Pediatr Nephrol (2016) 31:105–112
DOI 10.1007/s00467-015-3196-1

ORIGINAL ARTICLE

Insignificant impact of VUR on the progression of CKD in children with CAKUT

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Received: 7 February 2015 / Revised: 13 August 2015 / Accepted: 14 August 2015 / Published online: 24 September 2015

Abstract
Background Vesicoureteral reflux (VUR) is associated with an increased risk of kidney disorders. It is unclear whether VUR is associated with progression from chronic kidney disease (CKD) to end-stage kidney disease (ESKD) in children with congenital anomalies of the kidney and urinary tract (CAKUT).
Methods We conducted a 3-year follow-up survey of a cohort of 447 children with CKD (stage 3–5). Rates of and risk factors for progression to ESKD were determined using the Kaplan–Meier method and Cox regression respectively.
Results Congenital anomaly of the kidney and urinary tract was the primary etiology in 278 out of 447 children; 118 (42.4 %) had a history of VUR at the start of the cohort study. There were significantly more boys than girls with VUR, whereas the proportions were similar in children without VUR. The types of urinary anomalies/complications of the two groups were significantly different. Three-year renal survival rates of the groups were not significantly different, irrespective of CKD stage. Age <2 years and age after puberty, stage 4 or 5 CKD, and heavy proteinuria, but not history of VUR, were significantly associated with progression to ESKD.
Conclusions History of VUR at the start of follow-up was not associated with the progression of stage 3–5 CKD in children with CAKUT.

Keywords Chronic kidney disease · Cohort study · Congenital anomalies of the kidney and urinary tract · End-stage kidney disease · Vesicoureteral reflux

Electronic supplementary material The online version of this article (doi:10.1007/s00467-015-3196-1) contains supplementary material that is available to authorized users

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Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT), particularly hypoplastic and dysplastic kidneys, are the most common causes of advanced chronic kidney disease (CKD) in children. Children with hypoplastic and dysplastic kidneys often display other structural and physiological abnormalities in the urinary tract system, such as vesicoureteral reflux (VUR). It is probable that VUR predisposes children to urinary tract infections (UTIs) [1–4], although in children with normal kidneys, it was noted that childhood UTIs were unlikely to be associated with the development of CKD [3]. In some studies, VUR was reported to be associated with an increased risk of pyelonephritis, renal scarring, and end-stage kidney disease (ESKD) [5–7], while other studies have reported conflicting results [8–10]. Currently, however, the impact of VUR on the progression of CKD to ESKD in children remains controversial, and it is debated whether VUR is a benign or nonbenign condition [8, 9]. Indeed, some researchers have proposed that VUR is not a risk factor for renal scarring after UTI [10], while another report showed that children with higher-grade VUR (grade III or higher) were more likely to develop renal scarring than children with lower-grade VUR [5]. This controversy extends to the management and treatment of VUR and associated conditions/UTIs, and whether to administer prophylactic antibiotics to prevent recurrent UTIs or consider surgical correction of the ureterovesical junction responsible for VUR [11–13].

In 2010, we started a prospective study of a cohort of 447 Japanese children (aged 3 months to 15 years) with stage 3–5 CKD and reported that 62 % (n=278) of the children had CAKUT [14]. In a subsequent follow-up study, the 1-year renal survival rates, defined as cases that did not progress to ESKD or CKD-related death, were 98.3, 80.0, and 40.9 % for stage 3, 4, and 5 CKD respectively, and risk factors for ESKD were advanced CKD stage, age (<2 years and after the start of puberty), and severe proteinuria [15].

As part of the cohort study, we sent questionnaires to participating institutions to document the clinical characteristics of CKD patients, including history of VUR and its management, history of other urinary tract anomalies and subsequent complications, and history of UTIs. Therefore, the data obtained in this questionnaire provided a valuable opportunity to examine the association, if any, between congenital anomalies of the kidney and VUR. Because we have now accumulated 3 years of follow-up data for our initial cohort of 447 children with stage 3–5 CKD, we also assessed the outcomes of children with a history of VUR at 1 April 2010, namely at the start of the cohort study, in terms of the 3-year renal survival rate. Additionally, we evaluated whether VUR is associated with progression to ESKD. Our objective in this study was to examine the association between a history of VUR at the start of the follow-up and the progression of CKD to ESKD in children with congenital anomalies of the kidney.

Materials and methods

Study design and subjects

The study design and patient population are described in more detail in our previous reports [14, 15]. In August 2010, we sent surveys to 1,190 Japanese institutions asking them to report on their cases of pediatric CKD managed as of 1 April 2010. The first survey documented the number of children with stage 3–5 CKD at each institution. The respondents were asked to review their medical records to determine the numbers of patients with a confirmed diagnosis of CKD, or patients with abnormal serum creatinine values. A total of 925 out of 1,190 institutions (77.7 %) responded to the first questionnaire. In the second survey, questionnaires were sent to 130 institutions treating children with stage 3–5 CKD, as the remaining 795 institutions reported that they did not treat children with stage 3–5 CKD. Respondents were asked to record the clinical characteristics of their patients. In the second survey, 119 out of 130 institutions provided data for 479 children treated within 6 months of 1 April 2010. Of these, 447 children at 113 institutions were eligible, and 278 had a primary etiology of CAKUT [14, 15]. In the other 169 children, CAKUT was not the primary etiology. These children are referred to as children without CAKUT in this study. To determine pubertal stage, the patients were divided into three age groups for boys (<2, ≥2 to <10.8, and ≥10.8 years) and girls (<2, ≥2 to <10.0, and ≥10.0 years), where 10.8 and 10.0 years correspond to the mean age of Japanese boys and girls, respectively, at the start of puberty [16].

Survey on the etiology of chronic kidney disease

The second survey inquired about the factors that led to the discovery of CKD, the presence and type of CAKUT, what led to the detection of CAKUT, and the history/severity of VUR. Among 73 institutions that provided data on VUR assessments, 55 adopted the conventional bottom–up method, and 11 adopted the so-called top–down method (in which 99mTc-dimercaptosuccinic acid renal scans are performed before voiding cystourethrography) [17]. At the other 7 institutions, the method was selected based on the patient's situation. VUR was graded according to the International Classification of Vesicoureteral Reflux [4, 18], into unilateral VUR, low-grade bilateral VUR (with at least one side classified as mild, i.e., grades I and II), and high-grade bilateral VUR (with both sides classified as severe). For the purposes of the present study, we identified all patients with CAKUT and divided them according to the history of VUR, as documented in the second survey.

Survey on patient outcomes at 3 years

To assess the outcomes of patients at 3 years, another survey was sent to the participating institutions (n=113) in July 2013, with a deadline of September 2013. The survey recorded similar information to that recorded in our 1-year follow-up survey [15], and included patient characteristics, cardiac function, blood/urine parameters, renal outcomes, and CKD complications. The present survey also recorded urological complications and bladder dysfunction. As before, all surveys were to be returned using the envelopes provided and data entry was conducted by an independent data center (Japan Clinical Research Support Unit, Tokyo). In the third survey, 91 out of 113 institutions provided data for 384 of the 447 children covered by the second survey.

As previously described, stages 3, 4, and 5 CKD were defined as serum creatinine levels (measured enzymatically) more than twice, four times, and eight times respectively the median normal levels in age- and sex-matched Japanese children [14, 15, 19]. Using the Schwartz equation [20], we verified the accuracy of the classification, yielding a weighted κ-value of 0.71 (95 % confidence interval [CI] 0.65–0.77).

