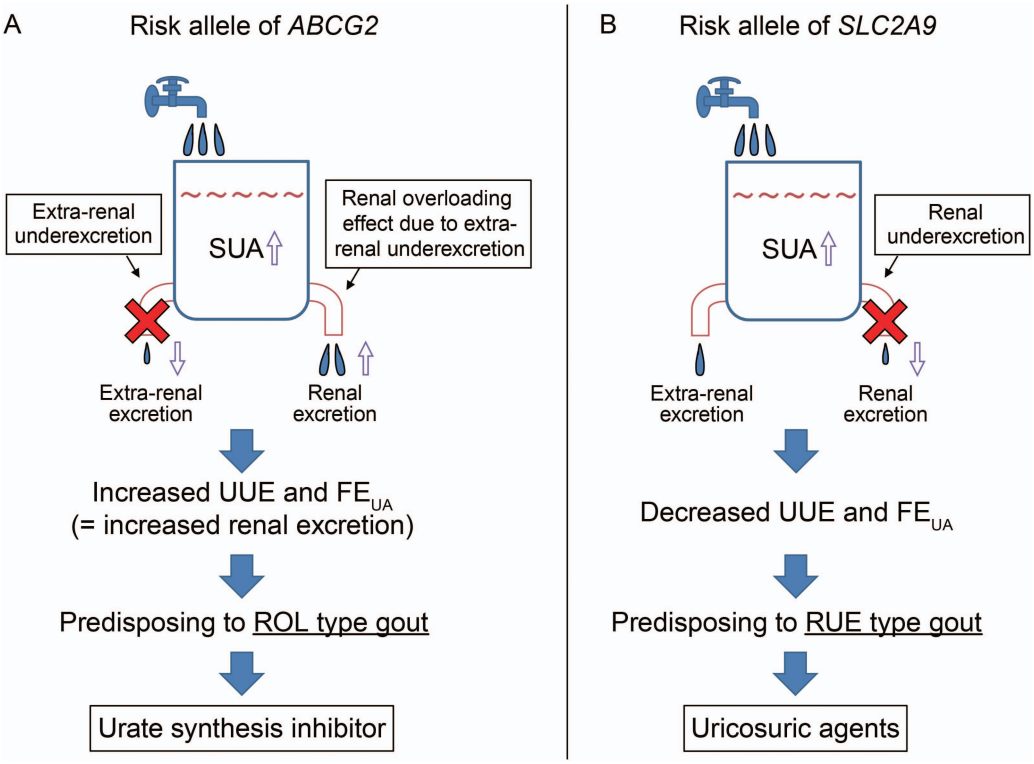

Figure 4 Differential effects by risk allele on clinical parameters and gout. (A) The risk alleles of *ABCG2* increase UUE and FE_{UA}, which leads to the overloading effect on renal urate excretion and increases the risk of the ROL-type gout. Therefore, patients with risk alleles for the ROL-type gout would be given urate synthesis inhibitors. (B) The risk allele of *SLC2A9* reduces UUE and FE_{UA}, which reflects a decreased renal urate excretion, thereby increasing the risk of the RUE-type gout. Patients with risk alleles for the RUE-type gout would be administered uricosuric agents. FE_{UA}, fractional excretion of urate clearance; ROL, renal overload; RUE, renal underexcretion; SUA, serum uric acid; UUE, urinary urate excretion.

We further investigated the cumulative effect of risk alleles of the five significant loci (*ABCG2*, *SLC2A9*, *MYL2-CUX2*, *GCKR* and *CNIH-2*) on gout risk. The result showed that individuals with five or more risk alleles had a higher risk for gout compared with those having four or fewer risk alleles. The more risk alleles in an individual, the higher became the risk of gout.

Furthermore, the relationship between genetic variation and clinical types of gout was investigated. The results of subtype analyses (table 2, figure 3 and online supplementary figure S6, table S9) indicate that the alleles closely associated with the risk of specific gout type represented differential effects on clinical parameters (FE_{UA} and UUE). This allows the estimation of disturbed urate excretion pathways. An increase of FE_{UA} and UUE by the risk alleles of *ABCG2* leads to the overloading effect on renal urate excretion and causes the ROL-type gout (figure 4A). These estimations are consistent with our previous finding obtained from *Abcg2*-knockout mouse models and hyperuricaemic patients.[4] In contrast, the reduction of FE_{UA} and UUE by the risk allele of *SLC2A9* reflects a decreased renal urate excretion, thereby increasing the risk of the RUE-type gout (figure 4B). The present study demonstrated that the combination of GWAS of patients with clinically defined gout with actual clinical data is an effective method to analyse genetic heterogeneity among different types of gout.

In summary, we conducted the first GWAS using patients with clinically defined gout only and identified five loci containing two novel loci. Moreover, identified SNPs showed differential effects on different gout types and affected clinical parameters underlying specific types. Thus, genetic testing for gout may well be introduced into future companion diagnostics. For example, patients with risk alleles for ROL-type gout would be given urate synthesis inhibitors[31 58] such as allopurinol and febuxostat, while patients with risk alleles for RUE-type gout would be administered uricosuric agents[31 58] including benzbromarone and lesinurad, a selective uric acid reabsorption inhibitor that has just finished its phase III study.[59 60] Exploring genetic heterogeneity among different gout types will deepen understanding of the aetiology of gout and serve to categorise patients for future personalised treatment.

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Contributors HM, KY, HNakaoka, AN, MS, TC, II and NS conceived and designed the experiments. ATakahashi, TN, HNakashima, YT, TT, YS, HS, YKanai, TH and MK assisted with research design. HM, AN, MS, TC, ATokumasu, KIchida, HOoyama and TS collected and analysed clinical data of cases. ATakahashi, GY, RO, EM, MN, NH and MK collected and analysed clinical data of controls. HM, KY, AN, MS, TC, YT, ID, SS, JA, YKawamura, STerashige, HOgata, STatsukawa, YN and NS performed genetic analysis. HNakaoka, ATakahashi, TN, HNakashima and YS performed statistical analysis. HM, KY, HNakaoka, AN, MS, TC, ATakahashi, TN, HNakashima, MK, and NS analysed the data. TN, HNakashima, HOnoue, KIwaya, TI, TT, KInoue, YKato and II provided intellectual input and assisted with the preparation of the manuscript. HM, KY, HNakaoka, AN, MS, TC, ATakahashi and NS wrote the paper.

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Competing interests HM, TT and NS have a patent pending based on the work reported in this paper. Other authors have declared that no competing interests exist.

Patient consent Obtained.

Ethics approval All procedures involved in this study were approved by the institutional ethical committees of National Defense Medical College, Nagoya University and RIKEN, and all procedures involved were performed in accordance with the Declaration of Helsinki.

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ORIGINAL ARTICLE

End-stage renal disease in Japanese children: a nationwide survey during 2006–2011

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Abstract
Background End-stage renal disease (ESRD) in children is considered a rare, but serious condition. Epidemiological and demographic information on pediatric ESRD patients around the world is important to better understand this disease and to improve patient care. The Japanese Society for Pediatric Nephrology (JSPN) reported epidemiological and demographic data in 1998. Since then, however, there has been no nationwide survey on Japanese children with ESRD.
Methods The JSPN conducted a cross-sectional nationwide survey in 2012 to update information on the incidence, primary renal disease, initial treatment modalities, and survival in pediatric Japanese patients with ESRD aged less than 20 years during the period 2006–2011.
Results The average incidence of ESRD was 4.0 per million age-related population. Congenital anomalies of the kidney and urinary tract were the most common cause of ESRD, present in 39.8 % of these patients. In addition, 12.2 % had focal segmental glomerulosclerosis and 5.9 % had glomerulonephritis. Initial treatment modalities in patients who commenced renal replacement therapy (RRT) consisted of peritoneal dialysis, hemodialysis, and pre-emptive transplantation (Tx) in 61.7, 16.0, and 22.3 %, respectively. The Japanese RRT mortality rate was 18.2 deaths per 1000 person-years of observation.

On behalf of the Japanese Society for Pediatric Nephrology.

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Conclusion The incidence of ESRD is lower in Japanese children than in children of other high-income countries. Since 1998, notably, there has been a marked increase in pre-emptive Tx as an initial treatment modality for Japanese children with ESRD.

Keywords End-stage renal disease · Children · Epidemiology · Renal replacement therapy · Japan

Introduction

End-stage renal disease (ESRD) in children is considered a rare, but serious condition [1]. Information on the epidemiology, demographics, treatment modalities, and mortality of pediatric patients with ESRD are essential for a better understanding of this disease and for improving patient care [2]. This information is also useful for patients, their families, physicians, and other healthcare providers. The epidemiology and demographics of pediatric ESRD have been analyzed in the USA [3, 4], Europe [5–8], and Australia and New Zealand [9, 10]. However, limited information is available on pediatric ESRD patients in other areas of the world.

The Japanese Society for Pediatric Nephrology (JSPN) reported epidemiological and demographic data on Japanese children with ESRD in 1998 [11]. Since then, however, there has been no nationwide survey on Japanese children with ESRD. International comparisons of the epidemiological and demographic characteristics of pediatric ESRD patients may improve outcomes [12, 13]. Therefore, the JSPN ESRD Survey Committee, in collaboration with the Japanese Society for Dialysis Therapy (JSDT) and the Japanese Society for Clinical Renal Transplantation (JSCRT), conducted a nationwide survey of Japanese children with ESRD in 2012. This report describes the basic epidemiological and demographic characteristics of Japanese children aged less than 20 years with ESRD over the period 2006–2011.

Patients and methods

Data collection

The JSPN conducted a cross-sectional nationwide survey in 2012, in collaboration with the JSDT and the JSCRT.

ESRD patients were defined as those with irreversible kidney function disorders requiring renal replacement therapy (RRT) to sustain life. This survey evaluated Japanese patients aged less than 20 years who were newly diagnosed with ESRD between January 1, 2006, and December 31, 2011, and who were followed up until December 31, 2011. Individual patient data included date of birth, gender, primary renal disease, date of starting RRT, treatment modality at the start of RRT, and important events such as death.

Questionnaires were collected in two steps. The first questionnaires, asking about patients newly diagnosed with ESRD, were sent to institutions at which members of the JSPN, JSDT, and JSCRT practiced, as well as to children's hospitals, and pediatric and nephrology departments of medical schools throughout Japan. The second questionnaires, which asked for data about individual patients, were sent to the institutions that reported having new pediatric ESRD patients.

This survey was in accordance with the ethical principles in the 1964 Declaration of Helsinki, and with the ethical guidelines for epidemiological studies issued by the Ministry of Health, Labour and Welfare, Japan. This survey was also approved by the central ethics board of Tokyo Women's Medical University (approval number; 2353) before study commencement.

Data analysis

The incidence of ESRD was defined as the number of new patients with ESRD per year, over the period 2006–2011, and was expressed as number per million age-related population (pmarp), with pmarp calculated using age-, sex-, and year-specific census data obtained from the Japanese data base [14].

Patient survival was analyzed by the Kaplan–Meier life table method. Mortality rates (deaths per 1000 person-years of observation) were also calculated. Data were analyzed using SAS system version 9 (SAS Institute, Cary, NC, USA).

Results

Patients with ESRD

The first questionnaires were sent to a total of 773 institutions, with 770 (99.6 %) responding; of these, 146 institutions had new pediatric ESRD patients during the period 2006–2011. The second questionnaires were therefore sent to these 146 institutions, with 136 (93.2 %) responding. These institutions reported a total of 540 new pediatric ESRD patients, including 322 male patients, 216 female patients and two patients not specified.

Incidence of ESRD

The per-year incidence of ESRD in the years 2006–2011 was 3.5, 3.9, 3.6, 4.7, 4.1, and 4.1 pmarp, respectively.

Table 1 Primary renal diseases of ESRD patients included in this survey

Renal diseases	Number of patients (%) Male/female/unknown				
	0–4 years	5–9 years	10–14 years	15–19 years	0–19 years
CAKUT	68 (43.0)	45 (45.0)	66 (40.2)	36 (30.5)	215 (39.8)
	39/29/0	33/12/0	46/19/1	29/7/0	164/67/1
Hereditary nephropathy	30 (19.0)	7 (7.0)	17 (10.4)	16 (13.6)	70 (12.9)
	12/18/0	3/4/0	13/3/1	11/5/0	39/30/1
FSGS	6 (3.8)	16 (16.0)	25 (15.2)	19 (16.1)	66 (12.2)
	3/3/0	11/5/0	14/11/0	14/5/0	42/24/0
Cystic kidney disease	17 (10.8)	10 (10.0)	19 (11.6)	6 (5.1)	52 (9.6)
	6/11/0	5/5/0	8/11/0	2/4/0	21/31/0
Glomerulonephritis	0 (0)	6 (6.0)	10 (6.1)	16 (13.6)	32 (5.9)
	0/0/0	1/5/0	6/4/0	2/4/0	19/13/0
HUS	4 (2.5)	1 (1.0)	2 (1.2)	2 (1.7)	9 (1.7)
	1/3/0	0/1/0	1/1/0	0/2/0	2/7/0
Ischemic renal failure	5 (3.2)	4 (4.0)	0 (0)	0 (0)	9 (1.7)
	1/4/0	1/3/0	0/0/0	0/0/0	2/7/0
Miscellaneous	20 (12.6)	7 (7.0)	12 (7.3)	8 (6.8)	47 (8.7)
	11/9/0	6/1/0	6/6/0	4/4/0	27/20/0
Unknown	0 (0)	4 (4.0)	6 (3.7)	8 (6.8)	18 (3.3)
	0/0/0	2/2/0	3/3/0	5/3/0	10/8/0
Missing	8 (5.1)	0 (0)	7 (4.3)	7 (5.8)	22 (4.2)
	4/4/0	0/0/0	5/2/0	4/3/0	13/9/0
Total	158 (100)	100 (100)	164 (100)	118 (100)	540 (100)
	77/81/0	62/38/0	102/60/2	81/37/0	322/216/2

Hereditary nephropathy includes Alport's syndrome, congenital nephrotic syndrome, and other specified types. Glomerulonephritis (GN) includes IgA nephropathy, membrano-proliferative GN, membranous nephropathy, crescentic GN, and other types of GN. Cystic kidney disease includes polycystic kidney disease, nephronophthisis, and other specified types

CAKUT congenital anomalies of the kidney and urinary tract, *FSGS* focal segmental glomerulosclerosis, *HUS* hemolytic uremic syndrome

The average incidence of ESRD over the 6-year period was 4.0 pmarp.

Primary renal disease in patients with ESRD

Primary renal diseases, categorized according to the European Renal Association and European Dialysis and Transplantation Association (ERA-EDTA) codes [5], with a minor modification, in the 540 pediatric ESRD patients evaluated in this survey are shown in Table 1. The most frequent primary renal disease was congenital anomalies of the kidney and urinary tract (CAKUT), including hypoplasia/dysplasia ± reflux nephropathy and obstructive uropathy, present in 39.8 % of these patients, followed by hereditary nephropathy (12.9 %), focal segmental glomerulosclerosis (FSGS; 12.2 %) and cystic kidney disease (9.6 %). Glomerulonephritis was observed in 5.9 % of these patients. CAKUT were the main causes of ESRD across all age groups and were more common in males than in females. Hereditary nephropathies including congenital nephrotic syndrome and Alport's syndrome were common in the youngest ESRD group and the adolescent ESRD group. FSGS and glomerulonephritis were less common in children aged less than 5 years as causes of ESRD. Cystic kidney disease caused 5–11 % of ESRD across all age group.

Initial treatment modalities of ESRD

Of the 540 patients newly diagnosed as having ESRD, six did not commence RRT because of their severe extra-renal comorbidities. In addition, RRT was not identified in four patients. Of the remaining 530 patients, 327 (61.7 %) were treated initially by peritoneal dialysis (PD), 85 (16.0 %) underwent hemodialysis, and 118 (22.3 %) underwent pre-emptive transplantation (Tx). The initial treatments by age group are shown in Fig. 1. Most children aged less than 5 years were treated initially by PD (89.0 %), with a small proportion undergoing pre-emptive Tx (2.6 %). In contrast, pre-emptive Tx was performed in 32.7, 29.2, and 30.2 % of children aged 5–9, 10–14, and 15–19 years, respectively. A comparison of initial treatment modalities in surveys of pediatric ESRD patients in 1998 [11] and 2006–2011 showed that the proportion of patients undergoing pre-emptive Tx markedly increased with time (Fig. 2).

Survival and cause of death

Survival analysis of the 530 patients who commenced RRT is shown in Fig. 3. The 1- and 5-year survival rates were 96.9 and 91.5 %, respectively. Patients were

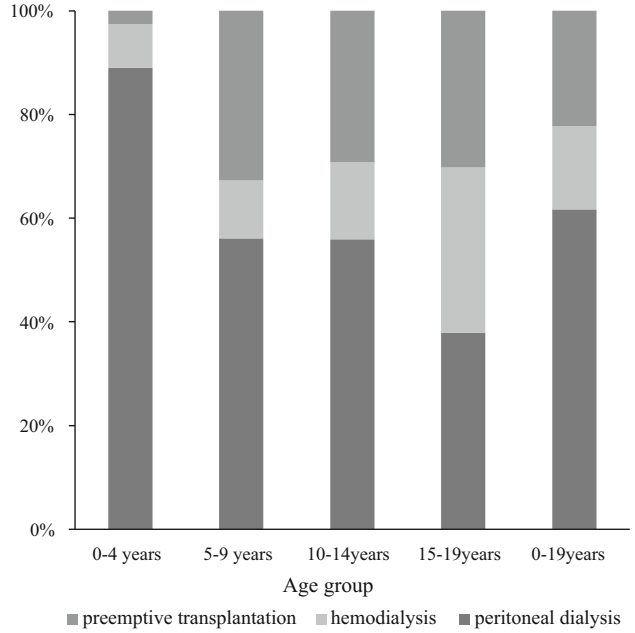

Fig. 1 Initial treatment modalities of end-stage renal disease by age group

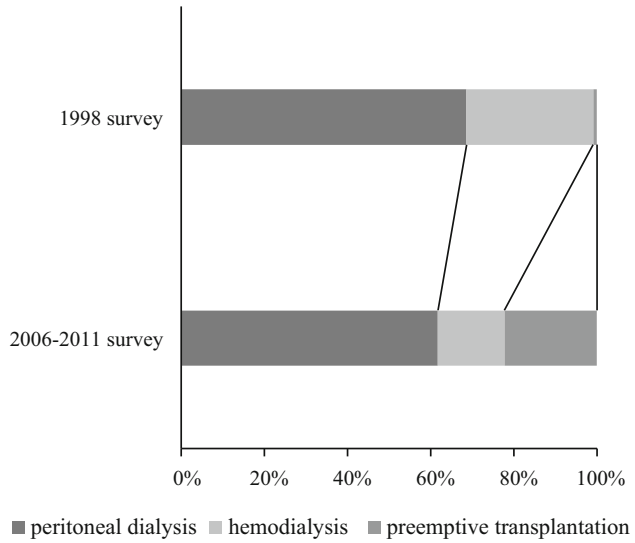

Fig. 2 Comparison of initial treatment modalities in Japanese patients with end-stage renal disease (ESRD) surveyed in 1998 [11] and in 2006–2011

followed up for a median 2.7 years (interquartile range 1.3–4.3 years). The mortality rate was 18.2 deaths per 1000 person-years of observation. During follow-up, 28 patients (5.4 %) died, all of whom were undergoing dialysis. Causes of death after the start of RRT, based on the United States Renal Data System [15] with a minor modification, are shown in Table 2. The main causes of death were infection ($n = 11$, 39.3 %) and cardiovascular causes ($n = 5$, 17.9 %).

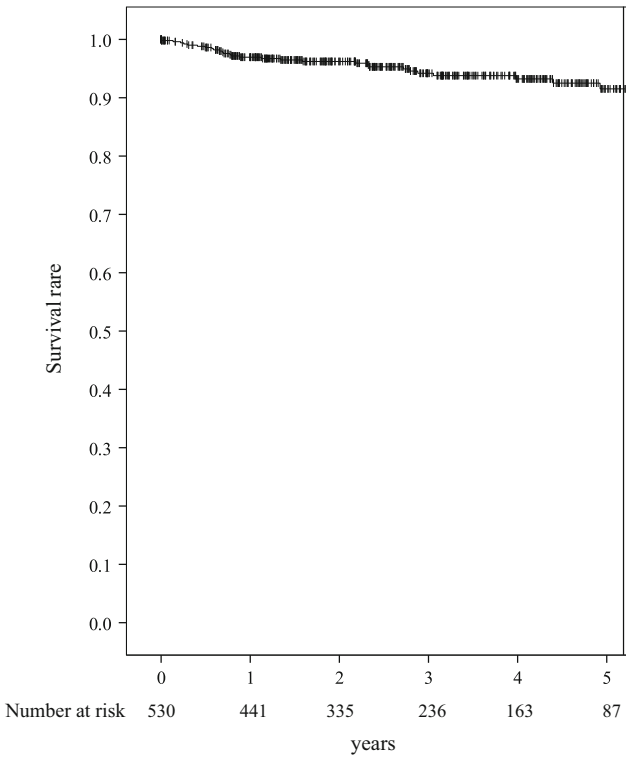

Fig. 3 Patient survival after the start of renal replacement therapy

Table 2 Causes of death after the start of renal replacement therapy

Renal diseases	Number of patients (%)
Infections	11 (39.3)
Cardiovascular	5 (17.9)
Cerebrovascular	0 (0)
Malignancy	0 (0)
Metabolic	0 (0)
Other	6 (21.4)
Unknown	4 (14.2)
Missing	2 (7.2)
Total	28 (100)

Discussion

The JSPN has updated the epidemiological and demographic information on the incidence, primary renal disease, initial treatment modalities, and survival in Japanese pediatric ESRD patients, aged less than 20 years, over the period 2006–2011. However, some important information, including the prevalence and probability of undergoing Tx, could not be updated in this survey.

There are marked variations in the incidence of pediatric ESRD across countries [13]. A previous survey by the JSPN, performed in 1998, reported that the incidence of ESRD in Japanese children aged less than 20 years was 4.0

pmarp [11], much lower than in other high-income countries, including 9.5 pmarp in 11 Western European countries and in Australia and 15.5 pmarp in the USA [13], despite Japan having one of the highest incidence rates of ESRD in adults [13, 16]. The current survey showed that the average incidence of ESRD in 2006–2011 was 4.0 pmarp, confirming the lower incidence of ESRD in Japanese children. Because the reasons for this lower incidence remain unclear [16], further research is needed to determine the specific factors responsible for the lower rate of ESRD in Japanese children.

Causes of ESRD also vary across races and countries. FSGS is more common in blacks than in whites, genetic diseases are more prevalent in the Middle East than in Europe, and infection-related renal diseases are more frequent in less-developed countries [12, 13]. In the USA, Europe, and Australia and New Zealand, CAKUT are the main cause, accounting for around 40 % of pediatric ESRD patients [13]. The current Japanese survey showed that CAKUT were also the most common cause of renal disease, accounting for 39.8 % of all pediatric Japanese ESRD patients. In contrast, it has been indicated that the proportion of pediatric ESRD caused by glomerulonephritis, including FSGS, was higher in Japan than in Europe or the USA [12, 13]. The 1998 Japanese survey reported that the proportions of pediatric ESRD patients with FSGS and glomerulonephritis were 21.0 and 13.3 %, respectively [11], whereas the current survey showed that these proportions were much lower, 12.2 and 5.9 %, respectively. The Australia and New Zealand Dialysis and Transplant Registry also showed a decline over time in glomerulonephritis as a cause of ESRD [10]. Additional surveys are needed to confirm this trend in the etiology of ESRD in Japanese children.