Statistical analyses

The characteristics of children with or without VUR were compared using unpaired t tests for continuous variables and Chi-squared tests for categorical variables. The 3-year renal survival rates were assessed using the Kaplan–Meier method, where death was also considered as an event. The date of measurement of serum creatinine closest to 1 April 2010 represented the starting point (i.e., t=0 years). The log-rank test was used to compare survival rates in patients with and those without VUR at each stage. Cox's proportional hazard regression model was used to identify possible predictors of CKD progression by calculating hazard ratios with 95 % confidence intervals. Values of $P<0.05$ were considered statistically significant. All statistical analyses were carried out using SAS system version 9 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the children with CAKUT

Of 278 children with CAKUT and stage 3–5 CKD, 60 children (21.6 %) had obstructive urological malformations, which included the posterior urethral valve, stricture of the urethra, hydronephrosis, hydroureter, and cloacal anomalies [14]. The characteristics of 278 children with CAKUT according to history of VUR are presented in Table 1. A history of VUR at 1 April 2010 was present in 118 children (42.4 %), and was absent in 115 children (41.4 %), or unknown/not evaluated in 45 children (16.2 %); data were not provided for 9 children (3.2 %). Among 118 children with VUR, 39 (33.3 %) were previously diagnosed with unilateral VUR, 10 (8.8 %) with low-grade bilateral VUR, and 59 (50.4 %) with high-grade bilateral VUR. The other 10 children were reported by the physician to have been diagnosed with VUR, but the classification was not stated. Among 169 children without a primary etiology of CAKUT, 6 had a history of VUR, of whom 3 had neurogenic bladder, 2 had nephronophthisis, and 1 had polycystic kidney disease. The mean ages at April 2010 were comparable in children with/without VUR. Furthermore, the distributions of stage 3–5 CKD were comparable in the two groups, as were the distributions of proteinuria, the use of antihypertensive drugs, and hypertension. However, there were significantly more boys than girls with VUR, whereas the proportions of boys and girls were approximately equal in children without VUR. Sixty children underwent surgical treatment for the correction of VUR. A large proportion of children with VUR had a history of UTI (66.9 %), and about half of the children with VUR had a history of≥2 episodes of UTIs. These percentages were significantly greater than those in children without VUR or in whom voiding cystourethrography was not performed. In addition, there were significant differences in the distribution of the types of urinary anomalies/complications between the groups, with hydronephrosis, megaureter, bladder dysfunction, and posterior urethral valve anomalies being significantly more frequent in children with VUR. Surgical treatment was performed in 58 children with VUR; the characteristics of children who did or did not undergo surgical treatment are presented in Supplementary Table 1.

The factors that led to the detection of CKD are listed in Table 1. Fetal/neonatal ultrasonography was the most common reason that led to the detection of CKD in both groups. However, the history of a UTI led to the detection of CKD in a significantly greater proportion of children with VUR than children without VUR (23.7 % vs 7.0 %, $P<0.001$). The rates of other factors that led to the detection CKD (e.g., incidental finding, failure to thrive, and blood analysis in the neonatal period) were comparable in the two groups.

Renal survival rates

The 3-year renal survival rates in children with CAKUT according to CKD stage are shown in Fig. 1a. As expected, renal survival rates declined with increasing CKD stage at the start of the survey. Figure 1b shows the renal survival rates according to CKD stage and history of VUR at 1 April 2010. The renal survival rates were not significantly different in children with VUR and children without VUR at each CKD stage. The renal survival rate was unaffected by the laterality or severity of VUR (Fig. 1c).

Table 1 Characteristics of children with congenital anomalies of the kidney and urinary tract (CAKUT) according to history of vesicoureteral reflux (VUR)

Variable	History of VUR		*P* value	Unknown VUR status
	Yes	No		
n	118	115		45
Sex, *n* (%)				
Male	91 (77.1)	58 (50.4)	<0.001*	26 (57.8)
Female	27 (22.9)	57 (49.6)		19 (42.2)
Age in April 2010, years	8.04±4.63	8.73±4.60	0.251**	7.23±4.27
Age at diagnosis, years	1.48±2.76	3.01±3.99	0.001**	2.08±3.25
CKD stage 3/4/5, *n* (%)				
Stage 3	77 (65.3)	79 (68.7)	0.432*	33 (73.3)
Stage 4	36 (30.5)	28 (24.4)		10 (22.2)
Stage 5	5 (4.2)	8 (7.0)		2 (4.4)
SCr (mg/dL)	1.66±1.13	1.66±1.24	0.960**	1.40±0.92
eGFR abbreviated (mL/min/1.73 m^2)[a]	38.30±16.98	39.79±16.69	0.513**	38.30±16.98
eGFR complete (mL/min/1.73 m^2)[b]	41.81±12.69	38.98±13.32	0.238**	41.81±12.69
History of UTI, *n* (%)	79 (66.9)	15 (13.0)	<0.001*	5 (11.1)
History of ≥2 UTIs, *n* (%)	56 (47.5)	9 (7.8)	<0.001*	2 (4.4)
Proteinuria (g/g creatinine)	1.23±2.66	0.94±1.01	0.376**	1.23±2.66
Heavy proteinuria, *n* (%)[c]	9 (7.6)	12 (10.4)	0.662*	4 (8.9)
Hypertension, *n* (%)[d]	21 (17.8)	20 (17.4)	0.916*	7 (15.6)
Use of antihypertensive drugs, *n* (%)	24 (20.3)	16 (13.9)	0.171*	1 (2.2)
Urinary anomalies/complications, *n* (%)				
Single kidney	18 (15.3)	19 (16.5)	0.791*	2 (4.4)
MCDK	3 (2.5)	9 (7.8)	0.068	2 (4.4)
Hydronephrosis	40 (33.9)	12 (10.4)	<0.001*	5 (11.1)
Megaureter	19 (16.1)	0 (0.0)	<0.001*	0 (0.0)
Bladder dysfunction	19 (16.1)	2 (1.7)	<0.001*	1 (2.2)
Posterior urethral valve	17 (14.4)	3 (2.6)	<0.001*	0 (0.0)
Duplication of pelvis and ureter	4 (3.4)	2 (1.7)	<0.426*	0 (0.0)
Factors leading to the detection of CKD, *n* (%)				
Fetal/neonatal ultrasonography	31 (26.3)	37 (32.2)	0.678*	11 (24.4)
UTI	28 (23.7)	8 (7.0)	<0.001*	3 (6.7)
Incidental finding	13 (11.0)	18 (15.7)	0.298*	8 (17.8)
Failure to thrive, weight loss, or general fatigue	9 (7.6)	13 (11.3)	0.337*	3 (6.7)
Blood analysis in the neonatal period, asphyxia, neonatal shock, or another event	9 (7.6)	9 (7.8)	0.955*	7 (15.6)

Values are *n* (%) or means±standard deviation

VUR vesicoureteral reflux, *CKD* chronic kidney disease, *SCr* serum creatinine, *eGFR* estimated glomerular filtration rate, *UTI* urinary tract infection, *MCDK* multicystic dysplastic kidney, *BUN* blood urea nitrogen

*Chi-squared test

***t* test

[a] Abbreviated Schwartz equation [20], eGFR=41.3 [height (m)/SCr (mg/dL)]

[b] Complete Schwartz equation [20], 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 if male]×[height (m)/1.4]$^{0.188}$