The initiation of RRT is highly dependent upon the economy and availability of healthcare resources [12, 17]. In countries where RRT is readily available, the most favored renal replacement modality in children is Tx because dialysis is associated with cardiovascular damage, access complications, infection, retarded linear growth and cognitive development in children [12]. Pre-emptive Tx is an especially attractive option for children with ESRD because pre-emptive Tx potentially avoids exposure to negative outcomes associated with dialysis [18]. While the previous Japanese survey in 1998 reported that only one patient (0.9 %) underwent pre-emptive Tx [11], this survey notably found that 22.3 % of patients were initially treated by pre-emptive Tx. In the USA, Europe, and Australia and New Zealand, around 15–20 % of children newly diagnosed with ESRD undergo pre-emptive Tx [13]. Thus, the use of pre-emptive Tx as an initial treatment modality for Japanese children with ESRD is comparable to that of USA, Europe, and Australia and New Zealand. The evolved immunosuppression protocols using calcineurin inhibitors, mycophenolate mofetil and basiliximab reduced acute rejection episodes and improved the patient and graft survival [19]. The improved treatment following Tx and the increased awareness of effectiveness of pre-emptive Tx seem to be responsible for the marked increase in pediatric pre-emptive Tx in Japan.

The 5-year survival rate of Japanese children with ESRD who received RRT was 91.5 %, which was similar to that reported from Europe (the 4-year survival rate was 92.9 % in European RRT children) [8]. The mortality rate of 18.2 deaths per 1000 person-years of observation was similar to that observed in the 1998 survey [11] and in pediatric ESRD patients in Australia and New Zealand [9]. The two main causes of death in Japanese ESRD patients receiving RRT were infections and cardiovascular disease, similar to findings in Western countries [12, 13]. Superior survival has been reported in patients with a functioning graft than in patients on dialysis, with the poorest survival rates observed in infants with ESRD [13]. Further studies are required to determine risk factors for mortality in Japanese pediatric ESRD patients.

Finally, epidemiological and demographic information on pediatric ESRD patients around the world is important to better understand this disease and to improve patient care. Because single country data may be underpowered to draw meaningful insights, international collaborations are required to improve the outcomes of children with ESRD [20, 21].

In conclusion, this survey of epidemiological and demographic information on Japanese children aged less than 20 years with ESRD over the period 2006–2011 confirmed that the incidence of ESRD is lower in Japan than in other high-income countries. Notably, there has been a marked increase in the use of pre-emptive Tx as the initial treatment modality for these patients.

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Conflict of interest The authors have declared that no conflict of interest exists.

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LETTER TO THE EDITOR

The first two cases of *MYH9* disorders in Thailand: an international collaborative study

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Dear Editor,

MYH9 disorders are the autosomal dominant platelet disorders characterized by giant platelets, thrombocytopenia, and granulocyte Döhle body-like cytoplasmic inclusion bodies and are due to mutations in *MYH9*, the gene encoding non-muscle myosin heavy chain IIA (NMMHC-IIA). Patients show variable expression of non-hematological complications, such as glomerulonephritis, hearing inability, and cataracts [1, 2]. Although granulocyte inclusion bodies are the laboratory hallmark, they are often overlooked due to their inconspicuous appearance on conventionally stained blood smears. Abnormal NMMHC-IIA protein accumulates in the granulocyte cytoplasm, and an immunofluorescence analysis of NMMHC-IIA localization is now used as a reliable diagnostic test [3–5]. Because the complications are progressive and there are strict genotype/phenotype correlations, an early genetic diagnosis is crucial to confirm and determine the prognosis, and if possible, to select appropriate treatment [6].

We conducted an international collaborative *MYH9* disorders study between Thailand and Japan. Patients were registered in the pediatric macrothrombocytopenia registry in Thailand. The criteria for registration were platelet counts <150,000/μL and large platelets. All patients were investigated for the common causes of macrothrombocytopenia, including the von Willebrand factor indices, flow cytometry for CD42b and CD41/CD61, and platelet aggregation studies. In the present study, peripheral blood smears from 10 patients were sent to Japan, where the immunofluorescence analysis for NMMHC-IIA was performed [3]. Two out of the 10 patients (patients 6 and 8) (20 %) were positive in the analysis, and *MYH9* gene sequencing was further performed [3]. Local institutional ethics committees approved the study, and informed consent was obtained from the patients.

Patient 6 was a 12-year-old female. The hematological examination showed a platelet count of 67,000/μL, MPV of 17.6 fL, giant platelets, and conspicuous granulocyte inclusion bodies (Fig. 1a). Blood chemistry and urinalysis were unremarkable. The hearing test showed a high-tone drop in the left ear. Her father was also hematologically affected (Fig. 1b). He had mild hearing loss in both ears. Cataracts were absent in both individuals. Patient 8 was a 12-year-old female presenting with persistent hypertension, nephritis, and thrombocytopenia. She had a history of intermittent epistaxis, gum bleeding, and menorrhagia. She had previously been treated with prednisolone and nifedipine without any response. The laboratory investigation showed a platelet count of 67,000/μL and MPV of 11.3 fL. A peripheral blood smear showed giant platelets and only faint inclusion bodies in neutrophils (Fig. 1c). The BUN was 64.5 mg/dL (normal range, 5–18 mg/dL), and creatinine was 1.63 mg/dL (normal range, 0.5–1.1 mg/dL). Urinalysis showed protein 2+ and blood 3+. Hearing test was not performed. Mild cataracts were observed. Her father and brother died from bleeding.

The immunofluorescence analysis for NMMHC-IIA revealed type II localization, consisting of several cytoplasmic spots with a circular or oval shape in both patients (Fig. 1d, e, f). Genomic DNA was extracted from the remaining smears.

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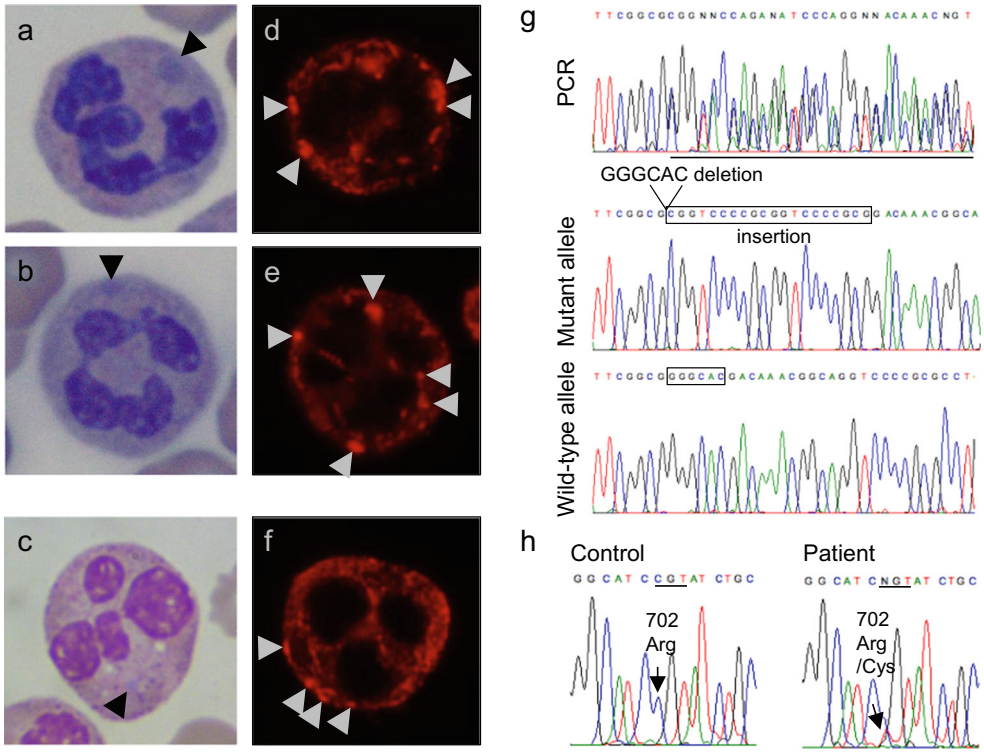

Fig. 1 The results of the NMMHC-IIA and *MYH9* analyses. Light micrographs of May-Grünwald-Giemsa-stained neutrophils from patient 6 (**a**), the father of patient 6 (**b**), and patient 8 (**c**), respectively. Inclusion bodies are indicated by *arrowheads*. Immunofluorescence micrographs of neutrophils immunostained with the anti-NMMHC-IIA antibody in patient 6 (**d**), the father of patient 6 (**e**), and patient 8 (**f**), respectively. The accumulations of NMMHC-IIA are indicated by *arrowheads*. Sequencing of the *MYH9* gene showed 5788_5793delinsCGCGGGGA CCGCGGGGACCG resulting in p.V1930_P1931fsX and 2104 C>T, resulting in p.R702C, in patients 6 (**g**) and 8 (**h**), respectively. Complementary sequences are shown

As type II NMMHC-IIA localization and visible inclusion bodies are mostly associated with exons 26 and 30 mutations [3], these exons were initially analyzed in patient 6. No mutations were found. An extended analysis revealed a novel exon 40 mutation, p.V1930_P1931fsX24 in both patient 6 and her father (Fig. 1g). The type II NMMHC-IIA localization and faint inclusion bodies in patient 8 suggested exons 1 and 16 mutations. Accordingly, a p.R702C was detected (Fig. 1h). This mutation is known as an early onset of glomerulonephritis and hearing disability [6–8]. Recent investigations have suggested that angiotensin receptor blockers and/or angiotensin converting enzyme inhibitors may have a protective effect against the progression of glomerulonephritis [7, 9]. We thus consider a careful follow-up and treatment plan.

We identified the first two Thai patients with *MYH9* disorders, who had not been definitely diagnosed. The present study should improve the diagnosis and treatment of patients with *MYH9* disorders in areas where a differential diagnosis is unavailable. Patients who present with macrothrombocytopenia, even without granulocyte inclusion bodies and non-hematological complications, should be screened by NMMHC-IIA immunofluorescence, and the presence of an *MYH9* mutation should be confirmed by genetic analysis. Close collaboration between hospitals in areas where these tests are unavailable and institutions with more advanced diagnostic capabilities should be established to facilitate the correct and prompt diagnosis and treatment of these cases [10].

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Conflict of interest The authors declare that they have no conflict of interest.

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Molecular Genetics & Genomic Medicine

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ORIGINAL ARTICLE

Alport syndrome caused by a *COL4A5* deletion and exonization of an adjacent *AluY*

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Keywords
Alu, collagen, exonization, intron, RNA processing

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Abstract

Mutation-induced activation of splice sites in intronic repetitive sequences has contributed significantly to the evolution of exon–intron structure and genetic disease. Such events have been associated with mutations within transposable elements, most frequently in mutation hot-spots of *Alu*s. Here, we report a case of *Alu* exonization resulting from a 367-nt genomic *COL4A5* deletion that did not encompass any recognizable transposed element, leading to the Alport syndrome. The deletion brought to proximity the 5′ splice site of *COL4A5* exon 33 and a cryptic 3′ splice site in an antisense *AluY* copy in intron 32. The fusion exon was depleted of purines and purine-rich splicing enhancers, but had low levels of intramolecular secondary structure, was flanked by short introns and had strong 5′ and *Alu*-derived 3′ splice sites, apparently compensating poor composition and context of the new exon. This case demonstrates that *Alu* splice sites can be activated by outlying deletions, highlighting *Alu* versatility in shaping the exon–intron organization and expanding the spectrum of mutational mechanisms that introduce repetitive sequences in mRNAs.

The mutation was found in a 14-year-old boy who developed macrohematuria at the age of 4 months. He was diagnosed with the Alport syndrome 14 months later following a confirmatory renal biopsy that showed a typical lamellation of the glomerular basement membrane and the absence of type IV collagen α5 chain by immunohistochemistry (data not shown) carried out as described (Oka et al. 2014). The patient developed mental retardation and autism; his severe proteinuria eventually culminated in renal failure at the age of 10 and he underwent preemptive renal transplantation using a kidney from his father. He did not show any detectable hearing loss or ocular abnormalities. His mother had hematuria and mild proteinuria since early childhood.

The disease-causing deletion was found by PCR amplifications of patient's DNA across exon 33, revealing a smaller fragment in the patient and in his heterozygous mother (Fig. 1A, left panel). DNA sequencing of the new fragment showed a 367-nt deletion (*COL4A5* c.2768-230_c.2904del367 at Xq22.3) encompassing most of the 150-nt exon 33 (Fig. 1B and C). Amplicons of cDNA samples reverse transcribed from blood or urine

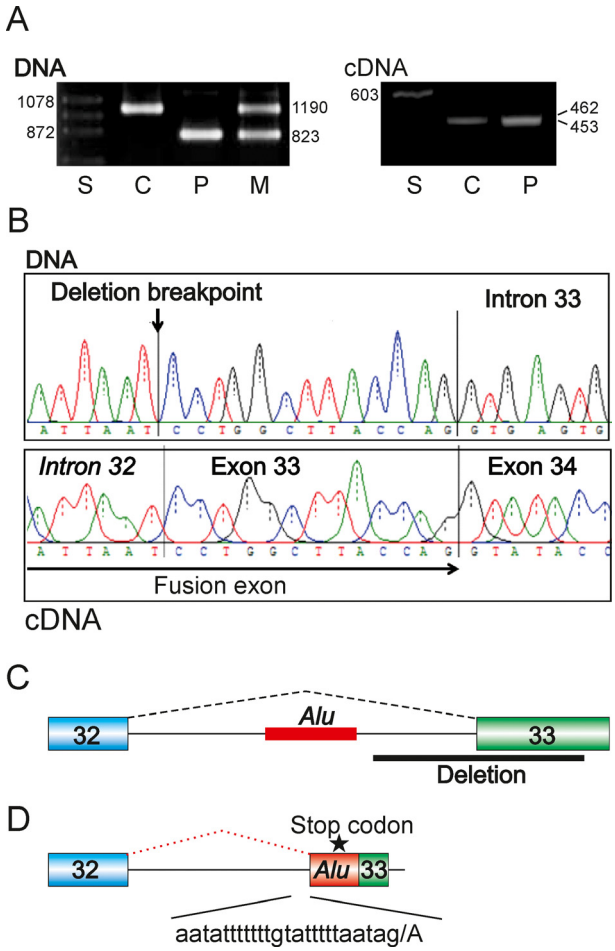

Figure 1. Deletion-induced exonization of *AluY* leading to Alport syndrome. (A) PCR amplifications of DNA (left panel) and cDNA (right panel) samples from a control (C), patient (P) and his mother (M). S, size marker; fragment sizes are shown in nts. DNA was amplified by primers 5′-AGTTTTCTGGTTGACATCTTA and 5′-ATAAGTCACTTTT CATGCTAT; cDNA was amplified by primers 5′-CAACCTGGTTTAC ATGGAAT and 5′-TCCAGGCAAACCCTGATAACC. (B) Sequence chromatogram of patient's DNA (upper panel) and cDNA (lower panel). (C, D) Schematic representation of the genomic deletion (C) and *AluY* exonization (D). Exons are shown as boxes, introns as horizontal lines, canonical (black) and aberrant (red) splicing by dotted lines above the primary transcripts. Sequence of the new 3′ splice is shown at the bottom, forward slash denotes the new intron–exon boundary. Location of the stop codon is shown by an asterisk.

RNA also showed a fragment with a slightly greater mobility (Fig. 1A, right panel). Sequencing of the cDNA fragment revealed the inclusion of a new exon of 141 nt, which contained a premature termination codon in an *AluY*-derived sequence of intron 32 (Fig. 1B and D). The adjacent deleted sequence was devoid of any transposed elements, as determined by the most sensitive option of the RepeatMasker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker, version 4.0.5), yet the deletion was capable of activating a distant cryptic 3′ splice site of the new exon 128-nt upstream of the deletion breakpoint in the left arm of the *AluY* copy. Thus, the fusion exon was composed of an *Alu*-derived sequence of intron 33, 15-nt linker between the *AluY* and the deletion breakpoint, and the 3′ end of exon 33 (Figs. 1D, S1 and S2).

Although cryptic exons derived from transposed elements causing genetic disease contain on average more splicing enhancers and less silencers than average human exons (Vorechovsky 2010), the new fusion exon was rich in pyrimidines and in splicing silencers and was depleted of purine-rich enhancers, the most potent exon recognition motifs. For example, the density of exon identity elements (Zhang et al. 2008) was only 68% of the average exon density and less than a half of the density calculated for the deleted exon counterpart (Table 1). This was also reflected in a lower predicted stability across the *Alu* portion of the new exon, consistent with a higher single-strandedness observed for exonizing *Alu*s than for nonexonizing *Alu*s (Schwartz et al. 2009). However, the 3′ splice site of the new exon was stronger than that of exon 33, most likely compensating the unfavorable nucleotide composition of the new exon (Table 1) in the context of a strong 5′ splice site (score 95.04). Finally, the inclusion of the new exon in the *COL4A5* mRNA (Fig. 1A) may have been assisted by shortening of the intron preceding exon 33 by a half and by a relatively short downstream intron, because long introns tend to associate with nonexonizing rather than exonizing *Alu*s (Schwartz et al. 2009).

Multiple sequence alignments of available primate orthologs coupled with evolutionary reconstruction of the insertion event (Krull et al. 2005) showed the presence of this *Alu* in Old World monkeys and the same 3′ splice-site consensus of the new exon across species (TAG/A).

Table 1. Comparison of sequence features of the new *Alu* exon and exon 33.

	%T	%C	%A	%G	EIE density[1]	3′ splice site score[2]	Free energy[3]
Deleted exon 33	18.0	24.0	26.7	31.3	660	75.96	−0.37
New *Alu* exon	25.5	29.1	22.0	23.4	324	82.18	−0.27

[1]Density of exon identity elements (EIEs) (Zhang et al. 2008) was computed as described (Divina et al. 2009).
[2]Shapiro–Senapathy score was calculated by an online tool at http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm.
[3]kcal/mol and nt at 37°C.

This *Alu* element was absent in New World monkeys (*Platyrrhini*), indicating that the insertion took place ~40–25 million years ago and that the 5' breakpoint (/) of the 367-deletion in the patient was in the target-site duplication sequence GGATTAAGCATTAAT/TTTTTT. Thus, the ancient *Alu* insertion was a prerequisite not only for the exonization event but also for the genomic deletion found in the family.

Alu exonization has facilitated gene regulation through alternative splicing during primate evolution and contributed to the expansion of proteomic interactions in humans (Makalowski et al. 1994; Sorek et al. 2002; Krull et al. 2005), however, only a very limited number of exonization mechanisms has been described, both for existing exons (Lev-Maor et al. 2003; Sorek et al. 2004) and disease-associated events (Meili et al. 2009; Vorechovsky 2010). This case demonstrates that a disease-causing *Alu* exonization can result from deletions not involving any transposed elements and reveals key sequence features promoting activation of the *Alu* exon with a poor splicing enhancer/silencer ratio, expanding the range of mutational mechanisms that introduce the most common human repeats in the mRNA.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of antisense *COL4A5* intronic *AluYm1* in patient ID45 and the *AluYm1* consensus. v, transversions; i, transitions. The 3′ splice-site AG is in red.

Figure S2. The genomic sequence of *COL4A5* across the fusion exon. Exons are in upper case, introns are in lower case. Deletion is highlighted in gray; sequence of the new fusion exon is in italics; target-site duplications are underlined.

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ORIGINAL ARTICLE

Selection of infants who potentially have congenital anomalies of the kidney and urinary tract from a large cohort for a more thorough examination

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Abstract
Background Although congenital anomalies of the kidney and urinary tract (CAKUT) are the most common cause of pediatric end-stage renal disease (ESRD), little is known about the characteristics exhibited in the infantile period by CAKUT patients who develop ESRD. Further, an efficient screening method for CAKUT diagnosis is not available currently. In the present study, we aimed to develop a method to select infants who potentially have CAKUT from a large group of infants.
Methods We retrospectively investigated the clinical characteristics of CAKUT patients in the infantile period. The medical records of 101 patients with CAKUT who had undergone dialysis or renal transplantation were reviewed. The data of gestational age, birth weight, oligohydramnios, poor body weight gain, asphyxia, and jaundice were recorded. We attempted to determine the ideal characteristics that could be used to select infants who potentially have CAKUT.
Results 14 % of patients were born prematurely, 18 % had low birth weight, 79 % had poor body weight gain, 18 % had asphyxia, 8 % had oligohydramnios, and 12 % had jaundice. We found that 82 % of patients had poor body weight gain or oligohydramnios among our patients and regarded these two symptoms as ideal markers for selecting those who potentially have CAKUT (specificity, 95 %; efficacy, 95 %). Further, the age of ≤7 months was the most appropriate time for the selection.
Conclusions For timely diagnosis of CAKUT, we recommend that ultrasound examination and the serum creatinine test be conducted for infants showing poor body weight gain or oligohydramnios at age ≤7 months.

Keywords Congenital anomalies of the kidney and urinary tract · Infantile period · Clinical characteristics · Chronic kidney disease · End-stage renal disease

Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT) are the most common cause of pediatric end-stage renal disease (ESRD); they have been identified as the underlying cause of ESRD in 34–43 % of the entire population of affected children [1–5]. Despite this, no report has determined the number of patients diagnosed with CAKUT during the neonate or infantile period. On the other hand, a few studies have reported that a considerably high percentage of CAKUT patients cannot be diagnosed until late childhood [6, 7]. Further, there is no appropriate screening method for newborns and infants to identify CAKUT patients among those who develop ESRD.

Studies have shown that chronic kidney disease (CKD) is associated with a high risk of cardiovascular events, morbidity, and mortality, not only in adults but also in children [8–11]. Moreover, the CKiD study recently

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showed that CKD in children is associated with impaired neurocognitive development and growth [12]. Given these findings about CKD in children, we considered it very important to ensure early selection of cases that potentially have CAKUT, which can progress to CKD and ESRD, using an effective method. A better understanding of the clinical characteristics of the disease in neonates and infants may lead to the development of a screening method for the early diagnosis of CAKUT. Thus, we retrospectively investigated ESRD patients with CAKUT and attempted to develop a method for seeking out the patients who have CAKUT in infants based on symptoms easily identifiable in routine medical care. To the best of our knowledge, thus far, no such long-term study on CAKUT patients has been conducted at a single, tertiary pediatric nephrology center.

Methods

This study enrolled 146 patients (91 male and 55 female) who were being followed up for CAKUT at Tokyo Metropolitan Kiyose Children's Hospital (Tokyo Metropolitan Children's Medical Center, at present) and in whom dialysis had been started or had undergone renal transplantation between January 1972 and June 2000. Since our previous study showed that poor body weight gain is the most important clinical symptom of CAKUT during infancy [13], 34 of the 146 patients (23 %) whose body weight gain data could not be obtained were excluded. Further, 4 among the remaining 112 patients were excluded because dialysis had been started for them during the neonatal period, and another 7 were excluded because they were diagnosed with CAKUT due to respiratory distress at birth (all patients with respiratory distress were immediately diagnosed with CAKUT). This latter group was excluded because we specifically wanted to examine patients who could not be diagnosed early in life despite the congenital nature of CAKUT.

For the remaining 101 patients (66 male and 35 female), the following clinical data were reviewed from the medical records: gestational age, birth weight, body weight gain, asphyxia, oligohydramnios, and jaundice. We selected these parameters because these were routinely recorded, easily accessible indicators. In developed countries, it is mandatory to record these data for all newborns. Information on gestational age was available for 99 patients; birth weight, for 98 patients; and both gestational age and birth weight, for 90 patients. Further, information on the presence of jaundice was available for 75 patients.