[c] Urine protein/creatinine ratio >2.0 g/g urine creatinine

[d] Systolic blood pressure >95th percentile

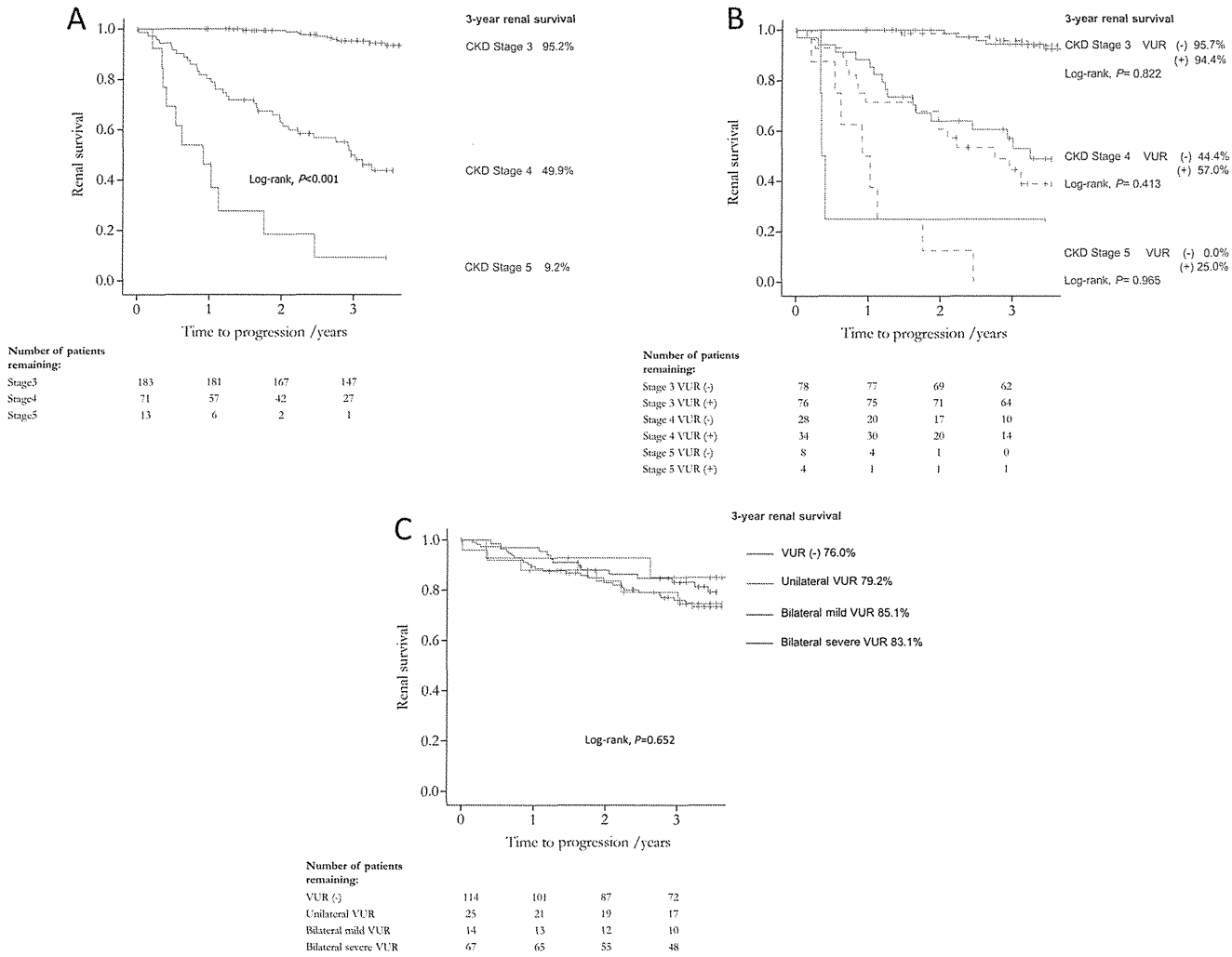

Fig. 1 Renal survival rates at 3 years according to the stage of chronic kidney disease (*CKD*) in patients with congenital anomalies of the kidney and urinary tract (CAKUT). **a** Renal survival rates according to CKD stage (n=267). **b** Renal survival rates according to the presence (n=114) or absence (n=114) of vesicoureteral reflux (*VUR*) for each CKD stage. *Solid lines* children with VUR [VUR (+)]; *dashed lines* children without VUR [VUR (−)]. **c** Renal survival rates according to the laterality and severity of VUR (n=220, those without information on VUR grade were excluded). Children were assessed for progression to end-stage kidney disease or death

Risk factors for progression to end-stage kidney disease

The risk factors for progression to ESKD at 3 years in 278 children with CAKUT, as determined by Cox regression, are shown in Table 2. Age <2 years and age after puberty (vs 2 years to the start of puberty), stage 4 or 5 CKD (vs stage 3 CKD), and heavy proteinuria (urine protein/creatinine ratio >2.0 g/g urine creatinine) were significant risk factors for progression to ESKD. Consistent with the Kaplan–Meier analysis of renal survival rates, the Cox regression model showed that the history of VUR was not associated with progression to ESKD (hazard ratio: 1.19; 95 % confidence interval: 0.53–2.64; P=0.675). Replacing history of VUR with the maximum severity of VUR (none, unilateral, bilateral mild, or bilateral severe) or latest VUR status at 1 April 2010 did not appreciably affect the associations observed (data not shown).

When we repeated this analysis only in children with a history of VUR, the risk factors for progression to ESKD included age and CKD stage, but not sex, heavy proteinuria, or history of surgical treatment of VUR (Supplementary Table 2).

Discussion

This 3-year prospective cohort study of children with stage 3–5 CKD caused by CAKUT revealed that the history of VUR at the start of follow-up did not influence the progression to ESKD in these children. However, there were significant differences in the sex distribution and the frequencies of urinary anomalies/complications between children with VUR and those without VUR. These features suggest that the main cause of kidney dysfunction and its progression in these

Table 2 Risk factors for end-stage kidney disease at 3 years (Cox regression model; n=278)

Variable	HR	95 % CI	P value
Female (vs male)	1.75	0.70–4.37	0.232
Age			
Age <2 years (vs 2 years to the start of puberty)	5.31	0.89–28.83	0.053
Age after puberty (vs 2 years to the start of puberty)	6.25	2.53–15.44	<0.001
CKD stage			
Stage 4 (vs stage 3)	37.45	11.56–121.31	<0.001
Stage 5 (vs stage 3)	249.38	43.20–1439.70	<0.001
History of VUR[a]	1.19	0.53–2.64	0.675
Heavy proteinuria[b]	5.08	1.98–13.05	<0.001

HR hazard ratio, *CI* confidence interval, *CKD* chronic kidney disease, *VUR* vesicoureteral reflux

[a] The associations did not change appreciably when history of VUR was replaced with the severity of VUR (none, unilateral, bilateral mild, or bilateral severe) or the maximum grade of VUR

[b] Urine protein/creatinine ratio >2.0 g/g urine creatinine

children might be the presence of a hypoplastic/dysplastic kidney, not VUR.

Notably, a history of VUR at the start of follow-up did not influence the progression of stage 3–5 CKD to ESKD in our cohort. However, it was of interest that there were several statistically significant and clinically relevant differences in the characteristics of children with VUR versus children without VUR. First, although the two groups of children were of similar age, there were significantly more boys than girls with VUR, whereas the proportions of boys and girls were approximately equal in children without VUR. Second, children with VUR were also more likely to have urinary tract anomalies/complications, including hydronephrosis and posterior urethral valve, compared with children without VUR. These differences, in addition to the nonsignificant effect of VUR on the progression of CKD, suggest that it might be important to consider the background etiology other than VUR, such as hypoplastic/dysplastic kidneys.

It is interesting that the majority of children with VUR had a history of UTIs, and about half of the children had a history of multiple UTIs, consistent with the notion that VUR might be a major risk factor for UTI [21–24]. However, despite the evidence supporting an association between UTIs and VUR, there is no conclusive evidence that either contributes to the progression of CKD to end-stage kidney disease in children. In addition, there is currently a limited consensus on how to treat UTIs, primary VUR, and associated anomalies in children [21, 23].

In the present study, risk factors for progression to ESKD included age <2 years and age after puberty (vs 2 years to the start of puberty), stage 4 or 5 CKD (vs stage 3 CKD), and heavy proteinuria (urine protein/creatinine ratio >2.0). These factors are identical to those identified in our previous study [15], which analyzed data on children with CKD with a 1.49-year follow-up period, and were confirmed to be risk factors in those with CAKUT with a longer follow-up period. Importantly, however, the 3-year renal survival rate was not markedly affected by a history of VUR at the start of follow-up in children with stage 3–5 CKD. These findings were also supported by the results of Cox regression, which further revealed that history of VUR was not a risk factor for progression to ESKD in this cohort of children. Our results suggest that VUR itself might not markedly affect the prognosis of children with CAKUT and CKD, addressing the controversy surrounding whether VUR is a benign or nonbenign condition, and helping to clarify the relationship between VUR and progression to ESKD in children with stage 3–5 CKD.

Some potential limitations warrant a mention. First, we did not observe the course of these children during stage 1–2 CKD. It is conceivable that VUR influences the emergence of CKD or progression through the early stages (i.e., stages 1–2). We must also consider that we used "history of VUR" (i.e., the diagnosis of VUR at any time) in the analyses examining the influence of VUR on the progression of CKD. Although similar results were obtained using the "severity of VUR" and the latest VUR status at the start of follow-up, many cases of VUR spontaneously resolve in clinical practice. Indeed, the severity of VUR is changeable over time; thus, the grade at any one time is difficult to know, especially given the invasiveness of the examination for VUR. Additionally, many children with a history of VUR underwent surgical treatment, which may have attenuated the pathological effects of VUR. However, the surgical treatment of VUR was not associated with progression to ESKD (Supplementary Table 2, Supplementary Figure). A similar limitation also applies to the analysis of UTIs, which was assessed as the history of UTIs before 2010. Another limitation is the duration of the survey period; 3 years may be too short to detect progression of CKD to ESKD in some children, depending on the cause of CKD.

In conclusion, about 40 % of children with CAKUT, who account for approximately 60 % of pediatric patients with stage 3–5 CKD, also had a history of VUR. However, the

history or severity of VUR was not associated with increased risk of progression of CKD to ESKD during a follow-up of 3 years. We found marked differences in the general and clinical characteristics of children with VUR vs children without VUR in terms of sex distribution and the proportions of children with urinary tract anomalies. Therefore, while children with CKD caused by CAKUT may have diverse backgrounds, the presence or absence of VUR may enable different backgrounds to be distinguished. Nevertheless, we observed no association between a history of VUR at the start of follow-up and the progression of CKD to ESKD in these children, which may implicate some background etiological factor other than VUR, such as the hypoplastic/dysplastic kidney itself, as the main cause of CKD and its progression to ESKD.

Acknowledgements The authors would like to thank Drs Takuhito Nagai (Aichi), Kenichi Satomura (Osaka), Tomoo Kise (Okinawa), Takuji Yamada (Aichi), Midori Awazu (Tokyo), Hiroshi Asanuma (Tokyo), Toshiyuki Ohta (Hiroshima), Takeshi Matsuyama (Tokyo), Hidefumi Nakamura (Tokyo), Mayumi Sako (Tokyo), Tomoyuki Sakai (Shiga), Yusuke Okuda (Shiga), Shunsuke Shinozuka (Saitama), Yoshinobu Nagaoka (Hokkaido), Shuichiro Fujinaga (Saitama), Hiroshi Kitayama (Shizuoka), Naoya Fujita (Shizuoka), Masataka Hisano (Chiba), Daishi Hirano (Tokyo), Yuko Akioka (Tokyo), Naoaki Mikami (Tokyo), Hiroshi Hataya (Tokyo), Hiroyuki Satoh (Tokyo), Tae Omori (Tokyo), Takashi Sekine (Tokyo), Yoshimitsu Goto (Aichi), Yohei Ikezumi (Niigata), Takeshi Yamada (Niigata), and Akira Matsunaga (Yamagata) of The Pediatric CKD Study Group in Japan for their contributions to the study. The authors would also like to thank all the institutions that participated in the surveys listed in the Supplement, and Mr Masaaki Kurihara, Ms Chie Matsuda, Ms Naomi Miyamoto, and Ms Takako Arai of the Japan Clinical Research Support Unit (Tokyo) for their help with data management; Dr Naoaki Mikami and Ms Sachiko Kawabe of Tokyo Metropolitan Children's Medical Center for their contribution to manuscript preparation; and Nicholas Smith, PhD, of Edanz Group Ltd., for providing language editorial support in the preparation of the manuscript. The results presented in this paper have not been published previously in whole or part, except in abstract format.

Ethics 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 the ethics committee of the Tokyo Metropolitan Children's Medical Center (ID: 23–49). Because data were reported using patient medical records, informed consent was not obtained in accordance with the above guidelines.

Funding This work was supported by a Health and Labour Sciences Research Grant for Research on Rare and Intractable Diseases from the Ministry of Health, Labour, and Welfare, Japan (H25-nanchitou(nan)-ippan-017 and H26-nanchitou(nan)-ippan-036) and the 2013 Tokyo Metropolitan Hospitals' Clinical Research Fund (Special Research).

Conflict of interest Kenji Ishikura has received lecture fees from Novartis Pharma and Asahi Kasei Pharma. Osamu Uemura has received lecture fees from Asahi Kasei Pharma, Kyowa Hakko Kirin, Takeda Pharmaceutical, 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. Hideo Nakai has received a research grant from Astellas Pharma. Ryojiro Yasuo Ohashi has received research grants from Kyowa Hakko Kirin and Chugai pharmaceutical. Tanaka has received lecture fees from Pfizer Japan and Asahi Kasei Pharma. Koichi Nakanishi has received lecture fees from Novartis Pharma, Asahi Kasei Pharma, and Astellas Pharma. Kazumoto Iijima has received research grants from Novartis and Pfizer Japan, and lecture fees from Novartis, Asahi Kasei Pharma, and Pfizer Japan. Masataka Honda has received lecture fees from Novartis Pharma, Asahi Kasei Pharma, Takeda Pharmaceutical, and Chugai Pharmaceutical. Drs Ito, Harada, Hattori, and Mr Kaneko have no conflicts of interest to declare.

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Clin Exp Nephrol
DOI 10.1007/s10157-015-1098-y

ORIGINAL ARTICLE

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

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Received: 14 May 2014 / Accepted: 15 February 2015

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

On behalf of The Pediatric CKD Study Group in Japan in conjunction with the Committee of Measures for Pediatric CKD of the Japanese Society of Pediatric Nephrology.

Electronic supplementary material The online version of this article (doi:10.1007/s10157-015-1098-y) contains supplementary material, which is available to authorized users.

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Published online: 26 February 2015

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 $\leq$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.

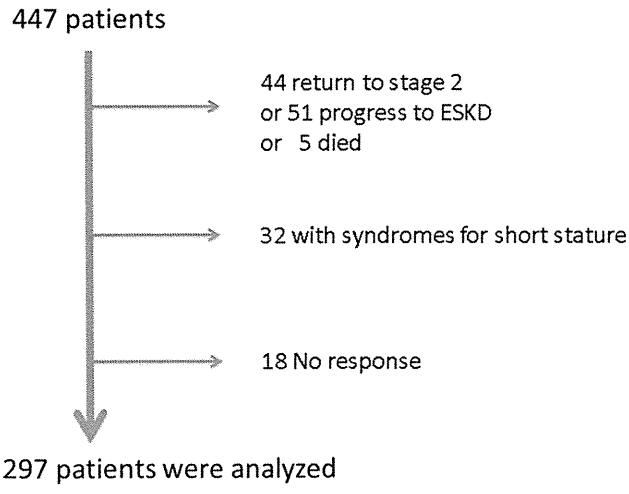

Fig. 1 Patient disposition

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]