Patients reported to have poor body weight gain according to pediatric physicians, who had referred the patients to our hospital, or according to the patients' caregivers were included in the poor body weight gain group. The diagnosis in all patients was confirmed according to the definition given below.

Table 1 Details of CAKUT in the study patients

Details of CAKUT	Cases	%
UTA with hypo/dysplastic kidney		
VUR total	36	36
VUR (grade IV–V)	9	9
VUR (grade I–III)	6	6
VUR (grade unknown)	18	18
VUR + stenosis	3	3
Stenosis only (UV, UP)	8	8
Urethral tract stenosis + VUR	6	6
Hypoplastic or dysplastic kidney without UTA	51	50
Total	101	100

UTA Urinary tract abnormalities, *VUR* vesico-ureteral reflux, *UV* ureterovesical junction stenosis, *UP* ureteropelvic junction stenosis

The age of the patients at the time of the investigation ranged from 9 to 37 years (median, 22 years). Premature births were defined as those occurring at gestational age $<$37 weeks. Low birth weight was defined as that less than 2,500 g. Poor body weight gain was defined as growth below the 3rd percentile or changes in growth that crossed two major growth percentiles of the growth curve in a short time. Asphyxia was defined as an Apgar score at 1 min of $<$7 points. Oligohydramnios was defined as an amniotic fluid volume of $\leq$100 mL. Jaundice was defined as the requirement of phototherapy or exchange transfusion. If adequate information on the neonatal and infantile period could not be obtained from the charts, a simple questionnaire was sent to the caregivers to request supplementary medical data.

Of the 101 patients, 51 (50 %) had hypoplastic or dysplastic kidneys (hypo/dysplastic kidney) without urinary tract abnormalities, 36 (36 %) had vesico-ureteral reflux (VUR), 8 (8 %) had ureter stenosis, and 6 (6 %) had VUR with urethral tract stenosis (Table 1). There were 46/101 (45.5 %) cases of bilateral hypoplastic kidney, 40/101 (39.6 %) of bilateral dysplastic kidney, and 15/101 (14.8 %) of unilateral hypoplastic and unilateral dysplastic kidney. We examined kidney size using ultrasound (US) (and compared it with age-matched data); dimercaptosuccinic acid uptake values and voiding cystogram were used for evaluation purposes if the patients had hypo/dysplastic kidney. Only one patient had abnormal prenatal US findings; she had hydronephrosis and hydroureter.

First, we investigated the age at which the patients were diagnosed with CAKUT, the signs and symptoms that led to the diagnosis, and the age at which dialysis or transplantation was performed. From the age at diagnosis, we determined how long disease discovery had been delayed

Table 2 The patients' age and the indication for diagnosis

Age	0–6 months	7–11 months	1–5 years	≥6 years
PBWG ($n = 12$)	9	1 (7 months)	2	
3-year exam ($n = 9$)			9	
School U/A ($n = 19$)				19
UTI ($n = 12$)	4	2	5	1
By chance ($n = 13$)	3	1	2	7
Short stature ($n = 3$)			3	
Other ($n = 9$)	3	1	3	2

n number of patients, *PBWG* poor body weight gain, *3-year exam* routine medical checkup at 3 years of age, *School U/A* routine annual urinary examination for school-age children, *UTI* urinary tract infection, *Other* a 6-month-old patient was diagnosed with renal dysfunction when he had an operation for anal atresia; two patients (1 and 6 months of age) were admitted for dehydration related to renal dysfunction; a 9-month-old patient had convulsions due to hypocalcemia and showed symptoms of renal failure; a 2-year-old patient had respiratory distress; a 6-year-old patient's face had a pale color; a 4-year-old patient had polyuria; a 4-year-old patient had light colored urine; and a 10-year-old patient had acute renal failure

in these patients. In Japan, routine medical checkups are frequent: there are checkups at age 1, 3–4, and 9–10 months as well as at age 3 years; additionally, routine urinary examinations are conducted at age 3 years and every year for school-age children. The results of these checkups were included in the above analysis.

Second, we studied the characteristics displayed by the patients during the neonatal or infantile period.

Lastly, we calculated the percentage frequency of each characteristic and ascertained the characteristics most useful for selecting patients who potentially have CAKUT.

Written informed consent was obtained from each patient or caregiver. This study was approved and registered by the Bioethics Committee of Dokkyo Medical University (accept no.: hosp-k25028).

Results

Age of CAKUT diagnosis and diagnostic indicators

The median age of diagnosis was 2.2 years (range 0–15 years) among the 101 patients, and the median age at which dialysis or transplantation was performed was 9.5 years (range 0.4–23.6 years). Further, 79(71 %) of 112 CAKUT patients could not be diagnosed during the neonatal period, which was a large number despite the congenital nature of CAKUT. To calculate the percentage of patients who could not be diagnosed during this period, we also included the number of patients who were excluded, because they had started undergoing dialysis during the neonatal period or they had been diagnosed with CAKUT due to respiratory distress at birth. Among these 79 patients, the median age of diagnosis was 3.4 years (range 0.1–15.1 years), and the median age at which dialysis or transplantation was performed was 10.1 years (range 0.5–23.6 years).

Table 3 Clinical abnormalities during infancy

	Study patients ($n = 101$)	General frequency [14–16, 23]
Gestational age (median)	39 weeks 2 days	38 weeks ± 2 weeks
Birth weight (g)	2922 ± 566	2979 ± 443
Light for date (*n*)	12/90 (13 %)	3.5 %
Heavy for date (*n*)	2/90 (2 %)	1.0 %
Premature baby	14/99 (14 %)	9.9 %
Low birth weight	18/98 (18 %)	12.7 %
Poor body weight gain (*n*)	80/101 (79 %)	4 % [23]
Asphyxia (*n*)	19/101 (19 %)	5.1 %
Oligohydramnios (*n*)	8/101 (8 %)	0.9 %
Jaundice (*n*)	9/75 (12 %)	8.0 %

The age of diagnosis and diagnostic indicators are shown in Table 2. It is noteworthy that in all but 2 cases, the poor body weight gain led to a diagnosis of CAKUT before 7 months of age (10/12, 83 %). Further, of the 2 exceptions (one patient was diagnosed at age 1 year and the other, at age 5 years), one patient had remarkably poor body weight gain from age 2 months, and the other showed this sign throughout the infantile period, but they were not diagnosed with CAKUT during the infantile period.

Only 5 patients of 101(4.9 %) were diagnosed with CAKUT at 7–11 months of age. For patients diagnosed at 1–5 years of age, the results of the routine medical checkup conducted at age 3 years were the most frequent diagnostic indicator(9/24, 37 %), followed by urinary tract infection (5/24, 21 %). Three patients were diagnosed on the basis of their short stature (3/24, 13 %). For those diagnosed at age 6 years or older, the results of routine urinary examinations conducted at school were the most common diagnostic indicators (19/29, 66 %).

Patient characteristics

The average gestational age at birth was 38 weeks (w) 5 days (d) ± 2 w 1 d and the average birth weight was 2922 ± 566 g. In terms of birth weight, among 90 patients who had the information, 76 (90 %), 12 (13 %), and 2 (2 %) were appropriate for date (AFD), light for date (LFD), and heavy for date (HFD), respectively.

Fourteen patients were born prematurely (14/99, 14 %), 18 had low birth weight (18/98, 18 %), 80 had poor body weight gain (80/101, 79 %), 19 had asphyxia (19/101, 19 %), 8 had oligohydramnios (8/101, 8 %), and 9 had jaundice (9/75, 12 %) (Table 3).

Among these abnormalities, LFD, poor body weight gain, asphyxia, and oligohydramnios showed unusually higher than normal frequency in the study population [14–16] (Table 3).

Table 4 Sensitivity and specificity of different combinations of patient characteristics in the infantile period

	Sensitivity (%)	Specificity (%)	Efficacy (%)	Size of the object (/10000)
PWG + AS + OL	84	90	90	1000
PWG + AS	83	91	91	910
PWG + OL	82	95	95	490

Size of the object: number of newborns considered as objects of examination among 10000 newborns

PWG poor body weight gain, *OL* oligohydramnios, *AS* asphyxia

Discussion

Recent studies have shown that children with CKD and childhood-onset ESRD show similar health problems as adults affected by these conditions [10–12, 17–19]. Early diagnosis of CAKUT could be greatly advantageous for affected children since it would enable timely administration of treatment or medical observation. These interventions may prevent various complications due to CKD, such as growth retardation, cardiovascular events, and severe secondary mineral bone disease. However, a useful screening method is currently unavailable, and CAKUT symptoms are overlooked in many patients, unless they have remarkable symptoms, such as respiratory distress. In fact, although neonatal US is used at many medical institutions and medical checkups at ages 1, 3, and 6 months are performed all over Japan, only 31 % of CAKUT patients are identified by US examination, whereas 2.5 % are identified by health checkups [20]. Further, 25 % of CAKUT patients cannot be diagnosed during the first 3 years of life [6]. Although mass US screening for infants is effective for identification of CAKUT patients [21, 22], the effort and costs are immense. The serum creatinine test may identify patients with hypo/dysplastic kidneys without formation abnormalities such as multiple cyst and pelvic dilatation, but it is not practical for use among neonates and infants. If the number of patients required to be screened can be limited using an easy method and if these patients can be examined by US and the serum creatinine test, the efficiency of screening can be dramatically improved.

Using a prospective method to accurately screen for CAKUT is difficult because many children with CAKUT cannot be diagnosed until their renal dysfunction has advanced. Thus, the present study is retrospective in nature. We excluded a considerable amount of patients with CAKUT (34/146), because sufficient data on body weight gain were not available since some patients were referred to our hospital during late childhood. We were usually careful when the patients had failure to thrive or when we suspected that the patient had hypo/dysplastic kidney; therefore, we recorded the body weight gain data when it was available. Although we did not expect that any bias would be present with this methodology, there may still be some bias in our report. Nonetheless, to our knowledge, it is the first study that investigates the clinical characteristics of CAKUT patients in the infantile period. Furthermore, a basic but effective method for selecting infants who potentially have CAKUT, based on their characteristics, has been suggested in this study. This method is easily applicable during routine medical care.

From the characteristics exhibited by our patients, we found that LFD, poor body weight gain, asphyxia, and oligohydramnios were exhibited at a higher frequency than normal [14–16] (Table 3). Using these data, we attempted to determine the most ideal symptoms for selecting patients who potentially have CAKUT. Obviously, those with one of the symptoms (poor body weight gain, asphyxia, or oligohydramnios) formed the largest group (85/101, 85 %). Those with poor body weight gain or asphyxia formed the second largest group (84/101, 83 %), while those with poor body weight gain or oligohydramnios (83/101, 82 %) formed the third largest group.

Generally, poor body weight gain is found in about 3–4 % of children until the age of 1 year [23]. In the present study, we used 4 % as the normal frequency of poor body weight gain and calculated the sensitivity and specificity of various symptoms assuming an annual birth rate of 10000 in Tokyo. As shown in Table 4, the condition of poor body weight gain, asphyxia, or oligohydramnios showed the highest sensitivity and the lowest specificity, and the number of infants with these symptoms was the highest. On the other hand, the condition of poor body weight gain or oligohydramnios showed the lowest sensitivity and the highest specificity, and the fewest number of infants had these symptoms. Therefore, we regarded the latter symptoms as ideal for selecting patients who potentially have CAKUT.

Next, we tried to determine the time appropriate for the selection test. As mentioned above, all patients who had a poor body weight gain showed this symptom before 7 months of age (Table 2). We believe that in cases of CAKUT, poor body weight gain may occur because of salt

loss, as breast milk and formula contain little salt. Therefore, in most cases, the poor body weight gain may be reversed by ensuring adequate salt levels in food. From the results, we recommend that the selection test for CAKUT be performed before 7 months of age.

Conclusion

Although CAKUT is known to be the most important cause of pediatric ESRD, identifying affected patients is difficult. Many CAKUT patients are diagnosed after the neonatal period, by which time they have already developed CKD and have not received the appropriate treatment. Our results indicate that CAKUT may be diagnosed earlier if infants with poor body weight gain or oligohydramnios are examined by US and serum creatinine levels are assessed before 7 months of age, through which the various risks of CAKUT may be reduced.

Conflict of interest The authors declare no conflicts of interest.

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X-Linked Alport Syndrome Caused by Splicing Mutations in *COL4A5*

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Abstract

Background and objectives X-linked Alport syndrome is caused by mutations in the *COL4A5* gene. Although many *COL4A5* mutations have been detected, the mutation detection rate has been unsatisfactory. Some men with X-linked Alport syndrome show a relatively mild phenotype, but molecular basis investigations have rarely been conducted to clarify the underlying mechanism.

Design, setting, participants, & measurements In total, 152 patients with X-linked Alport syndrome who were suspected of having Alport syndrome through clinical and pathologic investigations and referred to the hospital for mutational analysis between January of 2006 and January of 2013 were genetically diagnosed. Among those patients, 22 patients had suspected splice site mutations. Transcripts are routinely examined when suspected splice site mutations for abnormal transcripts are detected; 11 of them showed expected exon skipping, but others showed aberrant splicing patterns. The mutation detection strategy had two steps: (*1*) genomic DNA analysis using PCR and direct sequencing and (*2*) mRNA analysis using RT-PCR to detect RNA processing abnormalities.

Results Six splicing consensus site mutations resulting in aberrant splicing patterns, one exonic mutation leading to exon skipping, and four deep intronic mutations producing cryptic splice site activation were identified. Interestingly, one case produced a cryptic splice site with a single nucleotide substitution in the deep intron that led to intronic exonization containing a stop codon; however, the patient showed a clearly milder phenotype for X-linked Alport syndrome in men with a truncating mutation. mRNA extracted from the kidney showed both normal and abnormal transcripts, with the normal transcript resulting in the milder phenotype. This novel mechanism leads to mild clinical characteristics.

Conclusions This report highlights the importance of analyzing transcripts to enhance the mutation detection rate and provides insight into genotype-phenotype correlations. This approach can clarify the cause of atypically mild phenotypes in X-linked Alport syndrome.

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Introduction

Alport syndrome is a hereditary disorder of type IV collagen that progresses to CKD and usually develops into ESRD. Symptoms include sensorineural hearing loss and ocular abnormalities. X-linked Alport syndrome (XLAS) accounts for approximately 85% of patients with Alport syndrome. These patients have mutations in the *COL4A5* gene, which encodes the type IV collagen-α5 [α5(IV)] chain. *COL4A5* mutations result in abnormal α5(IV) expression, with a typically complete absence of α5(IV) in the glomerular basement membrane (GBM) and Bowman's capsule in men and a mosaic expression pattern in women (1).

To date, >500 different *COL4A5* mutations have been identified in patients with XLAS, but mutation detection rates vary widely, ranging between approximately 40%–82% (2–6). The low detection rate is unsatisfactory, because it hampers a deeper understanding of the genotype-phenotype relationship in patients with XLAS. Previous studies have reported that missense and in-frame mutations have milder phenotypes compared with truncating mutations (7–9). However, these studies did not fully consider the effect of splice site mutations on *COL4A5* expression. This study requires careful analysis of *COL4A5* transcripts and assessment of intronic mutations, which have been increasingly identified as a cause of aberrant splicing for various disorders, including Alport syndrome (10–12).

Recently, we reported that 29% of patients with XLAS showed α5(IV) expression on kidney glomeruli, and these patients displayed milder phenotypes (13). All of these patients positive for α5(IV) had nontruncating mutations, including in-frame deletions or in-frame mutations resulting from splice site mutations and exon skipping. This finding suggests that patients with XLAS with in-frame mutations can show a milder phenotype, even when derived from a splice site mutation (13). Therefore, it is important to clarify splice site mutations

as either in-frame or out-of-frame mutations after examining transcripts to estimate the phenotype.

It is well known among clinical nephrologists that some men with XLAS show a relatively mild phenotype, leading to the development of ESRD after the age of 60 years old. The reason for the mild phenotype has been attributed to missense or in-frame mutations or somatic mosaicism (8,9,13,14). One of our patients showed a novel mechanism leading to a mild phenotype revealed by transcript analysis extracted from the kidney. These patients showed both normal and abnormal spliced transcripts, and the normal transcript prevented progression to the typically severe phenotype of XLAS.

In this study, we report on 11 patients showing aberrant splicing of primary transcripts, providing additional insight into genotype-phenotype correlations in XLAS.

Materials and Methods

Ethical Considerations

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

Inclusion Criteria

Clinical and laboratory findings of Japanese patients with XLAS were obtained from their medical records. Patients were referred to our hospital for clinical evaluation or genetic analysis. Most patients were followed in various local hospitals in Japan. DNA and data sheets were sent to our laboratory after acceptance of the request for mutational analysis. We routinely conduct genetic analysis of clinically diagnosed patients with XLAS. When we detect mutations that may possibly affect RNA processing, we routinely analyze transcripts to confirm the mutation-induced splicing abnormalities. When we fail to detect mutations in typical patients with XLAS using DNA-based mutation assays, we conduct transcript analyses as well. All patients with splice site mutations that led to an atypical splicing pattern were included in this study.

One hundred fifty-two patients were genetically diagnosed with XLAS at our laboratory; 22 patients had splice site mutations, and 11 of them showed atypical splicing patterns. These 11 patients were included in this study.

The degree of urinary protein excretion was evaluated using the urinary protein-to-creatinine ratio. eGFR was calculated using the Schwartz equation for patients ≤17 years old (15,16) and GFR-estimating equations for Japanese for patients ≥18 years old (17). Images of kidney α5(IV) staining were sent to us for evaluation of the staining patterns and assessed by the same person (K.N.). eGFR was measured on the basis of the data on data sheets.

Mutational Analyses

Mutational analyses of *COL4A5* were carried out using the following methods. (*1*) PCR and direct sequencing of genomic DNA of all exons and exon-intron boundaries were performed. (*2*) When we detected a suspected splicing site mutation or failed to detect mutations with step 1 analysis, RT-PCR of mRNA and direct sequencing of abnormal mRNA products were carried out.

Genomic DNA was isolated from peripheral blood leukocytes from patients using the Quick Gene Mini 80 System (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's instructions. For genomic DNA analysis, all 51 specific exons of *COL4A5* were amplified by PCR as described previously (5). PCR-amplified products were then purified and subjected to direct sequencing using a Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) with an automatic DNA sequencer (ABI Prism 3130; Perkin Elmer Applied Biosystems, Foster City, CA). Total RNA was extracted from blood leukocytes, kidneys, and/or hair roots. RNA from leukocytes was isolated using a Paxgene Blood RNA Kit (Qiagen Inc., Chatsworth, CA) and then reverse transcribed into cDNA using random hexamers and a Superscript III Kit (Invitrogen, Carlsbad, CA). RNA from kidneys and hair roots was isolated as described previously (14). cDNA was amplified by nested PCR using primer pairs for *COL4A5* as described previously with slight modifications (sequences available on request) (18). PCR-amplified products were purified and subjected to direct sequencing.

Immunohistochemical Analyses

Immunohistochemical analyses were performed using frozen sections of kidney tissue. The immunohistochemical procedure has been described previously (19–21). A mixture of fluorescein isothiocyanate–conjugated rat mAb for the human α5(IV) chain (H53) and Texas red–conjugated rat mAb for the human α2(IV) chain (H25) was purchased from Shigei Medical Research Institute (Okayama, Japan). The epitopes were EAIQP at positions 675–679 of the α2(IV) chain and IDVEF at positions 251–255 of the α5(IV) chain.

Results

Patients' Clinical Features and Pathologic Findings

In total, 11 patients were included in the study. Mutations creating or eliminating the 3′ splice site consensus sequences were detected in six patients (patient IDs 7, 17, 27, 28, 128, and 158), including two patients who had relatively deep intron mutations (patient IDs 128 and 158 with mutation of intervening sequence (IVS)27–18 and IVS29–8, respectively). One patient had a mutation at the last nucleotide of exon 41, altering the 5′ splice site consensus (patient ID 21). These patients proceeded to transcript analysis to determine the influence of the mutations on splicing. Detection of mutations in another four patients failed with genomic DNA–based assays, prompting transcript analyses that detected intronic exonization in patient IDs 19, 48, 126, and 217. All mutations together with patients' clinical, laboratory, and pathologic data are shown in Table 1. All patients showed typical clinical features of XLAS.

Acceptor Splice Site Mutations

Six patients had mutations at 3′ splice sites (Figure 1, A–F). Four of them had mutations in the last intron dinucleotides (patient IDs 7, 17, 27, and 28) that did not result in skipping of complete exonic sequences but produced new 3′ splice sites downstream of authentic sites, creating small deletions in COL4A5 mRNA (19, 1, 1, and 18 bp, respectively) (Figure 1, A–D). One patient had an IVS27–18a>g

Table 1. Clinical and pathologic findings

Patient ID	Sex	Age (yr)	ESRD Age (yr)	Hearing Loss (Detected Age)	sCr (mg/dl)	U-P/Cr (g/g)	eGFR	EM	α5	Family History	Mutation Position	Nucleotide Change	Transcript	Truncating Mutation	Expected Change
Splicing acceptor site mutations															
7	M	25	9	Mild	ESRD	—	—	BWC	Negative	Mother: OB	Intron 10	IVS11−2a>t	19-bp deletion (frame shift)	Yes	Exon 11 skipping (36 bp)
17	W	11	—	—	0.35	1	151	BWC	Mosaic	None	Intron 38	IVS39−1g>a	1-bp deletion (frame shift)	Yes	Exon 39 skipping (99 bp)
27	M	5	—	—	0.21	0.71	155	BWC	Negative	None	Intron 18	IVS18−1g>a	1-bp deletion (frame shift)	Yes	Exon 18 skipping (42 bp)
28	W	6	—	—	0.32	0.2	158	TBM	Mosaic	Sister: OB	Intron 27	IVS28−2a>g	18-bp deletion	No	Exon 28 skipping (98 bp)
128	M	46	—	Mild	1.53	1.07	41	ND	Negative (skin)	Brother: 40 yr ESRD; daughters: OB	Intron 26	IVS27−18a>g	105-bp deletion (exon 27 skipping)	No	—
158	W	14	—	—	0.53	0.35	114	BWC	Mosaic	None	Intron 28	IVS29−8t>a	6-bp insertion	No	Exon 29 skipping (151 bp)
Exonic mutation															
21	M	41	24	Mild	ESRD	—	—	BWC	Negative	Grandfather: 20 yr ESRD; mother: OB	Exon 41	c.3790G>A	186-bp deletion (exon 41 skipping)	No	Missense mutation
Deep intronic mutations															
19	M	22	—	Mild	0.56	0.55	98	BWC	Negative	Mother: pro/OB	Intron 25	IVS25+894c>g	106-bp insertion with stop codon[a]	Yes	—
48	M	6	—	—	0.3	1.87	122	ND	Negative (skin)	Mother: 28 yr ESRD	Intron 47	IVS48−345a>g	74-bp insertion with stop codon	Yes	—
126	M	12	—	Severe	0.79	0.24	96	BWC	Negative	Mother: OB; sister: OB	Intron 10	IVS10+875g>t	123-bp insertion with stop codon	Yes	—
217	M	4	—	—	0.88	0.5	50	BWC	Negative	Mother and sisters: pro/OB	Intron 47	IVS47+1754t>g	84-bp insertion with stop codon	Yes	—

M, man; W, woman; sCr, serum creatinine; U-P/Cr, urinary protein-to-creatinine ratio; EM, electron microscopic findings; BWC, basket-weave change; ND, not determined; OB, occult blood; pro, protein; TBM, thin basement membrane.