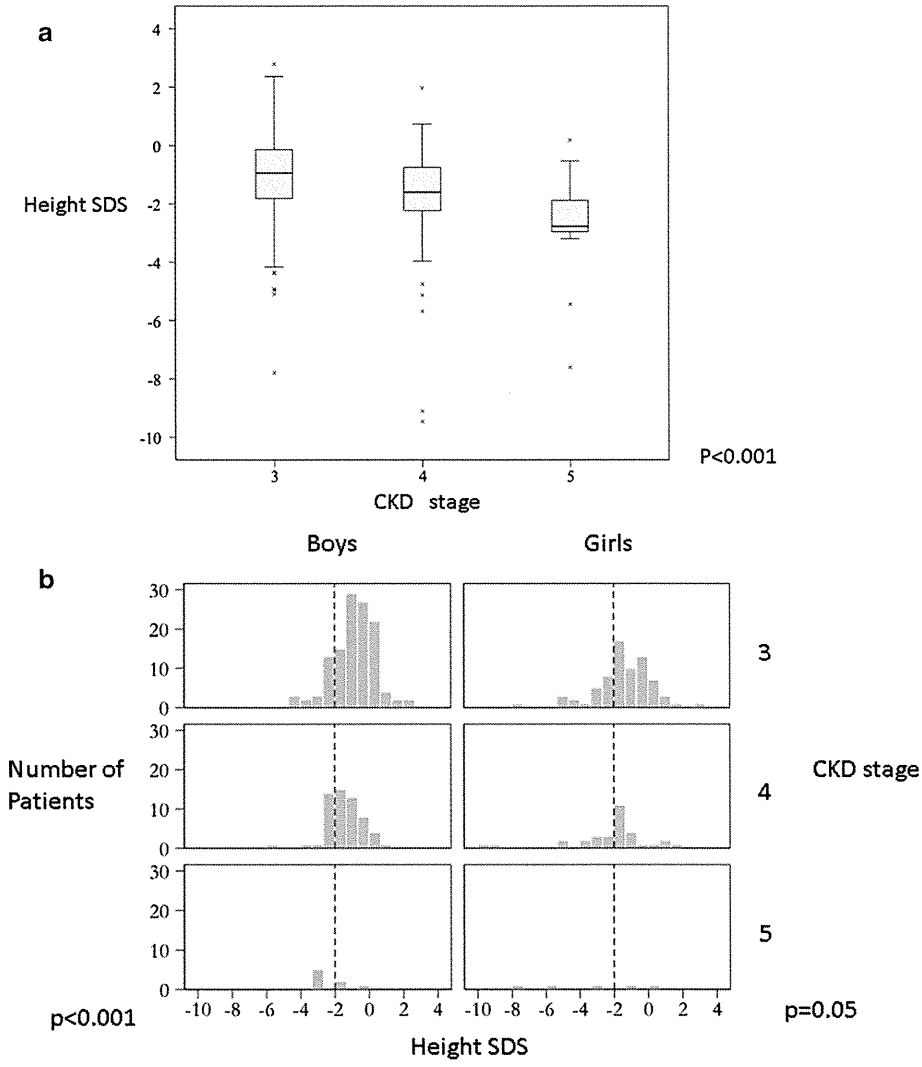

Fig. 2 **a** Height SDS according to CKD stage. The *bottom*, *middle*, and *top lines* of each box represent the 25th, 50th (median), and 75th percentiles of height SDS, respectively. The ends of the whiskers represent the range from 1.5 times the interquartile range (IQR) added to the 75th percentile 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).

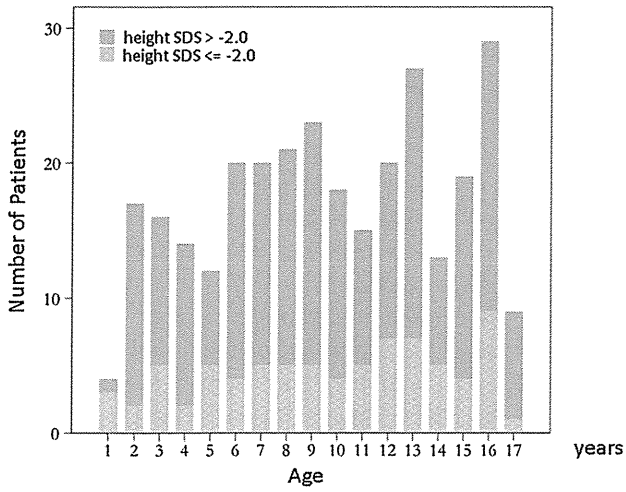

Fig. 3 Age distribution of patients by gender. The numbers of patients are plotted against age in years. *Blue bars* height SDS > -2, *Pink bars* height SDS ≤ -2

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

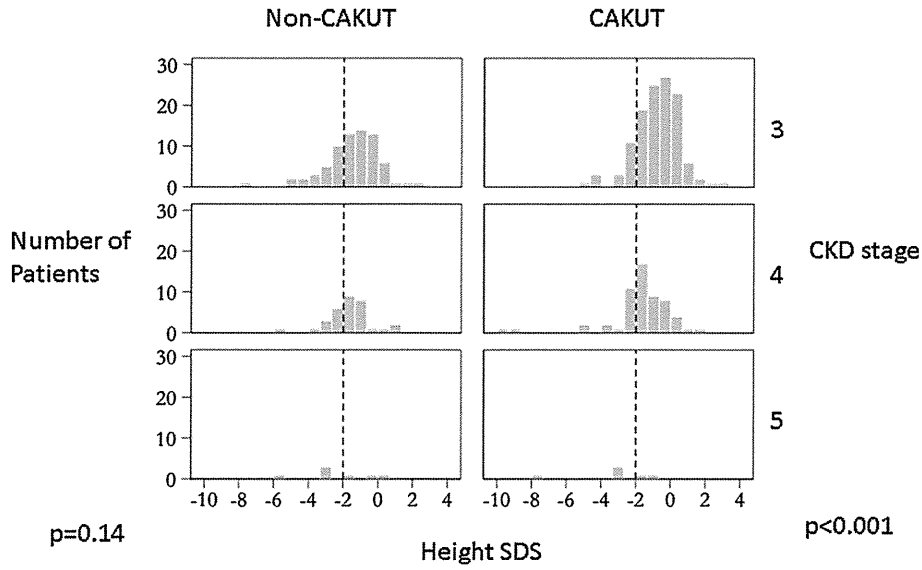

Fig. 4 Distribution of height SDS according to CKD stage and primary disease (CAKUT/non-CAKUT). *SDS* standard deviation score, *CKD* chronic kidney disease, *CAKUT* congenital anomalies of the kidney and urinary tract

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

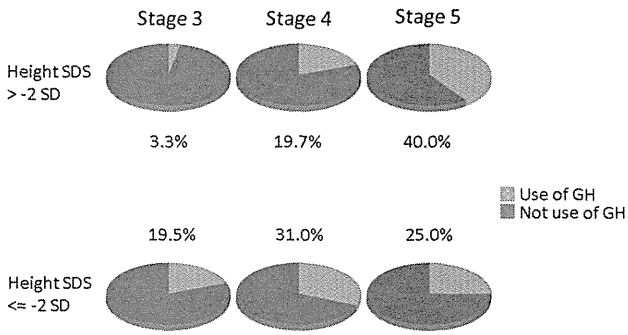

Fig. 5 Use of growth hormone according to CKD stage and height SDS. *SDS* standard deviation score, *CKD* chronic kidney disease

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 $\leq$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.

Acknowledgments This work was supported by 'Research on Rare and Intractable Diseases, Health and Labour Sciences Research Grants' from the Ministry of Health, Labour and Welfare, Japan. The results presented in this paper have not been published previously in whole or part, except in abstract format. The authors would like to thank Drs. Takuhito Nagai (Aichi), Kenichi Satomura (Osaka), Midori Awazu (Tokyo), Toshiyuki Ohta (Hiroshima), Kazumoto Iijima (Hyogo), Takeshi Matsuyama (Tokyo), Mayumi Sako (Tokyo), Hidefumi Nakamura (Tokyo), Shuichiro Fujinaga (Saitama), Hiroshi Kitayama (Shizuoka), Naoya Fujita (Shizuoka), Masataka Hisano (Chiba), Yuko Akioka (Tokyo), Daishi Hirano (Tokyo), Hiroshi Hataya (Tokyo), Shunsuke Shinozuka (Tokyo), Ryoko Harada (Tokyo), Yoshinobu Nagaoka (Hokkaido), Takashi Sekine (Tokyo), Yoshimitsu Goto (Aichi), Takuji Yamada (Aichi), Yohei Ikezumi (Niigata), Takeshi Yamada (Niigata), and Akira Matsunaga (Yamagata) of The Pediatric CKD Study Group in Japan for their contributions to the study. The authors also thank the institutions listed in the Supplementary Table for participating in the surveys, and Mr. Masaaki Kurihara, Ms. Chie Matsuda and Ms. Naomi Miyamoto of the Japan Clinical Research Support Unit (Tokyo) for their help with data management.