[a]RT-PCR results showed both normal and abnormal transcripts only in cDNA extracted from the kidney, and this case showed an extremely mild phenotype for a man with X-linked Alport syndrome with a truncating mutation.

mutation, which resulted in exon 27 skipping (patient ID 128) (Figure 1E). The IVS29–8t>a mutation (patient ID 158) produced a new splice acceptor site, resulting in a 6-bp insertion in the mature transcript (Figure 1F).

Donor Splice Site Mutation

One patient had a single nucleotide substitution at the last position of exon 41 (Figure 1G). Although the mutation is a missense change (p.G1263S), cDNA analysis revealed skipping of exon 41 (186 bp). This finding is consistent with previous studies pointing to a substantial fraction of missense mutations that alter pre-mRNA processing (22).

Deep Intronic Mutations

A failure to detect mutations using genomic DNA analysis in four patients with typical features of XLAS (patient IDs 19, 48, 126, and 217) prompted us to conduct transcript analyses. RT-PCR showed insertions of cryptic exonic sequences in each case (Figure 1, H–K, Supplemental Figure 1). We then conducted genomic DNA analysis of intronic sequences and found point mutations that produced new splice sites (Figure 1, H–K). Interestingly, patient ID 19 showed a milder phenotype than a typical patient with XLAS with a truncating mutation. This patient had both normal and abnormal transcripts of the kidney that led to his phenotype being milder than that usually seen with a man with XLAS with a truncating mutation. Patient ID 217 had a mutation at position +5 of the new exon (Figure 1K). All cryptic exons had stop codons in them and thus, turned out to be truncating mutations (Supplemental Figure 1). From these results, we could group these four patients as having truncating mutations.

Discussion

This study provides the first case series report on aberrant splice site mutations. We identified six splice consensus sequence mutations, including two relatively deeper intronic nucleotide substitutions, one exonic mutation, and four deep intron mutations.

It is well established that genotype-phenotype shows a strong correlation in XLAS (7–9). Jais *et al.* (9) reported that large deletions and nonsense mutations confer a 90% probability of ESRD by the age of 30 years old compared with a 70% risk with splice site mutations and a 50% risk with missense mutations. Gross *et al.* (8) grouped men with XLAS into three groups as follows. (*1*) Large rearrangements, frame shift, nonsense, and splice donor site mutations had a mean ESRD age of 19.8±5.7 years. (*2*) Nonglycine or 3′ glycine missense mutations, in-frame deletions/insertions, and splice acceptor site mutations had a mean ESRD age of 25.7±7.2 years, and (*3*) 5′ glycine substitutions had an even later onset of ESRD at a mean of 30.1±7.2 years (8). Recently, Bekheirnia *et al.* (7) reported the average onset of ESRD as 37 years old for those with missense mutations, 28 years old for those with splice site mutations, and 25 years old for those with truncating mutations. Although these reports very clearly show genotype-phenotype correlations, all studies have grouped splice site mutations together without considering their diverse consequences for collagen transcripts.

Recently, we reported that 29% of men with XLAS were positive for α5(IV) on the GBM and showed significantly milder phenotypes, including milder proteinuria, later onset of ESRD, and less occurrence of hearing loss (13). All of the α5(IV)-positive group had nontruncating mutations, including three deletion mutations (9, 36, and 384 bp) and one splice site mutation, which led to exon 9 (81 bp) skipping. From these results, it was suggested that in-frame mutations could show a milder phenotype, even if derived from a splice site mutation (13). One patient (patient ID 128) in this study had a 105-bp deletion (full exon 27 skipping) and showed an extremely mild phenotype for a man with XLAS. He is now 46 years old and has not developed ESRD (eGFR=41 ml/min per 1.73 m^2).

One man (patient ID 19) showed an atypically mild phenotype of slight proteinuria with normal renal function at the age of 22 years old. The intron mutation in this patient created a cryptic exon that included a stop codon, creating a truncating mutation. Patients with truncating mutations usually show a severe disease course. Bekhernia *et al.* (7) reported that patients with truncating mutations develop to ESRD at an average age of 25 years old, and Gross *et al.* (8) reported that patients with nonsense or other large mutations develop to ESRD at an average age of 19.8 years old. To clarify the reason why our patient showed a milder phenotype while possessing a truncating mutation, we conducted additional transcript analysis using cDNA extracted from the kidney and hair roots. Interestingly, cDNA from the kidney showed both a larger-sized band and a normal-sized band on electrophoresis and confirmed the presence of normal transcripts lacking the cryptic exon. This finding suggests that this patient's intronic exonization does not occur completely in the kidney and that some transcript escaped from producing the cryptic exon. This result could explain why this patient shows a milder phenotype of XLAS.

Patient ID 217 had a mutation at IVS cryptic exon position +5T>G that produced a cryptic splice donor site. The IVS+5G position is susceptible to aberrant splice site activation, and this point mutation of G to another nucleotide always changes the splice site, usually showing exon skipping (23). However, this patient created a cryptic splice site with the IVS+5T>G mutation and produced the cryptic splice site. This kind of mutation has been rarely reported (23).

In a recently published study, we reported that some men with XLAS were positive for α5(IV) at the GBM and that all were diagnosed with XLAS using a genetic approach (13). However, four patients in this study who showed no mutations using standard genome DNA direct sequencing analysis (patients ID 19, 48, 126, and 217) had abnormal α5(IV) expression, and we proceeded to transcript analysis and detected aberrant splicing in a deep intron. From these results, it is suggested that the genetic approach and immunohistochemical analysis provide some clues as to diagnosis of XLAS for patients with atypical α5(IV) expression and patients with an absence of any mutation by standard direct sequencing, respectively.

A recent case study reported a high sensitivity for detecting somatic mosaic mutations in the *COL4A5* gene using next-generation sequencing (24). High-throughput sequencing technologies may not only lower the cost of DNA

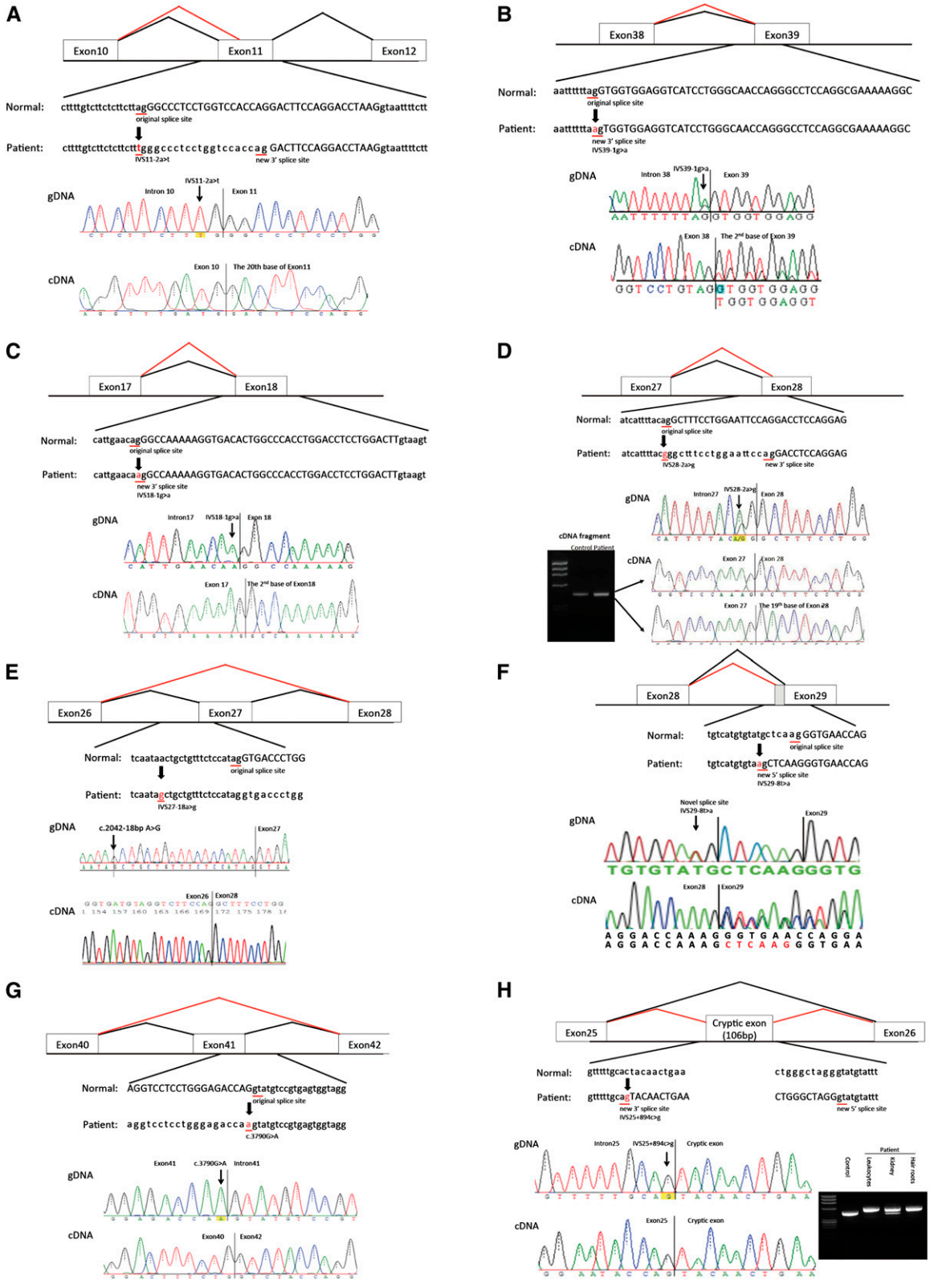

Figure 1. | **Mutations and their consequences.** Upper panels show schematics of aberrant splicing (red lines). Normal splicing is indicated by black lines. The original and new splice sites and flanking sequences are shown below. A patient's flanking genomic DNA and cDNA sequences are shown in lower panels. (A) Patient ID 7. IVS11–2A>T eliminated the splice acceptor site of intron 10 to activate a new splice site 19 nucleotides upstream. (B) Patient ID 17. IVS39–1G>A eliminated the splice acceptor site of intron 38, producing a transcript with a 1-bp deletion. (C) Patient ID 27. IVS18–1G>A removed the splice acceptor site of intron 17, generating a transcript with a 1-bp deletion. (D) Patient ID 28. IVS28–2A>G altered the splice acceptor site of intron 27, producing an 18-bp deletion in mature transcripts. (E) Patient ID 128. IVS27–18A>G potentially

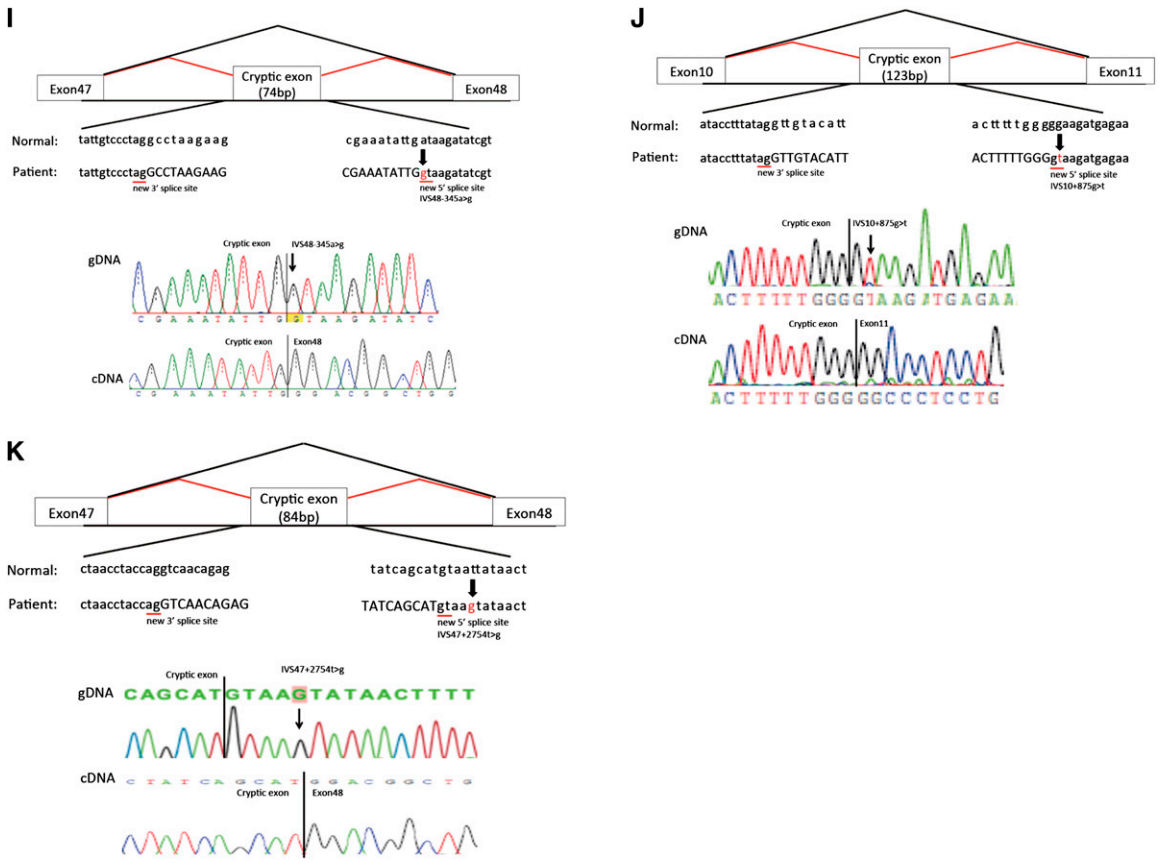

Figure 1. *(Continued)* | disrupted the splice acceptor site of intron 26, resulting in exon 27 skipping, which creates a transcript 105-bp deletion. (F) Patient ID 158. IVS29–8T>A changed the splice acceptor site of intron 28 to IVS29–7, which creates a transcript 6-bp insertion. (G) Patient ID 21. The last nucleotide of exon 41 mutation, C3790G>A, disrupted the splice donor site of intron 41, resulting in exon 41 skipping, which creates a transcript with a 186-bp deletion. (H) Patient ID 19. IVS25+894C>G produced a new splice acceptor site, resulting in a cryptic exon activation between exons 25 and 26 and creating a transcript with a 106-bp insertion that contains a stop codon. Lower right panel shows RT-PCR products from leukocytes, kidneys, and hair roots. Only the transcript from the kidney shows a normal-sized band, meaning that the kidney produced both abnormal and normal transcripts without a cryptic exon. (I) Patient ID 48. IVS48–345A>G made a new splice donor site, resulting in the production of a cryptic exon between exons 47 and 48, which creates a transcript with a 74-bp insertion that contains a stop codon. (J) Patient ID 126. IVS10+875G>T made a new splicing donor site, resulting in the production of a cryptic exon between exons 10 and 11 and creating a transcript with a 123-bp insertion that contains a stop codon. (K) Patient ID 217. IVS47+2754T>G made a new splicing donor site, resulting in the production of a cryptic exon between exons 47 and 48, which creates a transcript 84-bp insertion that contains a stop codon. IVS, intervening sequence.

sequencing but also help identify those low-percentage somatic mosaic mutations, which may help increase the mutation detection rates for XLAS as well as transcript analysis.

The results of this study may have implications in future therapeutic trials of XLAS. In muscular dystrophies, clinical trials using antisense oligonucleotides to induce exon skipping of specific mutations or drugs developed to allow read through of nonsense mutations are ongoing and have the aim of changing truncating mutations into nontruncating mutations (25). Similar therapeutic strategies may be available for patients with genetically diagnosed XLAS in the future, and accurate descriptions of the transcriptomic consequences of somatic or germ-line DNA changes in relevant tissues should be a prerequisite for inclusion in any clinical trials.

In conclusion, we report 10 intronic mutations and one exonic mutation that produce aberrant splicing, including four deep intronic mutations that produced cryptic exons. One patient showed a milder phenotype, although he had a truncating mutation because of different splicing patterns in different tissues. With transcript analysis, we can determine splice site mutations as either truncating or nontruncating mutations. This study provides valuable data, which will help with future analysis of genotype-phenotype correlations, because only transcript analysis makes it possible to divide splice site mutations into two groups of either truncating or nontruncating mutations. This report also illustrates that the low mutation detection rate in XLAS can be improved by thorough transcript analysis and highlights the importance of distinct tissue-specific exon inclusion levels that may explain the severity of genetic diseases in general.

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ORIGINAL ARTICLE

Beta-2 microglobulin-based equation for estimating glomerular filtration rates in Japanese children and adolescents

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Abstract
Background Although the creatinine (Cr)-based equation is widely used for estimating glomerular filtration rate (GFR), this equation is not ideally suited for children with low body weight or aged <2 years. Therefore, we established a new equation using serum beta-2 microglobulin (β2MG) levels for Japanese children with chronic kidney disease (CKD).
Methods Inulin clearance and standardized serum β2MG and Cr levels were measured in 137 CKD patients aged 1 month–18 years. Using the previously established normal β2MG levels, Cr reference values, and Cr-based equation of estimated GFR (eGFR) in Japanese children, receiver operating characteristics (ROC) analyses were performed to compare the diagnostic accuracy between β2MG- and Cr-based estimations of GFR.
Results Serum β2MG concentrations progressively increased as GFRs reduced. The correlation coefficients between GFR and β2MG, and between GFR and 1/β2MG were -0.74 ($p < 0.001$) and 0.86 ($p < 0.001$), respectively. The inulin clearance, as based on 1/serum β2MG expression, in pediatric CKD patients resulted in the equation: inulin GFR (mL/min/1.73 m^2) = 149.0 × 1/serum β2MG (mg/L) +9.15. ROC analyses indicated that the ability of serum β2MG-based GFR <95 mL/min/1.73 m^2 in children >2 years was better than the Cr-based estimated GFR (areas under the ROC curve 0.960 vs. 0.948, respectively).
Conclusion The new β2MG-based eGFR formula is useful for clinical screening of renal function in Japanese children and adolescents, and measurement of serum β2MG and Cr levels as markers for predicting glomerular function may aid the early detection of mildly reduced GFR in this population.

Keywords Beta-2 microglobulin-based equation · Children · Chronic kidney disease · Estimated glomerular filtration rate · Kidney function

Introduction

Serum creatinine (Cr) is currently the most widely used marker for predicting glomerular filtration rate (GFR) [1], and inulin clearance is the gold standard for evaluation of GFR. Based on this gold standard method, we recently established a serum Cr-based estimated GFR (eGFR) equation for use in children aged 2–11 years as follows: eGFR (mL/min/1.73 m^2) = 0.35 × body length (cm)/serum Cr level (mg/dL) [2], as well as complex eGFR equations using polynomial formulas for reference serum Cr levels according to body length in Japanese children and adolescents aged 2–18 years [3]. However, in children, serum creatinine levels vary according to age, gender, and muscle mass; and estimating the GFR with Cr in pediatric patients is not always accurate, and serum Cr-based formulas cannot generally be used in children <2 years, or in those with severe muscle loss [4, 5].

Beta-2 microglobulin (β2MG) serum concentrations correlate with GFR, and β2MG levels have been shown to

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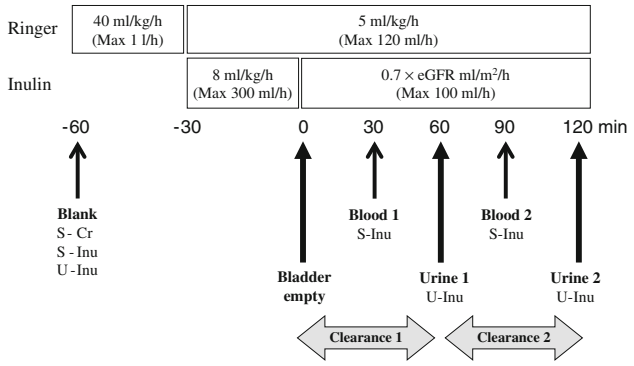

Fig. 1 Inulin clearance method standardized according to the Committee of Measures for Pediatric Chronic Kidney Disease. Inulin was administered intravenously to achieve extracellular fluid levels of 20 mg/dL upon testing. For this purpose, the rates of inulin infusion must equal the rates of loss in the urine, which was calculated using the Schwartz formula based on the serum creatinine levels. The estimated glomerular filtration rate was calculated using the previous Schwartz formula, and the body surface area was calculated using the Haycock method. *S–Cr* serum creatinine concentration, *S-Inu* serum inulin concentration, *U-Inu* urinary inulin concentration

be age independent in children, including infants [6]. Previously, we have reported on the normal reference values of creatinine and β2MG in Japanese children aged 0–17 years, and we demonstrated that muscle-mass independency of β2MG is an essential advantage compared with the conventionally used serum creatinine values [7]. Therefore, β2MG has been advocated as a better predictor of GFR than Cr [6]. In the current study, we present a novel serum β2MG-based eGFR equation for use in all children and adolescents, including infants, in Japan.

Materials and methods

Study population

A total of 174 children (113 male and 61 female; age 1 month–18 years) with chronic kidney disease (CKD) presenting at the facilities of the members of the Committee of Measures for Pediatric Chronic Kidney Disease between 2008 and 2011 were included in this study. The exclusion criteria included severe obstructive uropathy; infection during treatment; inflammatory disease; dehydration; severe cardiac, hepatic, or pancreatic disease; pregnancy or the possibility of pregnancy; nursing; and refusal or inability to give informed consent. Thirty-seven patients were excluded from the analysis, and 137 cases (89 male and 48 female) were assessed to establish the β2MG-based eGFR. The study was approved by the local ethics boards of each institution (ethics committee approval numbers: Niigata University Medical and Dental Hospital, 1878 and Aichi Children's Health and Medical Center, 200810), and written informed consent was obtained from the parents of each child.

GFR and serum β2MG measurements

GFR was measured using inulin levels [8, 9] and was adjusted to the body surface area standardized to 1.73 m^2. The inulin clearance (Cin) was measured from samples taken twice over 2 h under fasting and hydrated conditions by the continuous infusion method, as previously described (Fig. 1) [2, 3]. Since urine collection may be insufficient in children, a higher value of Cin was used for assessment in this study.

Serum β2MG and Cr levels were measured at the same time, and the GFR was estimated from serum Cr by the Schwartz formula to calculate inulin loss [4, 10–12]. The dose of inulin load was calculated by the Haycock method using the equation: 0.7 × eGFR mL/m^2/h [13].

The serum samples were stored at −70 °C at SRL Inc. (Tokyo, Japan) until further measurements were performed. The serum concentrations of β2MG and Cr were determined using latex agglutination immunoassay and enzymatically, respectively, using a Bio Majesty automated analyzer (JCA-BM8060; JEOL Ltd, Tokyo, Japan).

In this study, the dose of inulin was determined on the premise that the blood concentration was constant during the test. Therefore, cases in which the ratios of urine inulin excretion and intravenous inulin administration were <0.5 or >1.5 were excluded from this study, since this may indicate failure to collect all urine. Further, cases with GFR >150 mL/min/1.73 m^2 were excluded to increase the reliability of determining cases with GFR <120 mL/min/1.73 m^2.

The equation for eGFR was determined by univariate linear regression using measured GFR as the dependent variable and 1/β2MG as the independent variable.

We recently established the following polynomial formulas which were used to calculate the reference serum Cr levels in children aged 2–18 years:

For boys: $y = -1.259x^5 + 7.815x^4 - 18.57x^3 + 21.39x^2 - 11.71x + 2.628$

For girls: $y = -4.536x^5 + 27.16x^4 - 63.47x^3 + 72.43x^2 - 40.06x + 8.778$,

where y is the reference serum creatinine (mg/dL) and x is body length (m). The equation: eGFR = 110.2 × (reference Cr (y)/patient's serum Cr) + 2.93 was used to estimate their kidney dysfunction [3, 14].

Statistical analyses

All statistical analyses were performed with the GraphPad Prism software package (Ver. 5.0; GraphPad Software, San Diego, CA, USA), SigmaPlot (Ver. 12; Hulyncs Inc., Tokyo, Japan), and the JMP 9 statistical software package (SAS Institute Inc., Cary, NC, USA). Linear regression analyses

Table 1 Characteristics of patients

	Median (IQR)	*N* (%)
Number of patients		137
Gender		
Male		89 (65.0)
Female		48 (35.0)
Age (years)	10.6 (6.9–13.8)	137
<12 years	7.8 (5.8–9.7)	81 (59.1)
≥12 years	14.6 (13.4–16.4)	56 (40.9)
Height (cm)	133.4 (111.0–152.1)	137
<12 years	113.4 (105.0–127.9)	81 (59.1)
≥12 years	153.4 (145.2–163.3)	56 (40.9)
Weight (kg)	29.2 (18.8–41.6)	137
<12 years	19.9 (16.2–26.6)	81 (59.1)
≥12 years	46.3 (37.7–50.7)	56 (40.9)
Body surface area (m^2)	1.04 (0.76–1.30)	137
<12 years	0.80 (0.68–0.97)	81 (59.1)
≥12 years	1.41 (1.23–1.51)	56 (40.9)
Serum creatinine (mg/dL)	0.63 (0.50–0.87)	137
<12 years	0.56 (0.42–0.72)	81 (59.1)
≥12 years	0.78 (0.62–1.06)	56 (40.9)
Serum β2MG (mg/dL)	2.4 (1.7–3.1)	137
<12 years	2.4 (1.8–3.1)	81 (59.1)
≥12 years	2.4 (1.6–3.1)	56 (40.9)
Maximum inulin GFR (mL/min/1.73 m^2)	71.8 (52.9–97.2)	137
<12 years	70.3 (52.3–99.9)	81 (59.1)
≥12 years	76.4 (53.4–93.9)	56 (40.9)

IQR interquartile range

were performed to evaluate the relationships between the ratios of 1/serum β2MG and Cin. The significance of the differences between the correlations was analyzed using Fisher's *z* transformation statistics. The Mann–Whitney test was used to compare the differences between the two age groups.

The diagnostic value of β2MG and Cr for identifying GFR of <95 mL/min/1.73 m^2 in comparison to the Cin was evaluated by receiver operating characteristic (ROC) analysis using previously established normal reference values from healthy subjects as controls [7, 15]. The area under the curve and the sensitivity/specificity data at certain cutoffs were calculated. For all analyses, $p < 0.05$ was defined as statistically significant.

Results

Characteristics of the study population

Of the 174 children studied, 32 cases with ratios of urine inulin excretion and intravenous inulin administration <0.5 or >1.5, and 5 cases with GFR >150 mL/min/1.73 m^2 were excluded from this study. Therefore, a total of 137 cases (89 male and 48 female) were included in our analysis (Table 1). Among these, 41.6 % were diagnosed with congenital anomalies of the kidney and urinary tract (Table 2), and two patients with nephrotic syndrome had severe muscle loss due to high-dose steroid treatment.

Table 2 Primary diseases of patients

Diagnosis	*N* (%)
Congenital anomalies of the kidney disease (CAKUT)	57 (41.6)
Reflux nephropathy	16 (11.8)
Idiopathic nephrotic syndrome	15 (10.9)
Post-renal transplantation	7 (5.1)
Chronic glomerulonephritis	5 (3.6)
Nephronophthisis	5 (3.6)
Neurogenic bladder	4 (2.9)
Polycystic kidney disease	3 (2.2)
Alport's syndrome	3 (2.2)
Miscellaneous	22 (16.1)

The median values of serum Cr, β2MG, and maximum inulin GFR were 0.63, 2.4 mg/dL, and 71.8 mL/min/1.73 m^2, respectively.

Correlation of serum β2MG and inulin GFR

Serum concentrations of β2MG progressively increased as kidney function reduced (inulin GFR) (Fig. 2). A correlation between β2MG concentration and inulin GFR was noted, thus allowing nonlinear regression lines (exponential one phase decay) to be drawn. Moreover, a significant correlation between inulin GFR and the inverse of serum β2MG was observed, with a linear correlation coefficient of $r = 0.86$ ($p < 0.0001$); which resulted in the following equation:

Inulin GFR = 149.0 × 1/serum β2MG (mg/L) +9.15.

The 95 % confidence intervals for the slope and intercept were 133.9–164.0 and 1.742–16.56, respectively.

The performance of the β2MG-based eGFR formula was analyzed in two age groups (<12 and 12–18 years) and by gender (Fig. 3; Table 3). Bias was characterized as the absolute value of measured GFR by the inulin clearance method minus eGFR calculated using the β2MG-based formula, and is reported as mean ± standard deviation. The root mean square error was calculated to show the differences between β2MG-based eGFR and actual measured GFR values (Fig. 3). The correlation efficient was slightly higher in patients aged <12 years, although there was no statistical difference ($p = 0.50$). Moreover, there was no statistical difference in the bias ($p = 0.65$) or in the correlation coefficient between male and female children ($p = 0.13$).

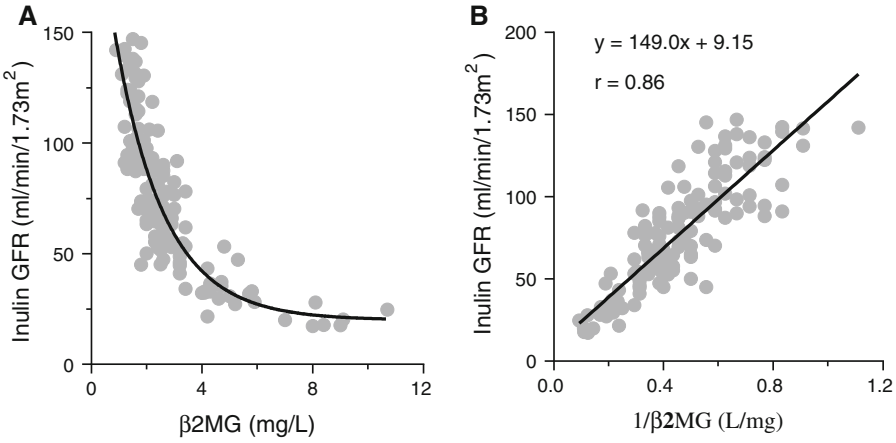

Fig. 2 Relationship between the inulin glomerular filtration rate (GFR) and serum beta-2 microglobulin (β2MG) concentration (**a**), and 1/β2MG (**b**) in children with chronic kidney disease

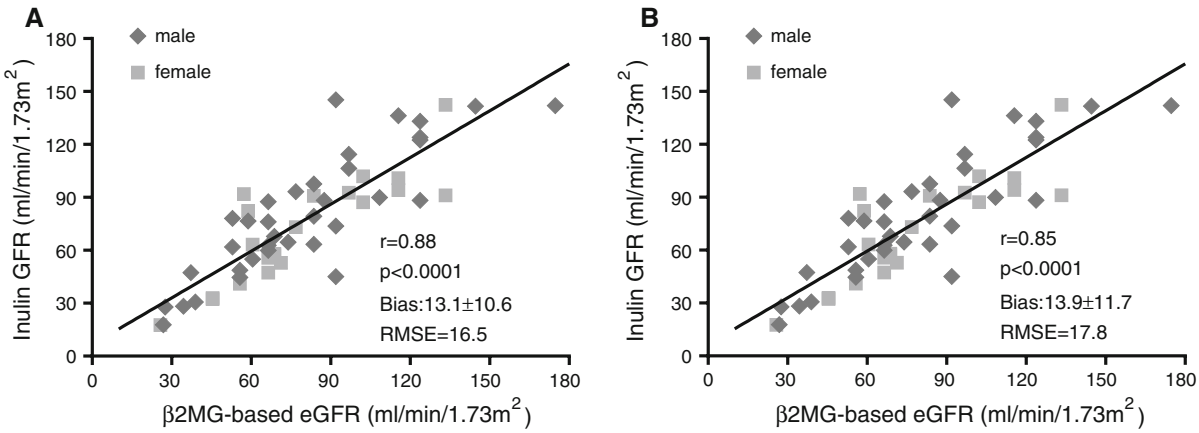

Fig. 3 Relationship between serum beta-2 microglobulin (β2MG)-based estimated glomerular filtration rate (eGFR) and inulin GFR in children with chronic kidney disease aged <12 years (**a**) and 12–18 years (**b**). Male patients are denoted by rhombuses and female patients by squares. *RMSE* root mean square error

Table 3 Performance of the GFR-estimating β2MG-based equation

	All ($n = 137$)	Aged <12 years ($n = 81$)	Aged ≥12 years ($n = 56$)
Bias (mL/min/1.73 m^2)	13.4 ± 11.0	13.1 ± 10.6	13.9 ± 11.7
Correlation coefficient (95 % CI)	0.86 (0.81–0.90)	0.88 (0.82–0.92)	0.85 (0.76–0.91)
RMSE (mL/min/1.73 m^2)	17.5	16.5	17.8
	Male ($n = 89$)	Female ($n = 48$)	
Bias (mL/min/1.73 m^2)	14.0 ± 11.9	12.4 ± 9.2	
Correlation coefficient (95 % CI)	0.84 (0.76–0.89)	0.90 (0.83–0.94)	
RMSE (mL/min/1.73 m^2)	18.4	15.3	

95 % CI 95 % confidence interval, *RMSE* root mean square error

Comparison of diagnostic performance between β2MG-based and Cr-based eGFR formulas

To evaluate the diagnostic accuracy of the β2MG-based and Cr-based formulas in estimating GFR, ROC analysis was performed on the data from the patients aged 2–18 years with Cin <95 mL/min/1.73 m^2 and healthy subjects (≥2 years) previously assessed for establishing reference values for β2MG and Cr levels in normal Japanese children [7]. The ROC plots are summarized in Fig. 4. The ROC area under the curve was slightly better for the β2MG-based formula [0.961; 95 % confidence interval (CI) 0.940–0.982] than that of the Cr-based formula (0.948; 95 % CI 0.916–0.980), although there was no statistical difference. However, when we excluded the two patients with severe muscle loss, the value increased to 0.968 (95 % CI 0.951–0.985) for the Cr-based formula, while that for the β2MG-based formula did not change substantially (0.964; 95 % CI 0.944–0.984).

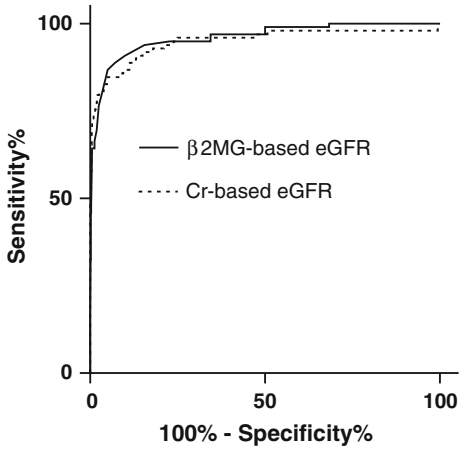

Fig. 4 Receiver–operating curves (ROC) of the serum beta-2 microglobulin (β2MG)-based and creatinine (Cr)-based formulas to diagnose inulin glomerular filtration rate (GFR) <95 mL/min/1.73 m^2

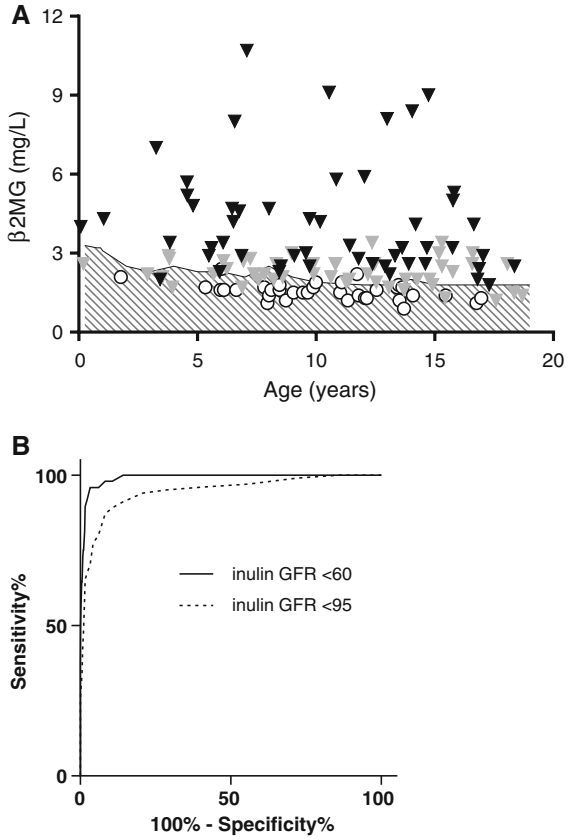

Fig. 5 Serum beta-2 microglobulin (β2MG) and age distribution of subjects (**a**), and receiver–operating curves (ROC) of the β2MG reference value to detect patients with inulin GFR <95 or <60 mL/min/1.73 m^2 (**b**). The *shaded area* and the *solid line* in **a** represent the age-specific reference range (the 2.5–97.5th percentile) for β2MG. *Open circle* indicates patients with glomerular filtration rate (GFR) ≥95 mL/min/1.73 m^2, *filled down-pointing triangle* patients with inulin GFR <95 mL/min/1.73 m^2, and *filled down-pointing triangle* patients with inulin GFR <60 mL/min/1.73 m^2

Table 4 Diagnostic efficiency values for β2MG to detect mild renal dysfunction

	GFR <95 mL/min/1.73 m^2	GFR <60 mL/min/1.73 m^2
Cutoff value	1.95 mg/L	2.25 mg/L
Sensitivity (95 % CI)	87.1 (79.0–93.0) %	95.8 (85.8–99.5) %
Specificity (95 % CI)	91.7 (89.9–93.2) %	96.7 (95.5–97.7) %
Positive likelihood ratio	10.5	29.3

95 % CI 95 % confidence interval

Assessment of the β2MG reference value in children with renal disorders

Using the previously established reference value for β2MG as the normal control [7], the accuracy of the reference range of serum β2MG was tested by plotting β2MG values over the normal range area (below the 97.5th percentile), and in comparison to the inulin clearance by ROC analysis. The values plotted in the normal range area detected 82.3 % patients with inulin GFR <95 mL/min/1.73 m^2 and 95.9 % patients with inulin GFR <60 mL/min/1.73 m^2 (Fig. 5a). The area under the curve was 0.946 (95 % CI 0.919–0.972) for patients with inulin GFR <95 mL/min/1.73 m^2, and increased to 0.991 (95 % CI 0.985–0.998) for patients with inulin GFR <60 mL/min/1.73 m^2 (Fig. 5b).

The diagnostic efficacies of β2MG to detect kidney dysfunction are summarized in Table 4. The cutoff level with the best sensitivity and specificity was chosen. To detect kidney dysfunction (<95 mL/min/1.73 m^2), the cutoff value was determined to be 1.95 mg/L, with 87.1 % sensitivity and 91.7 % specificity; to detect moderate kidney dysfunction (<60 mL/min/1.73 m^2), the optimal cutoff was 2.25 mg/L, with 95.8 % sensitivity and 98.3 % specificity.

Discussion

In the present study, we analyzed the linear regression coefficients between serum β2MG concentration and inulin clearance in children and adolescents with CKD. As a result, we established a new β2MG-based formula for estimating GFR: eGFR = 149.0 × 1/serum β2MG (mg/L) + 9.153 with correlation coefficient ($r = 0.86$, $p < 0.0001$). To our knowledge, although there are previous reports on equations of β2MG-based estimates of GFR for adults [16–18], this is the first study to establish an equation that estimates GFR based on β2MG and Cin measurement in children.

β2MG is a subunit of the major histocompatibility class I molecule produced by all nucleated cells [19]. Although

numerous studies have observed a very high correlation between plasma β2MG levels and GFR and β2MG is increasingly considered useful for estimating GFR and predicting prognosis in kidney disease, cardiovascular outcomes, and death independently of GFR [16–18, 20–26] thus far, plasma β2MG has received little attention as a marker of GFR. Cin is the usual gold standard for evaluating GFR; however, technical difficulties, particularly for children, prevent its routine use, and may therefore prevent studies establishing equations estimating GFR using serum concentration of β2MG in children. From this point of view, the current study, in which we established a β2MG-based eGFR equation using the gold standard method in children, is of great significance.

Furthermore, we also evaluated the performance of our β2MG-based formula for estimating GFR in children with CKD compared to the performance of the widely used Cr-based formula. Our data from the ROC analyses suggest that the β2MG-based formula is not inferior for detecting impaired kidney function compared to the Cr-based formula. Moreover, a great advantage of the β2MG-based formula is that it can be used in children under 2 years old, and in patients with severe muscle loss and/or suffering from malnutrition.

Our data showed that there was no statistical difference for the correlation efficient between patients aged <12 years old and 12–18 years. The ROC areas analyzed using β2MG- or Cr-based formulas were 0.957 and 0.948, respectively, in children with CKD, including two patients with severe muscle loss. When these two cases were excluded, the ROC value obtained by the Cr-based formula increased to 0.968. Thus, although Cr is the most widely used marker for detecting kidney dysfunction and for estimating GFR, the use of the Cr-based formula is limited for patients with low muscle mass.

We recently developed Cr-based eGFR equations for use in Japanese children [2, 3]. However, these equations must be adjusted according to body length, and cannot be used in children <2 years since GFR varies to some extent among children, and since it generally increases from approximately 30 % of the level in adults during the first 2 years after birth. Conversely, serum β2MG concentrations do not require adjustments for body length, are not complicated by muscle mass, and can estimate GFR as a single-sample measurement. Thus, we believe that serum β2MG measurement is a more attractive marker for estimating GFR in children with CKD, particularly for those with severe malnutrition due to uremia or those treated with high-dose glucocorticoid therapy.

Although β2MG levels are increased in patients with several malignancies, infectious diseases, and lymphoproliferative disorders [27–32], in general, which is a disadvantage, serum β2MG concentrations are primarily determined by GFR. Thus, β2MG is an ideal marker, as it is produced at a constant rate and freely filtered by the glomerulus before being reabsorbed and almost completely metabolized by the proximal tubule with no appreciable entry into the circulation [33].

Serum cystatin C (CysC) is another molecule that also offers potential advantages over Cr because of its age, gender, and muscle-mass independence [23, 24, 34], and we have recently established a CysC-based formula for estimating GFR [35]. However, the concentration of CysC is influenced by high-dose glucocorticoid therapy, thyroid function, diabetes mellitus, systolic blood pressure, smoking, hypoalbuminemia, and serum concentrations of other analytes independent of GFR [36–41]. Moreover, measurement of CysC is expensive, and in Japan, the cost of serum CysC measurement is covered by insurance once in three months.

In contrast, there is currently no such limitation on the measurement of serum β2MG concentration. Moreover, we have recently conducted a multicenter study to establish the reference value for serum β2MG in Japanese children [7]. In this study, we found a weak but significant correlation between β2MG concentration and age, which was expressed by the following simple formula: β2MG (mg/L) $= -0.0341 \times$ age (years) $+ 1.72$, with a regression coefficient of $r = -0.47$. Therefore, it can be argued that the independent relation of β2MG with age and body mass, which is one of the advantages for its use as a marker, is not applicable in studies on children. However, the slope of the regression line for β2MG with age is gradual and quickly reaches a plateau. Moreover, β2MG and age are negatively correlated, and therefore, elevations in β2MG concentrations relative to age can be easily detected. Indeed, in the current study, the ROC analyses testing the clinical validity of our established β2MG reference in children with CKD revealed that β2MG was a highly sensitive marker for detecting kidney dysfunction, as determined by GFR <95 or <60 mL/min/1.73 m^2.

The limitations of our study include the small size of the study population, particularly few subjects of <2 years of age. Therefore, we were unable to validate our β2MG-based formula by comparing it to the Cr-based formula in patients younger than 2 years. Therefore, further studies validating our new β2MG-based formula are warranted.

In summary, this study showed that our novel β2MG-based estimation had high diagnostic accuracy, similar to that of the serum Cr-based formula for identification of impaired GFR in children. The main advantage of the β2MG-based formula for estimation of GFR is that it can be used in patients with severe muscle loss.

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Conflict of interest The authors have declared that no conflict of interest exists.

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IDENTIFICATION OF A HYPOURICEMIA PATIENT WITH SLC2A9 R380W, A PATHOGENIC MUTATION FOR RENAL HYPOURICEMIA TYPE 2

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□ *Hypouricemia is characterized by low serum uric acid (SUA) levels (≤3.0 mg/dL) with complications such as urolithiasis and exercise-induced acute renal failure. We have previously reported that urate transporter 1 (*URAT1/SLC22A12*) and glucose transporter 9 (*GLUT9/SLC2A9*) are causative genes for renal hypouricemia type 1 (RHUC1) and renal hypouricemia type 2 (RHUC2), respectively. In the series of experiments, two families have been revealed to have RHUC2 due to* GLUT9 *missense mutations R198C or R380W, respectively. Thus far, however, no studies have reported other RHUC2 families or patients with these pathogenic mutations. This study is aimed to find other cases of RHUC2.*

We performed mutational analyses of GLUT9 *exon 6 (for R198C) and exon 10 (for R380W) in 50 Japanese hypouricemia patients. Patients were analyzed out of a collection of more than 2000 samples from the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study).*

We identified a novel male patient with heterogeneous RHUC2 mutation R380W. The SUA of this hypouricemia patient was 2.6 mg/dL, which is similar to that of our previous report (SUA: 2.7 mg/dL).

This is the second report indicating RHUC2 patient due to GLUT9 mutation R380W. This mutation occurs in highly conserved amino acid motifs and is reported to be an important membrane topology determinant. R380W is a dysfunctional mutation which completely diminishes the urate

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transport activities of GLUT9. Our study revealed a second hypouricemia patient with GLUT9 *R380W, a pathogenic mutation of RHUC2, which may help to expand our understanding of RHUC pathogenesis.*

Keywords: SLC transporters; GLUT family; GLUT9L; GLUT9S; renal urate reabsorption

INTRODUCTION

Renal hypouricemia is characterized by low serum uric acid (SUA) levels (≤3.0 mg/dL), and confers risk of severe complications such as exercise-induced acute renal failure or nephrolithiasis.[1, 2] Renal hypouricemia is mainly caused by impaired renal urate reabsorption. We previously reported that URAT1/SLC22A12[3] and GLUT9/SLC2A9[4] are key regulators of SUA, and play an essential role in urate reabsorption in the human kidney. The dysfunctional mutations of *URAT1* or *GLUT9* cause renal urate hypouricemia, called renal hypouricemia type 1 (RHUC1) and renal urate hypouricemia type 2 (RHUC2), respectively. [5] Previously, two families have been revealed to have RHUC2 due to *GLUT9* missense mutations R198C or R380W, respectively. Thus far, however, no studies have reported other RHUC2 families or patients with these pathogenic mutations. Here, we report another hypouricemia patient with the pathogenic RHUC2 mutation.

MATERIALS AND METHODS

For the hypouricemia patients, 50 Japanese patients with lower SUA (≤3.0 mg/dl) were identified out of more than 2000 samples from the Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study). We performed mutational analysis of *GLUT9* exon 6 (R198C) and exon 10 (R380W) in these 50 hypouricemia patients.