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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European Journal of Human Genetics (2015), 1–5

www.nature.com/ejhg

ARTICLE

Somatic mosaicism and variant frequency detected by next-generation sequencing in X-linked Alport syndrome

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X-linked Alport syndrome (XLAS) is a progressive, hereditary nephropathy. Although men with XLAS usually develop end-stage renal disease before 30 years of age, some men show a milder phenotype and develop end-stage renal disease later in life. However, the molecular mechanisms associated with this milder phenotype have not been fully identified. We genetically diagnosed 186 patients with suspected XLAS between January 2006 and August 2014. Genetic examination involved: (1) extraction and analysis of genomic DNA using PCR and direct sequencing using Sanger's method and (2) next-generation sequencing to detect variant allele frequencies. We identified somatic mosaic variants in the type VI collagen, α5 gene (*COL4A5*) in four patients. Interestingly, two of these four patients with variant frequencies in kidney biopsies or urinary sediment cells of ≥ 50% showed hematuria and moderate proteinuria, whereas the other two with variant frequencies of < 50% were asymptomatic or only had hematuria. *De novo* variants can occur even in asymptomatic male cases of XLAS resulting in mosaicism, with important implications for genetic counseling. This is the first study to show a tendency between the variant allele frequency and disease severity in male XLAS patients with somatic mosaic variants in *COL4A5*. Although this is a very rare status of somatic mosaicism, further analysis is needed to show this correlation in a larger population.
European Journal of Human Genetics advance online publication, 27 May 2015; doi:10.1038/ejhg.2015.113

INTRODUCTION

Alport syndrome (AS) is a hereditary disorder of type IV collagen, characterized by chronic kidney disease progressing to end-stage renal disease (ESRD), sensorineural hearing loss, and ocular abnormalities. Approximately 85% of AS patients show X-linked inheritance (XLAS: OMIM301050) and variants in *COL4A5*, which encodes the type IV collagen α5 (α5(IV)) chain. *COL4A5* variants result in abnormal α5 (IV) expression, typically with 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]

Male patients with XLAS can be classified as having either 'adult type', associated with mild deafness and the development of ESRD >30 years of age, or 'juvenile type', associated with hearing loss and often with lenticonus, and an onset of ESRD <30 years of age.[2] These two phenotypes are partially related to the genotype; for example, missense variants or in-frame variants of *COL4A5* were reported in cases of later-onset ESRD.[3–5] We recently reported that 29% of male XLAS patients expressed the α5(IV) chain in the glomerulus and showed milder clinical manifestations.[6] Interestingly, all α5(IV)-positive patients possessed non-truncating variants ($n = 13$) or somatic mosaic variants ($n = 2$) of *COL4A5*. One of these patients has been described in a previous case report.[7] This implies that men with XLAS and somatic mosaic variants show milder phenotypes; however, no case series has reported the correlation between variant frequency and disease severity in patients with somatic mosaic variants. The present study, therefore, examined the correlation between variant frequency and phenotype in a case series of male XLAS patients with somatic mosaic variants using next-generation sequencing (NGS). We provide herein the first report of an asymptomatic male XLAS case and also describe the first cases of somatic and gonadal mosaic variants in *COL4A5*.

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 all patients or their parents.

Data collection

Clinical and laboratory findings of 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.

Estimated glomerular filtration rates (eGFRs) were measured from the data in these data sheets. eGFRs were calculated using the Schwartz formula for patients aged ≤ 19 years, and the Cockcroft–Gault formula for patients aged ≥ 20 years.[8–10]

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Received 8 January 2015; revised 24 April 2015; accepted 29 April 2015

Mutational analyses using Sanger sequencing

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 and (2) reverse-transcription PCR of mRNA and direct sequencing of abnormal mRNA products when a suspected splicing-site variant was detected.

Genomic DNA was isolated from peripheral blood leukocytes, urinary sediments, kidney biopsies, skin and/or hair roots from patients, and their parents using the Quick Gene Mini 80 System (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's instructions. For genomic DNA analysis, all 51 *COL4A5* exons were amplified by PCR, as described previously.[11] PCR-amplified products were then purified and subjected to direct sequencing using a Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA) with an automatic DNA sequencer (ABI Prism 3130; Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Mutational analysis data were submitted to the Alport syndrome and *COL4A5* database (http://www.arup.utah.edu/database/ALPORT/ALPORT_welcome.php). For variant description, reference sequences were NC_000023.9 and NM_000495.3. Exons were numbered according to a previous report.[12]

Mutational analysis using NGS

A subset of exome-targeting genes with disease-causing variants were subjected to NGS using a commercially available kit (TruSight One, Illumina, San Diego, CA, USA) and targeted resequencing as a means of deep sequencing. Following the TruSight workflow, input genomic DNA was converted into adapter-tagged libraries by rapid Nextera (Nextera DNA Library Preparation Kit, Illumina)-based sample preparation. The libraries were then denatured into single-stranded DNA, and biotin-labeled probes specific to the targeted region were used for Rapid Capture hybridization. The pool was enriched for the desired regions by adding streptavidin beads that bound to the biotinylated probes. Biotinylated DNA fragments bound to the streptavidin beads were pulled down magnetically from the solution. The enriched DNA fragments were then eluted from the beads and hybridized for a second Rapid Capture. Sequence data generated from TruSight exome-enriched libraries were analyzed using the on-instrument MiSeq Reporter software (Illumina).

For deep sequencing of somatic mosaic variant analysis, 500-bp PCR products harboring each suspected mutation site were purified by gel extraction using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Each variant was then analyzed using the TruSeq PCR-free LT kit (Illumina). All procedures were conducted according to the manufacturers' instructions. The primer sequences were as follows:

COL4A5-exon25-F: 5′-CCCCAGTTGTATTCAGTA-3′ and COL4A5-exon 25-R: 5′-GAGCAAAATTAACAGTAA-3′; COL4A5-exon28-F: 5′-AAAAGCATA TGTTCCACA-3′ and COL4A5-exon28-R: 5′-GATGATTTGGGGTTAAAT-3′; COL4A5-exon44-F: 5′-ATTTATTCAGGGTAATCC-3′ and COL4A5-exon44-R: 5′-TAAAAGGTCTGCTATCAA-3′; and COL4A5-exon49-F: 5′-GGAGACA ATACTTAGCAAATG-3′ and COL4A5-exon49-R: 5′-ACACCAAGGGTAG TCAAA-3′.

To determine the limit of variant frequency detection, we made test samples containing mixtures of DNA from an XLAS patient with a hemizygous *COL4A5* c.1948+1G>A mutation and control DNA at variant frequencies of 0.5, 1, 2, 10, and 20%. Targeted resequencing was then conducted using the primer pair for *COL4A5* exon25.

RESULTS

Clinical, pathological, and mutational results are shown in Figures 1 and 2, Tables 1 and 2, and Supplementary Table 1. NGS analysis findings including the depth and forward/reverse reads are shown in Supplementary Table 1.

Patient ID14

The pedigree of patient ID14 is shown in Figure 1a. The precise clinical course of this patient has been reported previously.[7] At 16 years of age, the patient had microhematuria and moderate proteinuria with 0.74 g/g creatinine (Cr). Genetic analysis revealed the presence of an intron 43 splicing acceptor site variant (c.3998-2A>T, IVS44-2A>T). Transcriptional analysis showed that this variant caused skipping of exon44 (72 bp).

Patient ID28

A 38-year-old male was detected with microhematuria and proteinuria when he had a common cold; however, he had no urine abnormalities other than on that occasion. His pedigree is shown in Figure 1b. His older daughter also showed macrohematuria when she had a common cold at the age of 3 years, and subsequently demonstrated persistent microhematuria and mild proteinuria (0.2 g/g Cr). She underwent a kidney biopsy and was pathologically diagnosed with XLAS with a basket-weave change (BWC) on the GBM and mosaic α5(IV) expression. Genetic analysis revealed a *COL4A5* heterozygous variant at the intron 27 splicing acceptor site (c.2147-2A>G, IVS28-2A>G), which has been reported previously without precise clinical information.[13] Transcriptional analysis revealed this variant to cause skipping of part of exon28 (18 bp). Her mother was asymptomatic with no variants and her father was also asymptomatic, suggesting that she represents a sporadic case with a *de novo COL4A5* variant. However, the second daughter was also detected with hematuria at a screening test at 3 years of age, and was genetically diagnosed with XLAS with the same variant (IVS28-2A>G). Subsequent genetic testing of the father revealed the same variant with somatic mosaicism in genomic DNA extracted from leukocytes and urine sediments (Figure 2). He was confirmed to have a normal karyotype (46,XY). Because both daughters carry the same heterozygous variant, this indicates that their father also has the same variant in a mosaic state that includes germinal cells. The father was subsequently diagnosed with asymptomatic XLAS with a somatic and gonadal mosaic variant in *COL4A5*.