For the *GLUT9* sequence determination, we used following primers described previously:[4] for exon 6, forward 5′-GTCCTCTGAAATGCACCTCC-3′, and reverse 5′-GCACAGAAGATGCCTAAACAAACACA-3′; for exon 10, forward 5′-GGTGACCATATCCATCCAG-3′, and reverse 5′-GAAGGAG-CACCTTAAGGTTG-3′. High molecular weight genomic DNA was extracted from peripheral whole blood cells,[6] and was amplified by PCR. The PCR products were sequenced in both directions using a 3130xl Genetic Analyzer (Applied Biosystems).[7]

RESULTS

The human *GLUT9* gene consists of 14 exons (1 noncoding and 13 coding) and the alternative splicing of the *GLUT9* gene results in two main transcripts: GLUT9 isoform 1 (long isoform, GLUT9L) and isoform 2 (short isoform, GLUT9S). Two heterozygous missense mutations of R380W and

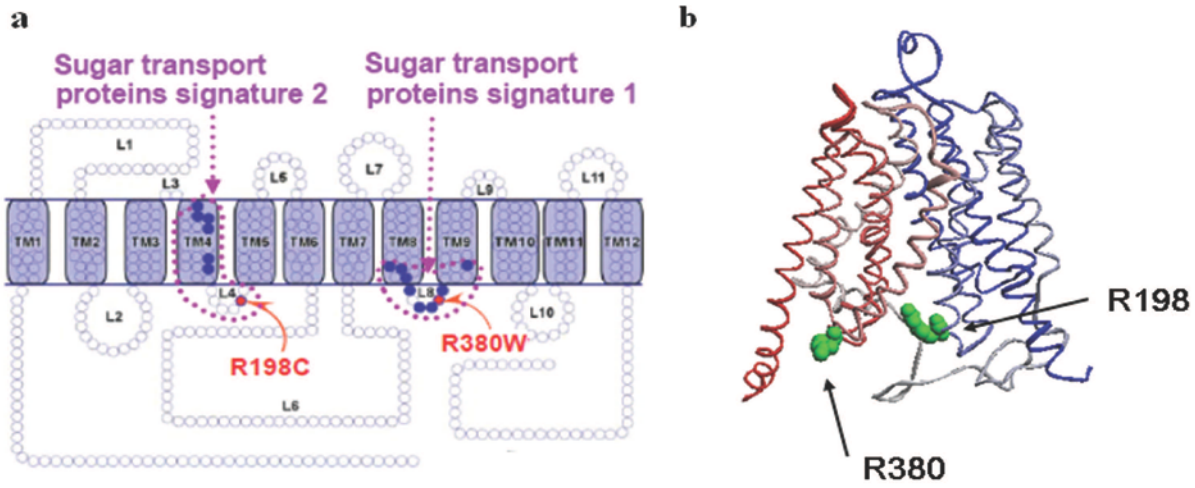

FIGURE 1 Pathogenic mutation sites of GLUT9 (Color figure available online).

R198C for GLUT9L have been identified in Japanese patients with renal hypouricemia. Both mutations are missense mutations from basic amino acid arginine to neutral amino acids, and are at equivalent positions within the cytoplasmic loops, which cause a loss of positive charge. These pathogenic mutation sites in two-dimensional and three-dimensional models are shown in Figure 1. No hypouricemia patient with the R198C mutation was identified among these 50 patients. However, we identified a novel male patient with heterozygous mutation R380W (Figure 2). SUA of this hypouricemia patient was 2.6 mg/dL (154.6 μmol/l), which is similar to that of our previous report (SUA: 2.7 mg/dL (160.6 μmol/l)).

DISCUSSION

GLUT9 mutations in renal hypouricemia patients may change its topology.

We have previously identified loss-of-function mutations of *GLUT9* in renal hypouricemic patients having no *URAT1* mutations.[4] Mutation sites in *GLUT9* (R380W and R198C for GLUT9L, corresponding to R351W and R169C for GLUT9S) locate in highly conserved amino acid motifs called "sugar transport proteins signatures," which is observed in GLUT family transporters. The corresponding mutations in *GLUT1* (R333W and R153C) are known to cause *GLUT1* deficiency syndrome.[8] Arginine residues in this motif are reported to be an important determinant of membrane topology of human GLUT1,[9] and the same may be true in GLUT9 on the basis of membrane topology.

Physiological Importance of GLUT9 in Human Urate Transport

The urate metabolism in humans is quite different from that in mice due to the lack of uricase.[10] In addition, hypouricemia is one of relatively rare

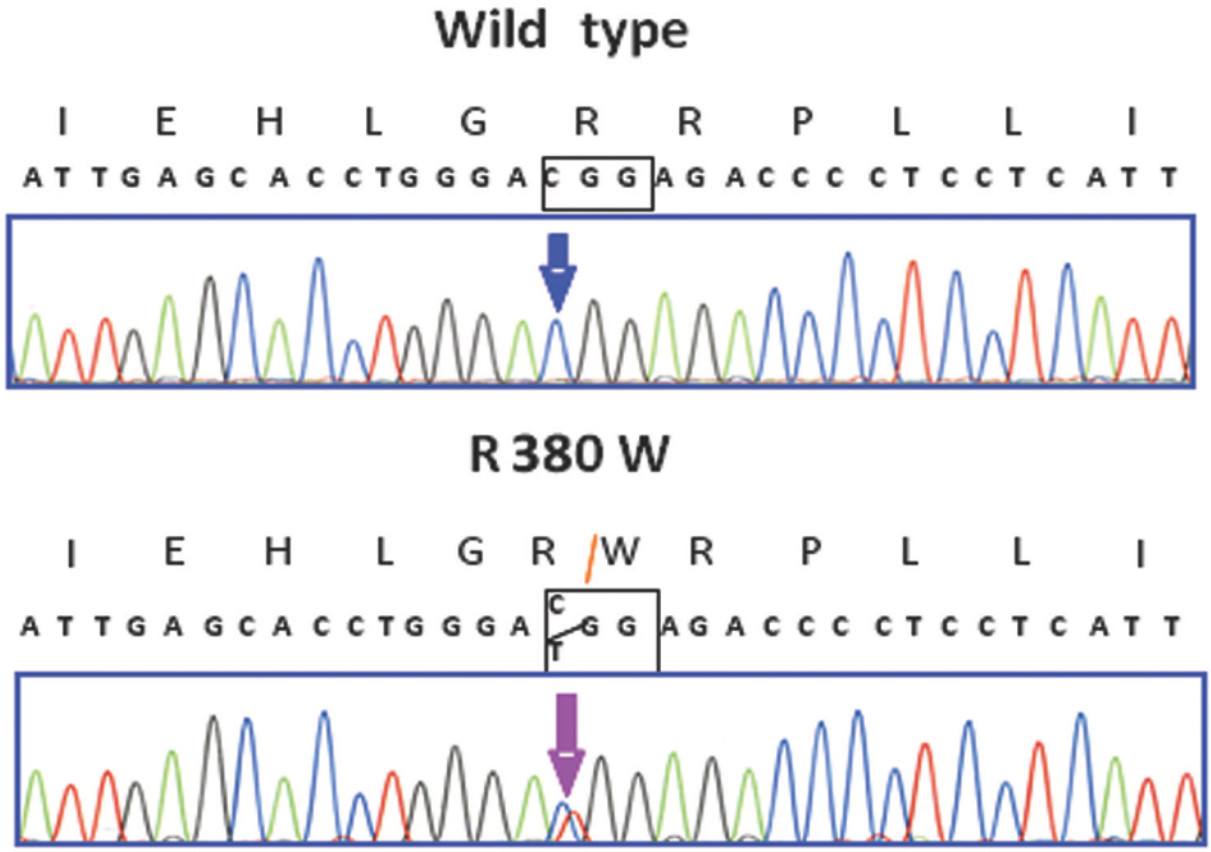

FIGURE 2 Heterozygous mutation (R380W) in a newly-identified renal hypouricemia patient (Color figure available online).

diseases compared with common diseases including hyperuricemia and gout. Therefore, it is of great significance to identify the dysfunctional *GLUT9* mutations in humans through a large population.

In MDCK cells, GLUT9L and GLUT9S show basolateral and apical localization, respectively. Since dysfunctional mutations of either GLUT9L or GLUT9S dramatically reduced the urate transport activity, renal hypouricemia caused by these mutations could be ascribed to the decreased urate reabsorption on both sides of the renal proximal tubules, where GLUT9 expresses. In the present study, we confirmed the importance of *GLUT9* as a causative gene for renal hypouricemia, which encodes a renal urate reabsorption transporter.

Identification of a Novel RHUC2 Patient

This is the second report indicating a RHUC2 patient due to *GLUT9* mutation R380W. Screening of large genome cohort samples revealed the second hypouricemia patient with *GLUT9* R380W, a pathogenic mutation of RHUC2. Our results confirm that GLUT9 can be a promising therapeutic target for hyperuricemia, gout, and related cardiovascular diseases. This finding may help to expand the understanding of RHUC pathogenesis.

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Review Article

Branchio-oto-renal syndrome: Comprehensive review based on nationwide surveillance in Japan

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Abstract Branchio-oto-renal (BOR) syndrome is an autosomal dominant disorder characterized by branchiogenic malformation, hearing loss and renal anomalies. The prevalence of BOR syndrome is 1/40 000 in Western countries, and nationwide surveillance in 2009–2010 identified approximately 250 BOR patients in Japan. Three causative genes for BOR syndrome have been reported thus far: *EYA1*, *SIX1*, and *SIX5*, but the causative genes for approximately half of all BOR patients remain unknown. This review article discusses the epidemiology, clinical symptoms, genetic background and management of BOR syndrome.

Key words branchio-oto-renal syndrome, *EYA1*, hearing loss, renal anomaly, *SIX1*.

Branchio-oto-renal (BOR) syndrome (OMIM 113650) is an autosomal dominant disorder characterized by branchiogenic malformation, hearing loss and renal abnormalities. BOR syndrome was first described by Melnick *et al*. in 1976.[1] Patients with BOR syndrome who do not present with renal abnormalities are also said to have branchio-otic (BO) syndrome (OMIM 602588). Both BOR and BO syndrome are allelic disorders. In the OMIM database, two BOR and three BO syndromes have been registered (Table 1). Due to recent advances in genetics, several of the causative genes of BOR syndrome have been identified (*EYA1*, *SIX1*, and *SIX5*), as well as the chromosomal region, microdeletions or microduplications within which can also lead to the syndrome. The causative genes for approximately half of all BOR patients, however, have yet to be identified.

This review article discusses the epidemiology, clinical symptoms, genetic background, differential diagnosis and management of BOR syndrome.

Epidemiology

Fraser *et al*. surveyed 421 children with hearing loss attending schools for hearing-impaired children in Montreal. They noted that 19 students had auricular pits, which led them to speculate that the prevalence of BOR syndrome is approximately one in 40 000 live births.[2] In Japan the prevalence of BOR syndrome was unknown until very recently. Our group carried out research in 2009–2010 to clarify the number of Japanese BOR patients.[3] An initial questionnaire was sent to the 1715 central hospitals throughout Japan. The collection rate of the questionnaire was 47.8%, and 85 patients with BOR phenotypes were identified. A second, more detailed questionnaire was then sent to each of these 85 patients and to their doctors. The collection rate was 37.6% (32/85) and 58.8% (50/85), respectively.

From this nationwide surveillance we estimated that there were approximately 250 BOR patients (95% confidence interval: 170–320) in the period 2009–2010 who were seen in clinics in Japan. These data suggest that the prevalence of BOR syndrome in Japan is much lower than in Western countries; the reason for this is unknown, but we suggest that BOR syndrome may be underdiagnosed in Japan because it is not well known. There may be many more patients in Japan who have not yet been accurately diagnosed.

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Clinical symptoms and diagnosis

Chen *et al*. produced a very comprehensive report on the clinical symptoms of BOR syndrome in the Western world.[4] That study has been very useful for increasing awareness of BOR syndrome. The present study comprising nationwide surveillance in Japan clarified the phenotype of BOR patients of Japanese descent (Table 2).

Second branchial arch anomalies

Second branchial arch anomalies include the branchial cleft sinus tract appearing as a pinpoint opening anterior to the sternocleidomastoid muscle, and the presence of a branchial cleft cyst as a palpable mass under the sternocleidomastoid muscle.[5] These symptoms were observed in approximately half of all BOR patients in both Western countries and Japan.

Hearing loss

Hearing loss is the most common symptom of BOR syndrome. Chen *et al*. reported that >90% of BOR patients have a hearing impairment.[4] According to our survey, hearing loss is also present in >90% of Japanese BOR patients. Various types of hearing loss were also observed, with mixed type being the most common cause (Table 3). In addition, two of the present patients had

Table 1 OMIM database entries for BOR and BO syndrome

	Name	OMIM	Locus	Gene
BOR1	branchio-oto-renal syndrome 1	113650	8q13.3	*EYA1*
BOR2	branchio-oto-renal syndrome 2	610896	19q13.3	*SIX5*
BOS1	branchio-otic syndrome 1	602588	8q13.3	*EYA1*
BOS2	branchio-otic syndrome 2	120502	1q31	Unknown
BOS3	branchio-otic syndrome 3	608389	14q23	*SIX1*

BO, branchio-otic; BOR, branchio-oto-renal.

different causes of hearing loss for each ear, and 12.8% of all cases involved progressive hearing loss.

Diagnosis of the cause of conductive hearing loss can be made on computed tomography (CT) or magnetic resonance imaging of the temporal bone. Various findings for BOR syndrome have been uncovered on temporal bone CT. Temporal bone anomalies include cochlear hypoplasia, absent or hypoplastic semicircular canals and large vestibular aqueduct syndrome.[6,7] Propst *et al*. reported that various characteristics of the 42 BOR patients surveyed in their study were significantly different from control patients; these were hypoplastic apical turn of the cochleae, division of the facial nerve toward the medial side of the cochleae, funnel-shaped internal auditory canals and patulous Eustachian tubes.[8]

Other otologic findings

Branchio-oto-renal patients present with various otologic anomalies (Table 2). Preauricular pits were the most frequently observed symptom in both the Chen *et al*. study,[4] and the present study. Branchial fistulae, pinnae deformities, external auditory canal stenosis and preauricular tags were also frequently observed.

Table 2 Clinical symptoms in Japanese BOR syndrome

Features	% (n = 50)
Hearing loss	92
Preauricular pit	53
Renal anomalies	40 (n = 35)†
Branchial fistulae	50
Pinnae deformities	38
External auditory canal stenosis	12
Preauricular tag	12
Others	
Retrognathia	2
Facial nerve paresis	3
Cleft palate	2
Bifid uvula	1
Congenital heart anomaly	1
Imperforate anus	1
Iris atrophy	1
Intracerebral hemorrhage	1

†Renal examinations were performed in 35 patients. BOR, branchio-oto-renal.

Table 3 Hearing loss in Japanese BOR syndrome

Side of hearing loss (%)	Type of hearing loss (%)
Bilateral 70.2†	Conductive 19.1
Unilateral 25.5	Sensorineural 29.8†
Unknown 4.3	Mixed 40.4†
	Unknown 14.9

†Two patients had different causes of hearing loss on either side (sensorineural and mixed). BOR, branchio-oto-renal.

Renal anomalies

Renal anomalies associated with BOR syndrome include renal hypoplasia, agenesis and hydronephrosis due to ureteropelvic junction (UPJ) or vesicoureteral reflux (VUR). Renal anomalies have been observed in approximately 67% of patients with BOR syndrome.[4] Krug *et al*. reported that in a large cohort of 140 BOR patients from 124 families, renal hypoplasia was the most frequent symptom with *EYA1* or *SIX1* mutations.[9] In the present survey, 40% of the 35 Japanese BOR patients who were examined for renal abnormalities presented with renal anomalies, with the most frequent symptom also being renal hypoplasia (Table 4). Some of the present patients had different types of anomalies in either kidney.

In rare cases, glomerular lesions have been observed in BOR patients. Gigante *et al*. reported an adult male patient with a BOR phenotype resulting from an *EYA1* mutation who presented with focal segmental glomerulosclerosis.[10] In the present study, a patient with BOR syndrome presented with membranous nephropathy resulting from an *EYA1* partial deletion. Although these glomerular findings were not presumably associated with the *EYA1* mutation, renal biopsy should be considered for all patients with proteinuria.

Renal examinations are usually not performed for BOR patients without any apparent renal symptoms. In the present study, 30% of BOR patients did not undergo any renal examination.

Table 4 Renal anomalies in Japanese BOR syndrome

Anomaly	n
Renal hypoplasia	10
Hydronephrosis	5
Renal agenesis	3
Others	
Multicystic dysplastic kidney	1
Hydroureter	1
Urethral stenosis	1
Membranous nephropathy	1
Renal malrotation	1
Nephroptosis	1
Chronic cystitis	1

BOR, branchio-oto-renal.

Other findings

Although heart disease is rare in BOR patients, mitral valve prolapse and bradycardia during anesthesia have been reported.[11,12] Although one patient with an *EYA1* mutation had large patent ductus arteriosus, the clinical diagnosis in that case was craniofacial syndrome.[13]

Intellectual disability (ID) and psychomotor delay (PD) are not frequently observed in patients with typical BOR syndrome. ID or PD may indicate the presence of another disease or contiguous gene syndrome due to microchromosomal deletions across genes such as *EYA1* or *SALL1*.

Clinical diagnosis

Chang *et al*. developed the diagnostic criteria of BOR syndrome in 2004.[5,14] These include major criteria such as second branchial arch anomalies, hearing loss, preauricular pits, auricular deformity and renal anomalies, and minor criteria such as external auditory canal anomalies, middle ear anomalies, inner ear anomalies, preauricular tags and other symptoms such as facial asymmetry and palate abnormalities. In patients with a family history, any single major criterion is sufficient for diagnosis of BOR syndrome. Without any family history, three major criteria or two major and two minor criteria are needed to make a confident diagnosis.

Genetic analysis

EYA1, *SIX1* and *SIX5* have been shown to be causative genes of BOR and BO syndrome (Table 1).

EYA1

EYA1 (8q13.3) is an ortholog of *Drosophila eya* (eyes absent) and acts as a protein phosphatase and transcriptional coactivator. *EYA1* is the most frequent causative gene of BOR syndrome (BOR1, OMIM 113650, BOS1, OMIM 602588) and was first reported in 1997 by Abdelhak *et al*.[15] *Eya1* homozygous-deficient mice lack ears and kidneys; in addition, *Eya1* heterozygous-deficient mice present with phenotypes resembling BOR syndrome.[16,17] Vertebrates encode four EYA proteins (EYA1–EYA4), and these proteins are involved in organ development, innate immunity, DNA damage repair, photoperiodism, angiogenesis and cancer metastasis.[18] *EYA1* has three isoforms and four transcript variants resulting from alternative splicing.[19,20] *EYA1* has 16 coding exons, and the EYA1 protein contains the eyes absent homologous region (EyaHR). Mutations are more frequently observed in the EyaHR, which is conserved across the EYA family.

Krug *et al*. recently reported that the causative gene could be identified for 42% of BOR patients, 93% of which were attributable to an *EYA1* mutation.[9] In an East-Asian cohort, *EYA1* was also identified as the major causative gene in BOR patients.[21–23] The types of mutations in *EYA1* associated with BOR syndrome are missense, nonsense, splice abnormalities and micro- or whole gene deletions. In the present study we were able to identify the causative gene in 21 Japanese BOR patients from 12 different families, and *EYA1* was observed in 11 of these families. *EYA1* was also therefore the major causative gene in this cohort.

SIX1

SIX1 is another causative gene of BOR syndrome (BOS3, OMIM 608389). *SIX1* (14q23.1) encodes a homeobox protein, which is similar to the *Drosophila* gene product, sine oculis. The genes in the *SIX* family all have a DNA-binding homeodomain (HD) and a protein interaction SIX domain (SD).[24] The HD is essential for protein-DNA binding, while the SD is involved in protein–protein interactions.[25] The EYA/SIX complex possesses tyrosine phosphatase activity,[26] and SIX1 interacts with EYA1 in the development of various organs.

In 2004, Ruf *et al*. first reported cases of BOR syndrome resulting from three types of *SIX1* mutation.[27] Kochhar *et al*. later reported five novel *SIX1* mutations identified across 247 BOR families.[28] The clinical phenotype of BOS3 mutations is similar to the BOR1 *EYA1* mutation, but renal anomalies are less frequent for the *SIX1* mutations.[28,29] DFNA23 (OMIM 605192), which is non-syndromic autosomal dominant hearing loss, is also attributable to *SIX1* mutations. *SIX1* mutations in BOR syndrome occur less frequently than the *EYA1* mutation. Krug *et al*. reported that *SIX1* mutations occurred in only 2.8% of their 140 BOR patients, while the *EYA1* mutation was present in 39.3% of the patients.[9] *SIX1* mutations in East-Asian BOR patients are also very rare.[22,23,29]

SIX5

SIX5, which is located on 19q13.32, has been reported to be the causative gene in 5% of BOR patients (BOR2, OMIM 610896).[30] *SIX5* has a high degree of homology to *SIX1* and directly interacts with *EYA1*, but there have been no additional reports since on the *SIX5* mutation and one of the patients initially reported by Klug *et al*. to have a *SIX5* mutation was later confirmed to have an *EYA1* mutation instead.[9] The role of *SIX5* in BOR syndrome is therefore still open to question and further study is essential.

Copy number variation in BOR syndrome

Brophy *et al*. recently reported that copy number variation (CNV) analysis from array-based comparative genomic hybridization (CGH) can identify the responsible lesion in BOR patients.[31] They analyzed 35 BOR patients without any mutations in *EYA1*, *SIX1* or *SIX5* and 17 patients were identified as having significant CNV (11 chromosomal microdeletions and six microduplications). These chromosomal abnormalities may be due to non-allelic homologous recombination (NAHR). Moreover, several new causative genes were suggested in their study: *SHARPIN* (8q24.3), *FGF3* (11q13) and the *HOXA* genes.

Other loci

Although Kumar *et al*. reported in 2000 that the locus associated with ontologic and branchial manifestations was 1q31,[32] which is the locus of BOS2 (OMIM 12052), it appears that this locus has since been withdrawn.[5]

Genotype–phenotype correlations

Genotype–phenotype correlations have not yet been confirmed for BOR patients. In the same consanguineous family, different

family members may present with different symptoms. ID is, however, infrequent in patients with distinctive BOR syndrome. Patients presenting with ID may have other syndromes or chromosomal microdeletions in contiguous genes including *EYA1* or *SALL1*.

Genetic analysis strategy for BOR diagnosis

The *EYA1* mutation is the most common cause of BOR syndrome, so *EYA1* direct sequencing should be performed first. In cases of *EYA1* point mutations, small deletions or insertions are not present, and multiplex ligation-dependent amplification (MLPA) analysis should be carried out. We identified 11 families with BOR syndrome resulting from an *EYA1* mutation, and in six cases the deletion of one or more exons in the *EYA1* gene was detected on MLPA analysis. We have reported that the *EYA1* partial deletion was due to replacement of the deleted region by the retrotransposon, LINE-1,as detected on MLPA analysis.[33] Patients without any *EYA1* mutations should be considered for *SIX1*, *SIX5* and array CGH analysis. For the approximately 50% of patients with BOR syndrome for which the causative gene cannot be detected, further investigations are needed to study novel candidate genes such as those proposed by Brophy *et al*.[31]

Genetic counseling

Genetic counseling is the process of empowering patients by providing them with medical information regarding their particular genetic abnormality.[5]

Approximately 90% of BOR patients have their affected parent in a Western country. BOR syndrome is inherited as an autosomal dominant disorder and *EYA1* penetrance is very high with an inherited relapse rate of 50%. When the proband is born to non-affected parents, the risk of the sibling having BOR is slightly higher than for the general population because of the possibility of germinal mosaicism. Generational progression in BOR syndrome has not been reported.

Differential diagnosis

Hearing loss is a symptom of many genetic syndromes so it may be difficult to reach a conclusive diagnosis based on this symptom alone, especially in cases of sporadic hearing loss. Genetic tests are required to distinguish BOR syndrome from other syndromes. Other syndromes that should be differentially diagnosed from BOR syndrome are as follows.

Townes–Brocks syndrome

Townes–Brocks syndrome (TBS, OMIM 107480) is an autosomal dominant disorder characterized by branchiogenic anomalies, hearing loss, congenital heart disease, preaxial polydactyly and imperforate anus. Renal anomalies are also seen. The causative gene of TBS is *SALL1* (16q12.1), which is a critical gene in renal generation in fetal development. The mechanism of *SALL1* mutations in TBS is dominant-negative based, therefore a heterozygous whole gene deletion leads to milder symptoms.

Engels *et al*. reported a family including an affected father and two daughters with a *SALL1* frameshift mutation.[34] These patients presented with hearing loss and pinnae deformities but without preaxial polydactyly or an imperforate anus. These symptoms are compatible with BOR syndrome rather than TBS. This report indicates that BOR syndrome patients without any detectable *EYA1*, *SIX1*, or *SIX5* mutations may benefit from genetic analysis of *SALL1*.

Branchio-oculo-facial syndrome

Branchio-oculo-facial syndrome (BOFS, OMIM 113620) is a rare autosomal dominant disorder resulting from a *TFAP2A* mutation.[35,36] There are phenotypic overlaps with BOR syndrome, but BOFS produces more distinctive facial features. BOFS is characterized by hearing loss, branchial anomalies and renal abnormalities. BOFS patients also have craniofacial and ocular abnormalities. Skin defects in the cervical or infra- or supra-auricle are observed in >90% of BOFS patients. Furthermore, ID is more common in BOFS than in BOR syndrome.

Oto-facio-cervical syndrome

Oto-facio-cervical syndrome (OFCS1 166780, OFCS2 615560) is an autosomal disorder characterized by facial dysmorphism, external ear malformations with hearing loss, branchial cysts or fistulae and anomalies of the vertebrate and shoulder girdle. In addition, mild ID is also seen. OFCS was first described by Fara *et al*. in an affected family.[37] OFCS has a similar phenotype to BOR; moreover, OFCS1 is an allelic disorder resulting from an autosomal dominant *EYA1* mutation.[38] OFCS2 is due to the mutation of *PAX1* in an autosomal recessive manner.[39] The difference between OFCS and BOR syndrome lies in the presence of ID and vertebral anomalies in OFCS patients.

CHARGE syndrome

CHARGE syndrome (OMIM 214800; coloboma, heart defects, choanal atresia, retarded growth and development, genital anomalies, and ear anomalies) has similar symptoms to BOR syndrome, including hearing loss, branchiogenic malformations and renal malformations. In addition, coloboma, mental and growth retardation, congenital heart anomalies, choanal atresia and cryptorchidism are often present. Facial asymmetry and hockey stick signs on the palms are often observed. The causative gene of CHARGE syndrome is *CHD7* (8q12.1-q12.2).[40] The less frequent developmental and growth delays observed for BOR can assist in differentiating this syndrome from CHARGE syndrome.

HDR syndrome

Hypoparathyroidism, with sensorineural deafness and renal dysplasia (HDR) syndrome is a rare autosomal dominant disorder characterized by sensorineural hearing loss, renal anomalies and hypothyroidism. HDR syndrome is due to haploinsufficiency of *GATA3* (10p14).[41] Patients with HDR syndrome often present with hypocalcemia. Historical interviews and genetic tests are helpful in distinguishing this syndrome from BOR.

Management

Second branchial anomalies and preauricular anomalies

Treatment for branchial anomalies is performed for supportive or cosmetic reasons. If auricular or branchial fistulae become infected, antibiotic therapy is applied and, in cases of recurrence, they are removed.

Hearing loss

Hearing loss requires early medical intervention because of its effect on language development. There are various causes of hearing loss in BOR syndrome, so it is important to provide treatment appropriate to each. In the present surveillance, 80.1% of BOR patients found the use of a hearing aid effective. Surgical operations such as tympanoplasty, cochlear implantation and tympanic ventilation tube therapy were performed in 11 patients and were effective in seven (63.6%). Cochlear implantation may be successful for improving the hearing ability of BOR patients.[42]

Renal anomalies

Renal anomalies are controlled in a standard manner. Patients with hydronephrosis including UPJ stenosis or VUR may need to undergo surgical therapy. Renal hypoplasia or dysplasia leads to end-stage renal failure, requiring renal replacement therapy. In the present surveillance, of the 14 BOR patients with renal symptoms, four had renal transplantation while three patients were given medical drugs. The prognosis of BOR syndrome mainly depends on the severity of the renal insufficiency.

Conclusion

Patients with BOR syndrome who receive adequate treatment can lead normal, productive lives. It is therefore essential to provide early diagnosis including the relevant genetic tests. Further studies are needed to clarify the molecular mechanisms and undiscovered causative genes of BOR syndrome.

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Original Article

Progression to end-stage kidney disease in Japanese children with chronic kidney disease: results of a nationwide prospective cohort study

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ORIGINAL ARTICLE

ABSTRACT

Background. The risk of progressing to end-stage kidney disease (ESKD) and factors associated with progression in children with chronic kidney disease (CKD) are unclear, especially in Asian children.

Methods. We started a nationwide, prospective cohort study of 447 Japanese children with pre-dialysis CKD in 2010, with follow-up in 2011. Progression to ESKD was analyzed by Kaplan–Meier analysis according to CKD stage. Cox regression analysis was used to identify risk factors for progression.

Results. Data were analyzed for 429/447 children. Five patients died, of which four died before progression to ESKD. Fifty-two patients progressed to ESKD (median follow-up 1.49 years), including 9/315 patients with stage 3 CKD, 29/107 with Stage 4 CKD and 14/25 with Stage 5 CKD. One-year renal survival rates were 98.3, 80.0 and 40.9%, for Stages 3, 4 and 5 CKD, respectively. Risk factors for progression to ESKD included CKD stage [versus Stage 3; Stage 4: hazard ratio (HR) 11.12, 95% confidence interval (CI) 4.22–29.28, $P < 0.001$; Stage 5: HR 26.95, 95% CI 7.71–94.17, $P < 0.001$], heavy proteinuria (>2.0 g/g urine creatinine; HR 7.56, 95% CI 3.22–17.77, $P < 0.001$) and age (< 2 years: HR 9.06; 95% CI 2.29–35.84, $P = 0.002$; after starting puberty: HR 4.88; 95% CI 1.85–12.85, $P = 0.001$).

Conclusions. In this cohort, 12.5% of children with pre-dialysis CKD progressed to ESKD with a median-follow-up of 1.49 years. Children with advanced (Stage 4/5) CKD were particularly likely to progress. To our knowledge, this is the first, nationwide, prospective cohort study of children with pre-dialysis CKD in Asia.

Keywords: Asia, child, chronic kidney disease, end-stage kidney disease, prognosis

INTRODUCTION

Chronic kidney disease (CKD) in children is a progressive and intractable disease [1]. In the CKD in Children study, children with a glomerular filtration rate (GFR) of <30 mL/min/1.73 m^2

showed significant growth failure and other clinically important disorders compared with children with a higher GFR (≥50 mL/min/1.73 m^2), and experienced greater progressive changes in their GFR [2]. The mortality rate in children with end-stage kidney disease (ESKD) is also quite high, and was reported to be 98.8/1000 person-years among children who started dialysis between 1990 and 2010 in the USA [3].

The prevalence of CKD in children/adolescents varies considerably among studies and countries [4–10]. Furthermore, the incidence of Stage 2–5 CKD in children was reported to range from 7.7 to 12.1 per million [6], based on data reported in six countries (Italy [11], Belgium [12], Spain [13], Sweden [14], France [15] and Turkey [16]). The broad range in the incidence of CKD was at least partly due to differences in the clinical definition of CKD used in each study. The differences in study design and possible differences in CKD characteristics among ethnic groups also mean it is difficult to compare the prevalence of CKD and ESKD among studies, or estimate the prevalence of severe kidney disease worldwide or in specific populations lacking current data. Furthermore, while the prevalence of CKD in adults is steadily increasing in many countries [8], the current situation in children is less clear, particularly in Asian children.

It was also suggested that the rate of decline in renal function in Japanese adults appears to be slow compared with that in other countries, and that hypertension, proteinuria and low GFR were significant risk factors for a faster decline of GFR in Japanese adults [17]. However, no studies have examined the decline in renal function in Japanese children with CKD, or sought to identify risk factors for progression to ESKD.

To address these issues and to help us to better understand the current status of CKD in Japan, we implemented a nationwide, prospective cohort study of pre-dialysis CKD in Japanese children [9], the first such study in Asia. We previously reported that the prevalence of Stage 3–5 CKD was 2.98 cases/100 000 children, and that most children with CKD presented with non-glomerular disease, including congenital anomalies of the kidney and urinary tract (CAKUT). As the original results were derived from a cross-sectional analysis, we could not determine the rate of disease progression in these patients at that time. Therefore, as planned, we conducted a follow-up survey to determine the rate of disease progression in these patients. From this context, the aims of the present analyses were (1) to investigate the progression of CKD to ESKD or death and (2) to identify factors associated with disease progression.

MATERIALS AND METHODS

Study design and population

The study design and patient population are described in more detail in our original report [9]. Briefly, we sent two surveys in August 2010 to 1190 institutions (all members of the Japanese Society for Pediatric Nephrology, all university and children's hospitals, and all general hospitals with >200 beds) in Japan inviting them to report on cases of pediatric CKD managed as of 1 April 2010. The first survey documented the number of children with Stage 3–5 CKD in each institution. The respondents were asked to search their medical records to determine the numbers of patients with a confirmed diagnosis of CKD, or patients with abnormal serum creatinine (SCr) values. In the second survey, the respondents were asked to record the clinical characteristics of each patient. A total of 925/1190 institutions (77.7%) responded to the first questionnaire. In the second questionnaire, the participating institutions provided data for 479 children. Of these, 447 children who met the following criteria were evaluable: (i) children with CKD aged 3 months to 15 years as of 1 April 2010; (ii) presence of Stage 3–5 CKD; (iii) no history of chronic dialysis or renal transplantation; (iv) renal failure lasting >3 months (cases with transient increases in SCr were excluded).

In September 2011, surveys were conducted for the 113 medical institutions that provided data for the cohort of children ($n = 447$) established in our original report [9]. The deadline for responding to this survey was November 2011. Data were provided for 429/447 children in the follow-up survey. The survey asked clinicians to record patient characteristics [e.g. height, weight, blood pressure, cardiac function and blood and urine parameters, including urine protein/creatinine ratio (g/g urine creatinine)], outcomes (start of dialysis, kidney transplantation and death), CKD complications, disease type and neonatal data (birth weight, gestational age and presence of asphyxia), as of 1 November 2011. All surveys were to be returned using provided envelopes and data entry was conducted by the data center.

CKD stage was assessed as previously described [9, 18]. Stages 3, 4 and 5 CKD were defined as SCr levels more than twice, four times and eight times, respectively, the median normal levels in age- and sex-matched Japanese children. In our previous report [9], we validated these reference levels by applying the abbreviated Schwartz equation [19], with Stages 3, 4 and 5 CKD being classified as GFR 30–59, 15–29 and <15 mL/min/1.73 m^2, respectively (<1/2, <1/4 and <1/8 of normal GFR, respectively), defined according to established guidelines [20–22]. All of the participating institutions reported using enzyme immunoassays to measure SCr. Heavy proteinuria was defined as urine protein/creatinine ratio >2.0 g/g urine creatinine. The patients were divided into three age groups for males (<2, ≥2 to <10.8 and ≥10.8 years) and females (<2, ≥2 to <10.0 and ≥10.0 years), where 10.8 and 10.0 years correspond to the mean age of Japanese males and females, respectively, at the start of puberty [23]. Hypertension was defined as systolic blood pressure >95th percentile [24].

The study was conducted in accordance with the principles of the Declaration of Helsinki and the ethical guidelines issued by the Ministry of Health, Labour and Welfare, Japan. The study was approved by a central ethics board at Tokyo Metropolitan Children's Medical Center (approval number: 23–49). Because data were reported using patient medical records, informed consent was not obtained in accordance with the above guidelines.

Statistical analysis

The primary outcome was the progression of CKD to ESKD. The cumulative proportion of progression was estimated by the Kaplan–Meier method, where death was also

Table 1. Patient characteristics according to CKD stage

	All patients	Stage 3	Stage 4	Stage 5	P-value*
n	447	315	107	25	
Age (years)	8.6 ± 4.5	8.6 ± 4.6	8.4 ± 4.2	9.9 ± 4.5	0.321
Sex, male/female (*n*)	272/175	192/123	67/40	13/12	0.618
Serum creatinine (mg/dL)	1.6 ± 1.2	1.1 ± 0.4	2.2 ± 0.8	5.3 ± 2.0	<0.001
Height (cm)	119.6 ± 27.8	120.5 ± 28.1	117.1 ± 26.9	118.1 ± 28.9	0.547
Height (SD)	−1.5 ± 1.8	−1.3 ± 1.5	−1.8 ± 2.1	−2.8 ± 3.2	<0.001
BUN (mg/dL)	35.5 ± 18.7	28.3 ± 9.7	48.4 ± 18.1	74.9 ± 31.5	<0.001
Cystatin-C (mg/L)	2.1 ± 0.8	1.9 ± 0.5	3.1 ± 1.0	4.1 ± 0.9	<0.001
eGFR abbreviated (mL/min/1.73 m^2)[a]	39.6 ± 15.9	47.3 ± 11.4	22.6 ± 5.3	10.4 ± 3.3	<0.001
eGFR complete (mL/min/1.73 m^2) [b]	39.9 ± 12.4	43.9 ± 10.0	24.7 ± 5.2	13.5 ± 4.0	<0.001

Values are means ± standard deviation. CKD, chronic kidney disease; SDS, standard deviation score; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate.
[a]Abbreviated Schwartz equation [19], eGFR = 41.3 [height (m)/SCr (mg/dL)].
[b]Complete Schwartz equation [19], eGFR = 39.1 [height (m)/Scr (mg/dL)]$^{0.516}$[1.8/cystatin C (mg/L)]$^{0.294}$ × [30/BUN (mg/dL)]$^{0.169}$ [1.099]^{male} [height (m)/1.4]$^{0.188}$.
*P-values were determined by analysis of variance for all variables except sex, which was analyzed by the χ^2 test.

considered as an event. The day on which SCr was measured that was closest to 1 April 2010 was used as the starting point (i.e. T = 0 years). Cox's proportional hazard regression model was used to identify possible predictors of CKD progression by calculating hazard ratios (HRs) with 95% confidence intervals (CIs). All statistical analyses were carried out using SAS system version 9 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Patient characteristics

The characteristics of the patients, as of 1 April 2010, are summarized in Table 1. Of the 447 children in this cohort, 405 were of Asian ethnicity and 3 were of another ethnicity; ethnicity was not reported by the institution for the remaining 39 children.

As would be expected, SCr, blood urea nitrogen and cystatin C levels increased significantly with increasing CKD stage, consistent with reductions in eGFR, as determined with the abbreviated and complete Schwartz equations [19]. Children with Stage 5 CKD tended to be older than children with Stage 3/4 CKD.

Progression to ESKD and renal replacement therapy

Table 2 shows the patient outcomes during this survey. Overall, 52 patients progressed to ESKD during the follow-up period [median follow-up period (interquartile range) 1.49 years (1.16–1.64 years); Stage 3, *n* = 9; Stage 4, *n* = 29; Stage 5, *n* = 14]. Of these, 1/9 patients in Stage 3, 21/29 patients in Stage 4 and 8/14 in Stage 5 had CAKUT. Five deaths (sepsis in two; acute encephalitis, graft versus host disease and acute heart failure and pulmonary edema caused by advanced uremia in one each) occurred during the study period, of which four occurred before and one occurred after progression to ESKD. The detailed characteristics of patients with progression to ESKD or who died are presented in Table 3. The Kaplan–Meier analysis for the time to ESKD or death (included as an event) is presented in Figure 1. Among 429/447 children with available data, the survival rates at 1 year were 98.3, 80.0 and 40.9% in children with Stage 3, 4 and 5 CKD, respectively. The Kaplan–Meier plot and survival rates were almost identical when deaths were censored instead of being included as an event; the survival rates at 1 year were 98.3, 80.9 and 43.1% in children with Stage 3, 4 and 5 CKD, respectively.

Table 2. Outcomes and renal replacement therapies according to CKD stage

	All patients	Stage 3	Stage 4	Stage 5
n	447	315	107	25
Data not provided by the participating institution	18	11	4	3
Death before progression to ESKD	4	1	2	1
ESKD	52[a]	9	29[a]	14
Renal replacement therapies				
PD	27	6	15	6
Preemptive kidney transplantation	16	1	11	4
Kidney transplantation after PD	3	0	1	2
HD	4[a]	2	1[a]	1
PD after HD	2	0	1	1
Change in CKD stage (excluding death before progressing to ESKD)				
To Stage 2		43	1	0
To Stage 3		210	6	0
To Stage 4		40	56	1
To Stage 5 (5D)		10 (9)	38 (29)	20 (14)

CKD, chronic kidney disease; ESKD, end-stage kidney disease; PD, peritoneal dialysis; HD, hemodialysis.
[a]Includes one death.

The most common chronic renal replacement therapy in children with ESKD was peritoneal dialysis, which was used in 27 children, followed by preemptive kidney transplantation in 16 patients (Table 2).

During the follow-up period, 40 and 10 of 315 children with Stage 3 CKD progressed to Stage 4 and Stage 5 (Stage 5D in 9/10 patients) CKD, respectively, while 38/107 patients with Stage 4 CKD progressed to Stage 5 (Stage 5D in 29/38 patients).

Factors associated with CKD progression

CKD progression was defined as ESKD or death occurring during follow-up. Table 4 shows the factors that were

Table 3. Characteristics of patients who progressed to ESKD or who died

CKD stage in 2010[a]	Age in 2010 (years)	Sex	Primary etiology	Method of detecting CKD	Recognizable syndrome
Deaths					
3	3.6	Male	Unknown	Urinary tract infection	Down syndrome
4	3.4	Male	Cortical necrosis (perinatal period)	Blood analysis in the neonatal period, asphyxia, neonatal shock	—
4	0.7	Male	CAKUT without obstructions	Fetal ultrasonography/ultrasonography in the neonatal period	—
4 Deaths after ESKD	8.3	Male	Drug induced	Detected during the management of other diseases(e.g. heart disease)	—
5	13.5	Female	CAKUT without obstructions	Failure to thrive, weight loss and general fatigue	—
Progression to ESKD					
3 ($n = 9$)	9.8 ± 4.9	6 males 3 females	CAKUT without obstructions (1); chronic glomerulonephritis (2); congenital nephrotic syndrome (1); focal segmental glomerulosclerosis (2); nephronophthisis (1); other inherited kidney damage (2)	Analysis by chance (4); annual urinalysis at school (3); blood analysis in the neonatal period, asphyxia, neonatal shock (1); fetal ultrasonography/ultrasonography in the neonatal period (1)	Bardet–Beadle syndrome (1); Lowe syndrome (1)
4 ($n = 28$)	9.5 ± 4.7	15 males 13 females	CAKUT with obstructions (4); CAKUT without obstructions (17); congenital nephrotic syndrome (1); hemolytic uremic syndrome (1); nephronophthisis (3); neurogenic bladder (1); other inherited kidney damage (1)	Analysis by chance (6); annual urinalysis at school (2); blood analysis in the neonatal period, asphyxia, neonatal shock (4); dysuria, including neurogenic bladder and nocturia (1); failure to thrive, weight loss and general fatigue (3); fetal ultrasonography/ultrasonography in the neonatal period (6); symptoms of glomerulonephritis (edema, oliguria or gross hematuria (1); unknown (1); urinalysis at 3 years (2); urinary tract infection (2))	15q syndrome (1); chromosomal anomalies (1); Ellis–van Creveld syndrome (1); Prune belly syndrome (1); renal coloboma syndrome (1)
5 ($n = 14$)	9.9 ± 1.2	9 males 5 females	CAKUT with obstructions (1); CAKUT without obstructions (7); cortical necrosis (perinatal period) (3); nephronophthisis (1); polycystic kidney disease (2)	Analysis by chance (2); annual urinalysis at school (2); blood analysis in the neonatal period, asphyxia, neonatal shock (1); failure to thrive, weight loss and general fatigue (2); fetal ultrasonography/ultrasonography in the neonatal period (5); unknown (1); urinary tract infection (1)	—

CAKUT, congenital anomalies of the kidney and urinary tract; CKD, chronic kidney disease; ESKD, end-stage kidney disease. Values in parentheses indicate the number of patients. Age is shown as the mean ± SD.
[a]Data are presented for individual patients (deaths) or groups by CKD stage (alive).

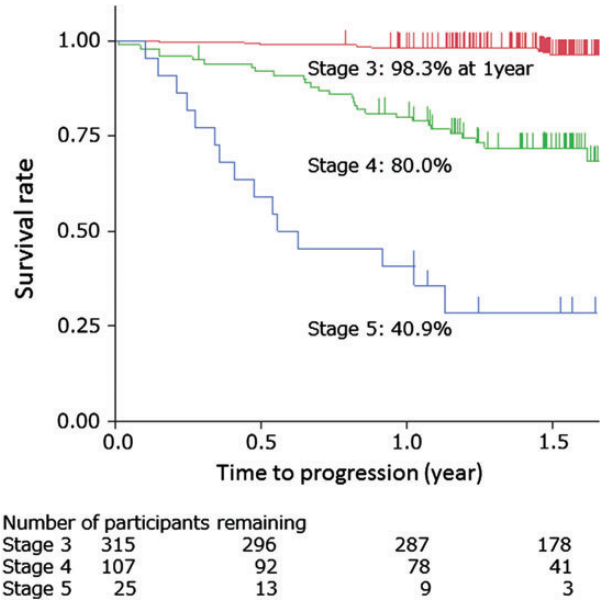

FIGURE 1. Kaplan–Meier plot showing time to ESKD according to CKD stage. $T = 0$ years was defined as the day on which serum creatinine was measured that was closest to 1 April 2010. The 1-year survival rates are shown for each stage.

Table 4. Risk factors for ESKD (Cox regression model)

Variable	HR	95% CI	P-value
Sex			
Female	1.56	0.67–3.62	0.306
Male	1.00	—	—
Age			
Age <2 years (versus 2 years to the start of puberty)[a]	9.06	2.29–35.84	0.002
After puberty (versus 2 years to the start of puberty)[a]	4.88	1.85–12.85	0.001
Recognizable syndrome[b]	2.54	0.75–8.58	0.133
CKD stage			
CKD Stage 4 (versus Stage 3)	11.12	4.22–29.28	<0.001
CKD Stage 5 (versus Stage 3)	26.95	7.71–94.17	<0.001
CAKUT	0.60	0.25–1.47	0.261
Preterm delivery (<37 weeks)	1.33	0.50–3.53	0.562
Heavy proteinuria[c]	7.56	3.22–17.77	<0.001
Hypertension[d]	0.53	0.19–1.46	0.219
Use of antihypertensive drugs	1.08	0.42–2.75	0.874

ESKD, end-stage kidney disease; HR, hazard ratio; CI, confidence interval; CKD, chronic kidney disease; CAKUT, congenital anomalies of the kidney and urinary tract.
[a]Age at the start of puberty was defined as 10.8 years for males and 10.0 years for females [23].
[b]Recognizable syndromes included Down syndrome, Kabuki syndrome, Townes–Brocks syndrome, VATER association, prune belly syndrome, Wolf–Hirschhorn syndrome and branchio-oto-renal syndrome, among others.
[c]Urine protein/creatinine ratio >2.0 g/g urine creatinine.
[d]Systolic blood pressure >95th percentile.

independently associated with CKD progression, as determined using Cox's proportional hazards model. As shown in this table, CKD stage and heavy proteinuria were significantly associated with disease progression. Age of <2 years and age at or above the start of puberty were significantly associated with increased risk of disease progression. In contrast, sex, the presence of a recognizable syndrome, disease (CAKUT or other disease), preterm delivery (<37 weeks), hypertension (systolic blood pressure >95th percentile) [24] and the use of antihypertensive drugs were not associated with disease progression. The results did not change when we included the duration of disease instead of age or eGFR calculated using the abbreviated Schwartz equation instead of CKD stage, or if deaths were censored instead of being included as an event (data not shown).