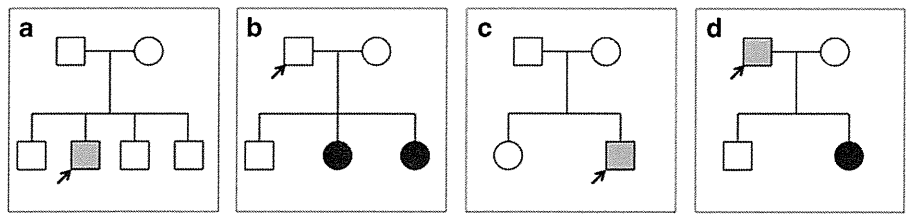

Figure 1 Patient pedigrees. (**a**) Patient ID14 possessing *COL4A5* mutation c.3998-2A>T in intron 43. This individual showed hematuria and moderate proteinuria. The parents are asymptomatic. (**b**) Patient ID28 possessing *COL4A5* mutation c.2147-2A>G in intron 27. This individual is asymptomatic although possesses a somatic and gonadal mosaic variant. One daughter has hematuria and mild proteinuria, whereas the second daughter has hematuria. (**c**) Patient ID52 possessing *COL4A5* mutation c.1912G>A in exon25. This individual has hematuria and moderate proteinuria. The parents are asymptomatic. (**d**) Patient ID 252 possessing *COL4A5* mutation c.4787G>T. This individual has hematuria without proteinuria, and the daughter has hematuria and mild proteinuria.

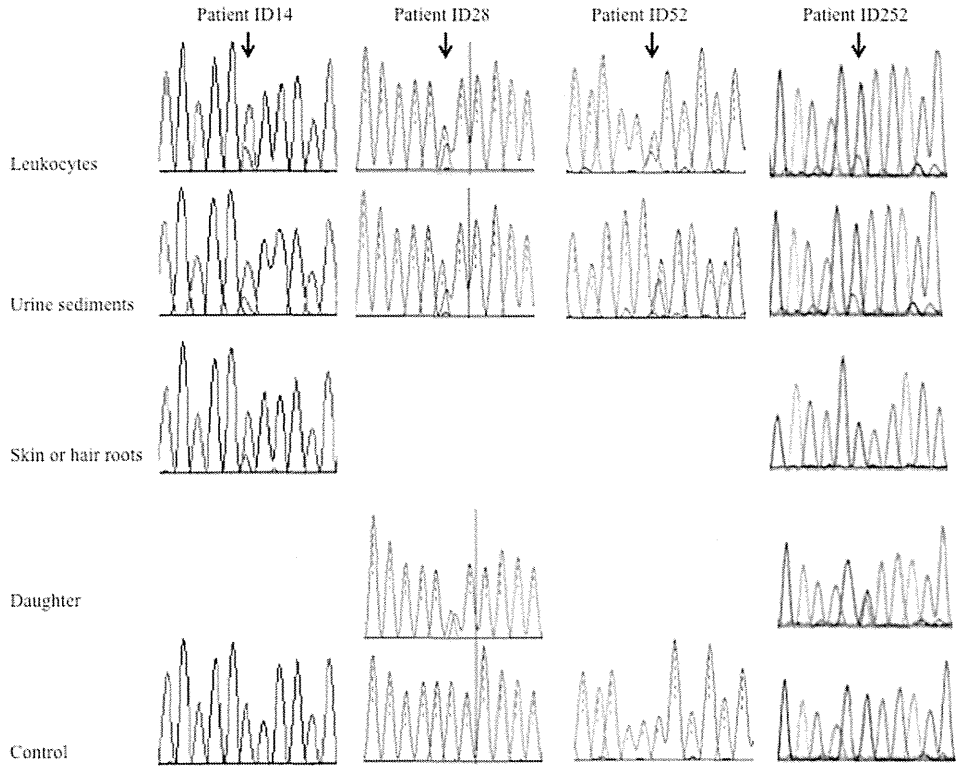

Figure 2 Direct sequencing of patients with somatic mosaic variants. Patient ID14: c.3998-2A>T, IVS44-2A>T. NGS analysis revealed variant allele frequencies of 57.1% in leukocytes, 61.3% in urinary sediment, cells and 75.3% in skin. Patient ID28: c.2147-2A>G, IVS28-2A>G. NGS analysis revealed variant allele frequencies of 31.3% in leukocytes and 33.3% in urinary sediment cells. His daughter shows heterozygous variant. Patient ID52: c.1912G>A, p.(Gly638Ser). NGS analysis revealed variant allele frequencies of 60.9% in leukocytes and 68% in urinary sediment cells. Patient ID252: c.4787G>T, p.(Gly1596Val). NGS analysis revealed variant allele frequencies of 20.6% in leukocytes, 24.1% in urinary sediment cells, and 0% in hair roots. His daughter shows heterozygous variant.

Table 1 Clinical characteristics and laboratory data

Patient ID	*Sex*	*Age (years)*	*ESRD (age)*	*Hearing loss (detected age)*	*sCr (mol/l)*	*eGFR (ml/min/1.73 m2)*	*Hematuria*	*U-P/Cr (g/g Cr)*	*EM*	*alpha-5*	*Family history*
14	M	15–20	—	—	68.9	144.7	3+	0.74	BWC	Mosaic	Sporadic
28	M	35–40	—	—	68.9	87.1	—	—	—	—	Two daughters pro/OB
52	M	15–20	—	—	80.4	125	3+	0.83	BWC	Mosaic	Sporadic
252	M	40–45	—	—	86.6	104.1	3+	—	—	—	Daughter pro/OB

Abbreviations: BWC, basket-weave change; EASRD, end-stage renal disease; eGFR, estimated glomerular filtration rate; EM, electron microscopic findings; M, male; ND, not determined; OB, occult; pro, proteinuria; blood sCr, serum creatinine levels; U-P/Cr, urinary protein–creatinine ratio.

Patient ID52

The pedigree of patient ID52 is shown in Figure 1c. He was an 18-year-old man who was first detected with hematuria and proteinuria by screening at the age of 3 years. Examination of a kidney biopsy taken at 10 years of age revealed AS with a BWC on the GBM. However, α5(IV) expression showed a mosaic pattern. His karyotype was 46,XY. Genetic analysis revealed an exon25 missense variant (c.1912G>A, p.(Gly638Ser)), which was reported previously without precise clinical information.[14] At the age of 18 years, he had microhematuria and moderate proteinuria of 0.83 g/g Cr.

Patient ID252

Patient ID252 was a 42-year-old man in whom hematuria was first detected at 6 years of age. His pedigree is shown in Figure 1d. His daughter showed macrohematuria and mild proteinuria (0.2 g/g Cr) when she was 6 years old. She underwent kidney biopsy and was pathologically diagnosed with XLAS with a BWC on GBM and mosaic α5(IV) expression. Genetic analysis revealed a heterozygous missense variant at *COL4A5* exon 49 (c.4787G>T, p.(Gly1596Val)). This amino-acid variant with a different amino-acid substitution was reported in a male patient who had not developed ESRD at the age of 19.[15] Her mother was asymptomatic with no variants, but her father showed persistent microhematuria without proteinuria and had the same variant with somatic mosaicism in genomic DNA extracted from both leukocytes and urine sediments. We confirmed his karyotype to be normal (46,XY). His daughter had the same heterozygous variant, indicating that their father also had a germline variant. The father was diagnosed with XLAS with a somatic and gonadal mosaic variant in *COL4A5*.

Table 2 *COL4A5* variants and variant allele frequencies

Patient ID	Variant position	Variants	Amino acid change	Methods	Variant frequency (%) Leukocytes	Urine sediments	Kidney	Hair roots	Skin
14	intron43	c.3998-2A>T	p.(Gly1333_Pro1356del)	Trusight One	57.1	61.3	—	—	75.3
		c.3998-2A>T		Targeted resequencing	60.8	63.0	62.9	—	64.8
28	intron27	c.2147-2A>G	p.(Gly716_Pro721del)	Trusight one	31.3	33.3	—	—	—
		r.2147_2164del		Targeted resequencing	43.7	45.5			
52	exon25	c.1912G>A	p.(Gly638Ser)	Trusight one	60.9	68.0	—	—	—
		r.(1912g>a)		Targeted resequencing	70.4	—	72.7	—	—
252	exon49	c.4787G>T	p.(Gly1596Val)	Trusight one	20.6	24.1	—	0	—
		r.(4787g>t)		Targeted resequencing	24.9	19.4	—	1.5	—

Table 3 Determining the limit of variant frequency detection

Variant frequency (%) Test sample	NGS result	Depth	Wild type Forward reads	Wild type Reverse reads	Mutant Forward reads	Mutant Reverse reads
0.5	1.1	459 772	140 068	310 841	1665	3441
1	1.9	504 779	149 572	340 835	2954	6680
2	2.6	463 811	124 329	323 702	3158	8805
10	10.7	399 956	98 220	255 492	11 222	31 623
20	19.4	440 254	350 826	118 714	26 670	58 759

Limit of variant frequency detection

Table 3 shows the results of our analysis to determine the limit of variant detection frequency. Targeted resequencing revealed that 1–2% was the lower limit of detection.

Comparison of variant frequencies between kidney biopsies and urinary sediments

We previously showed that urinary sediments can be used as an alternative cell source to kidney biopsies.[16,17] The present study compared the allele frequencies between DNA extracted from these two sources and obtained very similar findings (Table 2). Therefore, we compared the variant frequency of either kidney biopsies or urinary sediments with the phenotype in our analysis.

DISCUSSION

Male patients with XLAS sometimes show a milder, 'adult type' phenotype, with only mild deafness and an onset of renal failure >30 years old.[2] This milder phenotype is associated with unique genotypes such as missense or in-frame variants in *COL4A5*.[3–5] We previously reported a male XLAS patient with a missense *COL4A5* variant who showed only hematuria without proteinuria at the age of 33.[18] We also reported a male patient with a somatic *COL4A5* variant who showed hematuria and mild proteinuria at the age of 8 years. His kidney biopsy expressed α5(IV) mosaicism in the glomerulus, which was associated with the somatic mosaic variant.[7] To date, however, only six patients in four reports have been described with somatic mosaic variants in *COL4A5*, including our previous report (Table 4).[7,19–21] Although all six cases showed a milder phenotype and some of the female cases were asymptomatic, no asymptomatic male cases have previously been reported.

A recent publication by Beicht *et al.*[19] described an asymptomatic female XLAS patient with a somatic mosaic variant who had variant allele frequencies of 14, 7, 4, and 7% in leukocytes, urine sediments, hair roots, and oral mucosa, respectively, as shown by NGS. However, it is difficult to evaluate variant allele frequencies and phenotypes in female XLAS patients because skewed X-inactivation might affect the phenotype. The present study examined the correlation between the percentage of variant alleles in genomic DNA extracted from kidney biopsies and/or urinary sediments and renal symptoms in men with XLAS and somatic mosaic variants for the first time, revealing a tendency for an association between lower variant allele frequency and milder phenotype. Interestingly, two patients with variant frequencies in kidney biopsies and/or urinary sediment cells of ≥50% showed hematuria and moderate proteinuria, whereas two patients with frequencies <50% were asymptomatic or only had hematuria.

We recently reported a male XLAS patient with a mild phenotype caused by a unique intronic splicing variant, causing a cryptic exon in the transcript; however, mRNA extracted from the kidney showed both normal and abnormal transcripts, the former rescuing him from having the severe phenotype.[22]

The milder phenotype in men with XLAS is currently defined by the following five patterns: (1) missense variants in *COL4A5*;[4–6] (2); in-frame variants in *COL4A5*;[4,6] (3) somatic mosaic variants in *COL4A5*;[7,19–21] (4) α5(IV)-positive expression in the glomerulus;[6] and (5) aberrant splicing variants in *COL4A5*, leading to both normal and abnormal mRNAs.[22] In this study, we reported four cases with milder phenotypes: two with splice site variants (ID14 and 28) and two with missense variants (ID52 and 252). These variant types could contribute to a modulation of the phenotype. However, among these four patients, the influence of somatic mosaicism appears to be stronger because, of the two patients with missense mutations, ID252 with a lower variant frequency showed a much milder phenotype.

We previously used the techniques of semi-quantitative PCR analysis, restriction enzyme digestion, and electrophoresis to report variant frequencies for patient ID14 of 37% in leukocytes, 71% in urine sediments, and 32% in the skin.[7] Although at the time of this study (2008), we thought that our methods were highly efficient, it now appears that they were not reliable because the two techniques used in the current study (TruSight One and targeted resequencing) achieved almost identical frequencies, which differed from our previous data.

Patients ID28 and 252 of the present study also showed mosaic variants in germline cells. In these cases, we were unable to conduct an analysis of sperm cells because we were not given consent to do so. However, determining the mutation allele frequency in these cells would provide additional information about the genetic risk facing

Table 4 Previously reported cases with *COL4A5* mosaic variants

		Mosaicism							Variants		
First author	*Sex*	*Age (years)*	*Somatic cells*	*Germline cells*	*ESRD (age)*	*Urinary exam*	*Hearing loss*	*Ocular lesion*	*Exon*	*Nucleotide*	*Amino acid*
Plant KE	Female	ND	+	+	–	OB	–	–	26	c.2006G>C	p.Gly669Ala
	Female	ND	+	+	–	–	–	–	IVS12-3	c.848-3C>A	exon 12 skip
	Male	ND	+	+	43	ND	ND	–	25	c.1912G>A	p.Gly638Ser
Bruttini M	Female	ND	–	+	–	–	–	–	IVS44+1	c.4069+1G>C	exon44 skip
Krol RP	Male	8	+	ND	–	OB, mild pro	–	–	IVS44-2	c.3998-2A>T	exon44 skip
Beicht S	Female	ND	+	+	–	OB	+	Myopia	IVS30-1	c.2396-1G>A	exon 30 skip

Abbreviations: ESRD, end-stage renal disease; ND, not determined; OB: occult blood; pro: proteinuria.

offspring inheriting the mutated allele, which would be invaluable for genetic counseling.

NGS is a highly relevant tool for use in the diagnosis of AS. Moreover, early diagnosis of this disease is becoming increasingly important because AS is now a treatable disease.[23,24] The targeted resequencing technique that we used in the present study is both efficient and cost effective, and we propose that it should be adopted worldwide for the use in disease diagnosis.

The present study reports a tendency between variant allele frequency and the severity of renal symptoms in four men with XLAS with somatic mosaic variants. Although asymptomatic female cases with mosaic variants have been reported previously, the current study provides the first report of an asymptomatic male XLAS patient with a mosaic variant in *COL4A5*. We also describe the first male XLAS cases with somatic and gonadal mosaic variants in *COL4A5*. These results indicate that *de novo* variants can occur even in asymptomatic men with XLAS, and that the variant frequency may influence the severity of XLAS in patients with somatic mosaic variants. These cases highlight the fact that genetic counseling for asymptomatic parents of a child with AS should consider the possibility that one of the parents may carry a variant and show somatic and gonadal mosaicism.

CONFLICT OF INTEREST

KI received grants from Novartis Pharma K.K., Japan Blood Product Organization, Kyowa Hakko Kirion Co., Ltd, JCR Pharmaceuticals Co., Ltd, AbbVie Inc., Genzyme Japan K.K., Teijin Pharma Ltd, Daiichi Sankyo Co., Ltd, and Miyarisan Pharmaceutical Co., Ltd, and lecture fees from Kyowa Hakko Kirin Co., Ltd, Astellas Pharma Inc., Pfizer Japan Inc., Asahi Kasei Pharma Corp., Kowa Pharmaceutical Co., Ltd, Merck Sharp & Dohme Corp., Alexion, Meiji Seika Pharma Co., Ltd, and Novartis Pharma K.K.. KI is also an advisor for Zenyaku Kogyo Co., Ltd.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Health, Labour, and Welfare of Japan for Research on Rare Intractable Diseases in Kidney and Urinary Tract (H24-nanchitou (nan)-ippan-041 to KI) in the 'Research on Measures for Intractable Diseases' Project; a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Subject ID: 25893131 to KN); and a grant from the Mother and Child Health Foundation (Subject ID: 25-7 to KN).

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)