DISCUSSION

The main findings of this prospective cohort study in Japanese children with CKD Stages 3–5 are that (i) the prognosis of CKD in children is poor, as disease progression to a higher CKD stage or ESKD occurred in a sizeable number of children, particularly those with advanced (Stages 4/5) CKD, and (ii) advanced CKD stage and heavy proteinuria were independently associated with progression to ESKD. Age of <2 years and age at or above the start of puberty (≥10.8 years in males and ≥10.0 years in females) were also significantly associated with increased risk of disease progression. To our knowledge, this is the first nationwide, prospective cohort study of children with pre-dialysis CKD to examine the risk for progression to ESKD in Asia.

The present results are broadly consistent with those reported elsewhere, showing the poor outcomes of CKD in children [1, 3–6, 11, 12, 14–16, 25]. In a retrospective analysis of 176 children with dysplastic kidneys and ≥5 years of follow-up, Gonzalez Celedon *et al.* [1] reported that there was an early improvement in renal function, which lasted until ∼3.2 years of age, and was followed thereafter by maintained or deteriorating renal function, particularly after 7 and 11 years of age. They reported that hypertension, albuminuria, number of febrile urinary tract infections, eGFR at onset and puberty were significantly associated with disease progression. Sanna-Cherchi *et al.* [26] reported that the prognosis of CAKUT was also poor, as 58/312 patients required dialysis by 30 years of age. Elevated SCr and proteinuria were associated with worse outcomes, as were specific disorders (solitary kidney, posterior urethral valves and vesicoureteral reflux). In the present study, a sizeable proportion (12.5%) of children progressed from Stage 3 to 5 CKD to ESKD during the follow-up period (median 1.49 years). In addition, children with advanced stage CKD (4/5) are at particularly high risk of progressing to ESKD, irrespective of the primary etiologies of CKD. Furthermore, as in the study by Sanna-Cherchi *et al* [26], we found that proteinuria was a risk factor for progression to ESKD. We also found that age <2 years and age at or above the start of puberty were significantly associated with increased risk of progressing to ESKD relative to the risk in patients aged 2 to the start of puberty (10.8 years in males and 10.0 years in females). These results may reflect the risk of disease progression in very young patients with severe congenital complications and that disease progression may be more pronounced in puberty.

The CKD in Children cohort study in the USA [5, 6], as well as studies performed in France [15], Sweden [14], Italy [11] and Australia/New Zealand [25], consistently reported that many children with CKD ultimately require renal replacement

therapies. However, renal transplantation was reported to achieve better long-term outcomes and reduce the mortality rate compared with dialysis in children with ESKD [25]. Although the most common modality (51.9%) of renal replacement therapies was peritoneal dialysis in our cohort, ∼30% of children with ESKD received preemptive kidney transplantation, reflecting the current trends in Japan. The superiority and clinical benefits of preemptive kidney transplantation relative to dialysis should be confirmed in future studies.

The present study and the studies described above have consistently shown that heavy proteinuria is independently associated with CKD progression. Prior studies have also indicated that antihypertensive drugs, particularly angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), help to delay or prevent the progression to ESKD in children [27, 28]. These drugs not only lower blood pressure, but also have antiproteinuric, antifibrotic and anti-inflammatory properties. In the present study, 28.4 and 28.2% of patients were prescribed an ARB or ACEI, respectively, and 7.2% were prescribed a calcium channel blocker [9]. In contrast, the use of an antihypertensive drug and hypertension *per se* were not associated with progression to CKD in our cohort study. In the ItalKid project, also an observational study, the use of an ACEI did not significantly modify the progressive course of hypodysplastic nephropathy in children [29]. Therefore, in children with CKD, the effects of antihypertensive drugs, particularly ACEIs and ARBs, on modifying disease progression shown in adults need to be verified in future studies. We are now conducting a randomized controlled trial to prospectively examine the renoprotective effects of ARBs to address this issue (UMIN ID: UMIN000006917, http://indice.umin.ac.jp).

The strengths of this study are that the cohort was representative of children with CKD throughout Japan, as the information was obtained from ∼80% of the institutions that manage children with CKD at the time of establishment of the cohort, and the follow-up rate of this cohort was 96%.

Some limitations also warrant mention. We classified CKD using reference SCr levels determined enzymatically in Japanese children. These diagnostic criteria have not been validated globally and so the criteria may not be appropriate for other populations, particularly non-Asian children. However, as described in our prior report [9], this approach was necessary because of potential limitations of using the Schwartz equation in Japanese children or for screening purposes, where SCr is available, but height is not. The duration of follow-up, ∼1.5 years, is also relatively short in the context of CKD progression. The pubertal stage of patients was not assessed in this study. Therefore, to estimate the effects of puberty on disease progression, we stratified the patients according to the mean age of Japanese children at the start of puberty (10.8 years in males and 10.0 years in females [23]) in lieu of the actual pubertal stage.

In conclusion, this nationwide, prospective cohort study showed that 12.5% of children with pre-dialysis CKD (stages 3–5) ultimately progressed to ESKD in the follow-up period (median 1.49 years). In particular, children with Stage 4 or 5 were at very high risk of progression to ESKD. Heavy proteinuria was also significantly associated with progression to ESKD. A longer follow-up of this cohort is currently underway to explore outcomes of these children beyond adolescence and into adulthood.

SUPPLEMENTARY DATA

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

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CONFLICT OF INTEREST STATEMENT

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SPECIAL ARTICLE

Diagnostic criteria for atypical hemolytic uremic syndrome proposed by the joint committee of the Japanese society of nephrology and the Japan pediatric society

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Abstract Atypical hemolytic uremic syndrome (aHUS) is rare and comprises the triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury. Recently, abnormalities in the mechanisms underlying complement regulation have been focused upon as causes of aHUS. The prognosis for patients who present with aHUS is very poor, with the first aHUS attack being associated with a mortality rate of ~25 %, and with ~50 % of cases resulting in end-stage renal disease requiring dialysis. If treatment is delayed, there is a high risk of this syndrome progressing to renal failure. Therefore, we have developed diagnostic criteria for aHUS to enable its early diagnosis and to facilitate the timely initiation of appropriate treatment. We hope these diagnostic criteria will be disseminated to as many clinicians as possible and that they will be used widely.

Keywords Atypical hemolytic uremic syndrome · Thrombotic microangiopathy · Complement dysregulation · Alternative complement pathway · ADAMTS13

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Introduction

Hemolytic uremic syndrome (HUS) is characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury (AKI) [1]. Approximately 90 % of pediatric patients develop this syndrome after infection with *Shigella dysenteriae*, which produces true Shiga toxins, or *Escherichia coli*, some strains of which produce Shiga-like toxins. Shiga toxin was originally called verotoxin because Vero cells derived from the kidney epithelial cells of the African green monkey are hypersensitive to this toxin [2]. Subsequently, other toxins were called Shiga-like toxin because of their similarities to Shiga toxin in terms of their antigenicity and structure. Shiga-like toxin-1 differs from Shiga toxin by only 1 amino acid, whereas Shiga-like toxin-2 shares 56 % sequence homology with Shiga-like toxin-1. Although Shiga-like toxin-producing *E. coli*-HUS (STEC-HUS) strains most often trigger HUS, certain Shiga toxin-secreting strains of *S. dysenteriae* can also cause HUS. They are currently known as the Shiga toxin family, and the terms are often used interchangeably. HUS occurring from infection with STEC-HUS was formerly called diarrhea + HUS (D + HUS) or typical HUS.

In contrast, HUS that is not related to Shiga toxins and accounts for ~10 % of all HUS cases, is called atypical HUS (aHUS). Although STEC-HUS is relatively common in children, aHUS occurs in individuals of all ages and is often familial. The prognosis is very poor, with the first aHUS attack being associated with a mortality rate of ~25 %, and with ~50 % of cases resulting in end-stage renal disease requiring dialysis [3].

In recent years, abnormalities in the mechanisms underlying complement regulation have been focused on as causes of aHUS. Various genetic abnormalities in complement regulatory factors, including complement factor H, have been noted in 50–60 % of patients. The analysis of the pathology underlying this condition is currently progressing rapidly [4].

The differential diagnosis of aHUS from STEC-HUS or thrombotic thrombocytopenic purpura (TTP), another form of thrombotic microangiopathy (TMA) caused by a deficiency of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), is not necessarily easy at the early stages of disease onset. However, if treatment is delayed, there is a high risk of this syndrome progressing to renal failure. Therefore, the Joint Committee of the Japanese Society of Nephrology and the Japan Pediatric Society (JSN/JPS) has developed diagnostic criteria for aHUS to enable its early diagnosis and to facilitate the timely initiation of appropriate treatment [5, 6]. We hope that the diagnostic criteria presented in this report will become familiar to as many clinicians as possible and that they will be used widely.

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Definition of aHUS

aHUS is a type of TMA that differs from STEC-HUS and TTP, with the latter being caused by markedly reduced ADAMTS13 activity. aHUS is a syndrome characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and AKI, which is similar to STEC-HUS.

Guidelines for the diagnosis of aHUS

Definitive diagnosis

A definitive diagnosis of aHUS is made when the triad of microangiopathic hemolytic anemia, thrombocytopenia, and AKI is present. The disease should not be associated with Shiga toxins, and TTP should also be excluded.

The Joint Committee of the JSN/JPS defined microangiopathic hemolytic anemia based on a hemoglobin (Hb) level of <10 g/dL. The presence of microangiopathic hemolytic anemia should be confirmed based on increased serum lactate dehydrogenase levels, a marked decrease in serum haptoglobin levels, and the presence of red blood cell fragments in a peripheral blood smear.

Thrombocytopenia is defined as a platelet (PLT) count of <150,000/μL.

The definition of AKI has been updated, with the most recent definition given by the international guidelines group, the Kidney Disease: Improving Global Outcomes that integrates both the Risk, Injury, Failure, Loss, End-stage kidney disease and the Acute Kidney Injury Network classifications to facilitate identification. Thus, we recommend diagnosis based on the most recent guidelines, along with the following definitions. For pediatric cases, the serum creatinine should be increased to a level that is 1.5fold higher than the serum creatinine reference values based on age and gender issued by the Japanese Society for Pediatric Nephrology [7]. For adult cases, the diagnostic criteria for AKI should be used.

Guidelines for the diagnosis of aHUS

Definitive diagnosis

A definitive diagnosis of aHUS is made when the triad of microangiopathic hemolytic anemia, thrombocytopenia,

Table 1 Definitions of microangiopathic hemolytic anemia, thrombocytopenia, and AKI that have been established by the joint committee of the JSN/JPS

Microangiopathic hemolytic anemia	Thrombocytopenia	Acute kidney injury
Defined as an Hb level <10 g/dL Presence confirmed based on: Increased serum LDH levels Marked decreases in serum haptoglobin levels The presence of red blood cell fragments in a peripheral blood smear	Defined as a PLT count <150,000/μL	The most recent AKI definition is provided by the international guideline group, the KDIGO, integrating the RIFLE and AKIN classifications to facilitate identification. Thus, diagnosis should be based on the most recent guidelines, and the following definitions should be used. Pediatric cases: Serum creatinine should be increased to a level that is 1.5fold higher than the serum creatinine reference values based on age and gender issued by the Japanese Society for Pediatric Nephrology [7]. Adult cases: Diagnostic criteria for AKI should be used

Hb hemoglobin, *LDH* lactate dehydrogenase, *PLT* platelet, *AKI* acute kidney injury, *KDIGO* kidney disease: improving global outcomes, *RIFLE* risk, injury, failure, loss, end-stage kidney disease, *AKIN* acute kidney injury network

and AKI is present. The disease should have no association with Shiga toxins, and TTP should also be excluded. Table 1 presents the definitions of microangiopathic hemolytic anemia, thrombocytopenia, and AKI that are established by the Joint Committee of the JSN/JPS.

Probable diagnosis

A probable diagnosis of aHUS is made when 2 of the following 3 conditions are found: microangiopathic hemolytic anemia, thrombocytopenia, and AKI. The disease should have no association with Shiga toxins and TTP should be excluded.

Applicability of these diagnostic criteria

When we applied these diagnostic criteria to the Nara Medical University (NMU) TMA cohort, 15 out of 37 individuals who had all the data required for the assessment were diagnosed as having definitive aHUS. Since the data were recorded at one time point only, we speculate that the sensitivity of the diagnostic criteria would increase if we could assess data from multiple time points. The cut-off value for anemia, defined as an Hb level of <10 g/dL, and the cut-off value for thrombocytopenia, defined as a PLT count of <150,000/μL, are equivalent to those employed by the International Registry of Recurrent and Familial HUS/TTP [8]. We had considered using a cut-off value of a PLT count <100,000/μL for thrombocytopenia to reflect that used in the diagnostic criteria for STEC-HUS by the Japanese Society for Pediatric Nephrology (2000), but we only found 1 patient with a PLT count between 100,000 and 150,000/μL in the NMU cohort. Therefore, it is likely that this difference will not have a large impact on the sensitivity or specificity of our diagnostic criteria. Our diagnostic criteria include the category of "Probable" aHUS because we believe that this tentative diagnosis will help in the early diagnosis of aHUS and avoid delays in developing appropriate therapeutic approaches for patients with aHUS.

Evaluation of inappropriate complement activation

Abnormalities in complement regulation are among the main causes of aHUS. The diagnosis of aHUS that is caused by inappropriate complement activation has become more critical because eculizumab, a humanized anti-C5 monoclonal antibody, has been shown to be an effective therapeutic modality [9] that has been approved for the treatment of aHUS patients in Europe and the United States. Recently, Fan and colleagues evaluated genotype–phenotype relationships in 10 Japanese patients with aHUS and identified potentially causative mutations in complement factor H, C3, membrane cofactor protein, and thrombomodulin in 8 of the patients [10]. However, the definitive diagnosis of inappropriate complement activation in aHUS patients is difficult because some patients show normal serum levels of complement components [11] and there are a number of complement regulatory proteins, making it difficult to decide which complement regulatory protein is responsible for a particular patient developing aHUS.

Excluding Shiga toxin-producing *E. coli* infection

STEC-HUS is characterized by diarrhea accompanied by bloody stools. However, diarrhea may also be present in some aHUS cases. Diarrhea in aHUS can be a manifestation of ischemic colitis. In addition, enteritis that is not caused by STEC can trigger aHUS. Therefore, a diagnosis of STEC-HUS cannot be made based on symptoms alone, and the earlier nomenclature that used "D + HUS" to correspond with STEC-HUS and "D-HUS" to correspond

with aHUS is not used at present [11]. The involvement of Shiga toxins should be confirmed by stool culture, the direct detection of Shiga toxins, or the detection of anti-lipopolysaccharide-IgM antibodies.

Excluding TTP

Conventionally, TTP has been diagnosed based on the classic pentad (microangiopathic hemolytic anemia, thrombocytopenia, labile psychoneurotic disorder, fever, and renal failure). However, the discovery of ADAMTS13 led to the finding that 60–90 % of patients with TTP have a marked reduction in the activity of ADAMTS13, to a level of <5 %, regardless of race. Therefore, when diagnosing aHUS, patients who have markedly reduced levels of ADAMTS13 activity (<5 %) should be diagnosed as having TTP, thereby ruling out a diagnosis of aHUS. However, some patients may show the classic TTP pentad and have normal or slightly reduced levels of ADAMTS activity. Therefore, if a patient has levels of ADAMTS13 activity ≥5 %, a differential diagnosis of aHUS or TTP may be necessary to account for other clinical symptoms.

Excluding TMA caused by other distinct factors

Diseases that evidently cause a clinical state of TMA, including disseminated intravascular coagulation, sclerodermatous kidney, and malignant hypertension, should be excluded when diagnosing aHUS.

When a probable case of aHUS is suspected

When a probable case of aHUS is suspected, samples that are necessary to determine the appropriate diagnosis should be collected, and the therapeutic strategy should be established after consultation with an institution that has extensive experience of managing aHUS cases.

Cases where aHUS should be strongly suspected

If there are features that are characteristic of HUS, aHUS should be strongly suspected if the following criteria are fulfilled, regardless of the presence of diarrhea: the patient is younger than 6 months of age; time of onset is unclear (latent onset); the patient has a history of HUS (recurrent case); the patient has a history of anemia of unknown cause; recurrent HUS after kidney transplantation; the patient has a family history of HUS (excluding cases of food poisoning); and, the patient has no diarrhea or bloody stools.

Classification of aHUS causes, excluding TTP caused by the ADAMTS13 defect

Table 2 classifies the causes of aHUS and presents methods to determine the causes.

Discussion

Nineteen years after Gasser et al. [1] reported HUS, an interesting report was published in the *Lancet* [10]. This report indicated that although C3-predominant activity is initiated in the blood vessels in TMA patients, this is not observed in typical cases of HUS, suggesting that complement activation is involved in aHUS onset [12]. Subsequently, numerous researchers have elucidated further information on the pathology of aHUS. At present, the reported causes of aHUS include, complement regulation abnormalities, cobalamin metabolism disorder, infection with *Streptococcus pneumoniae* and other microorganisms, drugs, pregnancy, and autoimmune diseases.

The complement system plays an important role as part of the immune systems of living organisms. It is activated via 3 pathways, the classical, alternative, and lectin pathways. As a result of the activation of the host's alternative and classical pathways, C5b-9, a membrane attack complex, is generated and destroys cells by forming transmembrane pores. The alternative pathway is involved in the onset of aHUS. Unlike the classical and lectin pathways, activation of the alternative pathway does not require initiators; it is continuously activated by the spontaneous hydrolysis of C3.

When complement proteins are inappropriately activated, there is a risk of inducing cell dysfunction within the host itself. Thus, humoral factors in the circulating plasma and several plasma membrane-bound factors are involved in the regulation of complement activation and act at various stages, such as the inactivation of C3b or C4b, and the inhibition of the generation of membrane attack complexes. The regulators involved in the alternative pathway include complement factors H and I, which are humoral factors, and membrane cofactor protein and thrombomodulin, which are membrane-bound factors. If these factors are abnormal, the subsequent failure of regulation will hyperactivate the complement proteins, leading to the onset of aHUS. Some cases of aHUS develop after trigger events, for example, infections of the respiratory tract and the gastrointestinal tract, and it is likely that activation of the complement cascade by these trigger events and the

Table 2 Classification and determination of the causes of aHUS, excluding TTP caused by the ADAMTS13 defect

Cause of aHUS	Method to determine the cause
Complement regulation abnormality	Hemolysis test, quantification of complement proteins and complement regulatory proteins, and gene analysis. Even if the amounts of complement proteins and complement regulatory proteins are within the normal ranges, it does not serve as a basis for excluding complement-related aHUS
(i) Congenital	
Genetic mutations of complement proteins, factor H, factor I, membrane cofactor protein, C3, factor B, and thrombomodulin	
(ii) Acquired	
Production of autoantibodies, including anti-factor H antibody	Detection of anti-factor H antibody by ELISA, western blot, etc.
(2) Cobalamin metabolism disorder	Age at onset should be considered (<6 months old), and hypomethioninemia or hyperhomocysteinemia is detected on plasma amino acid analysis
(3) Infection	Definitive diagnosis by identification of pathogenic microorganisms and serological examination
(i) Pneumococcus	
(ii) Human immunodeficiency virus	
(iii) Pertussis	
(iv) Influenza	
(v) Varicella	
(4) Drug-induced	Identification of the drug
(i) Anticancer drugs	
(ii) Immunomodulatory drugs	
(iii) Antiplatelet drugs	
(5) Pregnancy-related	
(i) Hemolysis, elevated liver enzymes, low platelet counts (HELLP) syndrome	
(ii) Eclampsia	
(6) Autoimmune disease, collagen disease	Definitive diagnosis by autoantibody test, antiphospholipid antibody test, and serological examination
(i) Systemic lupus erythematosus	
(7) Bone-marrow transplant, organ transplant-related	
(8) Others	

aHUS atypical hemolytic uremic syndrome, *ELISA* enzyme-linked immunosorbent assay

subsequent amplification of complement activation by the alternative pathway cannot be regulated in patients with deficiencies in complement regulation. Gain-of-function mutations in C3 and complement factor B, which are complement-activating factors, also cause hyperactivation of complement proteins and, ultimately, aHUS.

It has been reported that ~50 % of aHUS patients have genetic abnormalities in complement regulatory factors, including complement factor H. The frequency of the presence of certain mutations among aHUS cases, responsiveness to plasma therapy, prognosis of kidney function, and the recurrence rate after kidney transplantation, vary depending on the type of genetic abnormalities present [13]. Although plasmapheresis within 24 h of confirmation of the diagnosis has been recommended as the initial treatment for aHUS [14], its effects are not always satisfactory. The mortality or incidence of end-stage renal disease is considered to be between 70 and 80 %, and the recurrence rate after kidney transplantation may be as high as 80–90 %, particularly in patients with abnormal complement factor H, which is the most frequent abnormality [15].

In 2011, eculizumab (Soliris®, Alexion Pharmaceuticals), a terminal complement inhibitor, was approved as a new drug for the treatment of aHUS in Europe and the US. Eculizumab is a humanized recombinant immunoglobulin G2/4 monoclonal antibody directed against the complement component C5, which was developed as a treatment for paroxysmal nocturnal hemoglobinuria. By binding to complement component C5, the drug inhibits the generation of C5a and C5b-9, and thus subsequently inhibits the complement system.

There are a number of reports stating that only HUS that is associated with complement regulation abnormalities is defined as aHUS. On the basis of the current diagnostic criteria, we have defined aHUS to include all types of HUS that are not related to Shiga toxins or other distinct causes. In cases where aHUS is associated with complement

dysregulation, the introduction of eculizumab may markedly change therapeutic strategies. It should be noted, however, that recommendations of specific therapeutic modalities are beyond the scope of the current diagnostic criteria. However, in cases where complement dysregulation is confirmed as the cause, treatment with eculizumab is established. Thus, it may be desirable to assign HUS associated with complement dysregulation a separate disease name rather than it being classified as "aHUS", as in the case of definitive "complement-mediated TMA".

As described in previous reports, aHUS is a disease that may frequently cause renal failure and be fatal if it is not appropriately diagnosed and treated at the early stages of disease onset. In Japan, aHUS may be misdiagnosed as HUS caused by Shiga toxins because clinicians are not sufficiently aware of aHUS, and consequently, treatment may be delayed. Thus, our diagnostic criteria include the category of "Probable" aHUS to ensure that the clinicians consider aHUS during diagnosis. Many issues should be addressed in the future, including the development of diagnostic strategies to diagnose cases of suspected aHUS, the establishment of insurance coverage for ADAMTS13 activity measurement testing that is necessary to differentiate aHUS from TTP, and the development of treatment guidelines. We hope that our diagnostic criteria will be used widely and will contribute to the diagnosis and treatment of aHUS patients.

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