the number of times urinalysis was conducted before detailed examination, consisting of urinalysis, medical history, physical examination, and laboratory tests, also depended on the arrangement with the local medical association. For these reasons, the literature on urinary screening for 3-year-old children was searched using the following criteria: urinalysis performed twice before detailed examinations; and positive criterion +/−, by the Japan Medical Abstracts Society using a web search system to analyze the prevalence of abnormal urinalysis results. Four such reports were identified, from Okinawa (10 752 children screened, 1984–1986),[3] Nagasaki (6637, 1982–1985),[4] Yokohama (8779, 1985–1990)[5] and Kanagawa (5384, 1990–1992).[6] Additionally, unpublished data from Chiba City in Japan from 1991 to 2011 were reviewed. For other analyses on diseases identified on urinary screening, the clinical course of children with urinary abnormalities, and screening for asymptomatic UTI as a way of identifying CAKUT, a computerized literature search, along with research papers reported by some study groups under the Ministry of Health, Labour and Welfare, and data of Akita City in Japan from 2006 to 2010, were also reviewed. The unpublished data of Chiba City and Akita City are summarized in Table 1. Akita City conducts urinalysis once before detailed examination.

Results

Prevalence of abnormal urinalysis

Data are shown as percentage of the number of children at the first urinalysis (Fig. 1a). The median prevalences of hematuria, proteinuria and leukocyturia at first urinalysis were 8.16%, 1.20%, and 1.01%, respectively. At the second urinalysis (Fig. 1b), these prevalences decreased to 1.24%, 0.05%, and 0.18%, respectively, with decreases of 1/6–1/20. At the third urinalysis and on detailed examination (Fig. 1c), the prevalences of hematuria and proteinuria were 0.48% and 0.02% respectively, which were equal to or slightly lower than those previously reported for elementary school children in school urinary screening.[7] Meanwhile, the prevalence of

Table 1 Urinalysis data of 3-year-old children from Chiba City and Akita City

		Chiba City 1991–2011				Akita City 2006–2010	
		First		Second			
Urinalysis	n	154 456		11 346		11 894	
	Proteinuria	2009	1.30%	52	0.03%	662	5.57%
	Hematuria	6300	4.08%	2649	1.72%	673	5.66%
	Glycosuria	52	0.03%	4	0.003%	30	0.25%
	Nitrite	1267	0.82%	188	0.12%	ND	
	Leukocyturia	2959	1.92%	471	0.30%	ND	
Detailed examination	n	2332				1220	
	Microhematuria	1923	1.25%			190	1.60%
	Hematuria	231	0.15%			114	0.96%
	HP	29	0.02%			20	0.17%
	Proteinuria	25	0.02%			71	0.60%
	UTI	111	0.07%			15	0.13%
	CAKUT	13	0.01%			4	0.03%

CAKUT, congenital anomalies of the kidney and urinary tract; HP, hematuria and proteinuria; ND, not done; UTI, urinary tract infection.

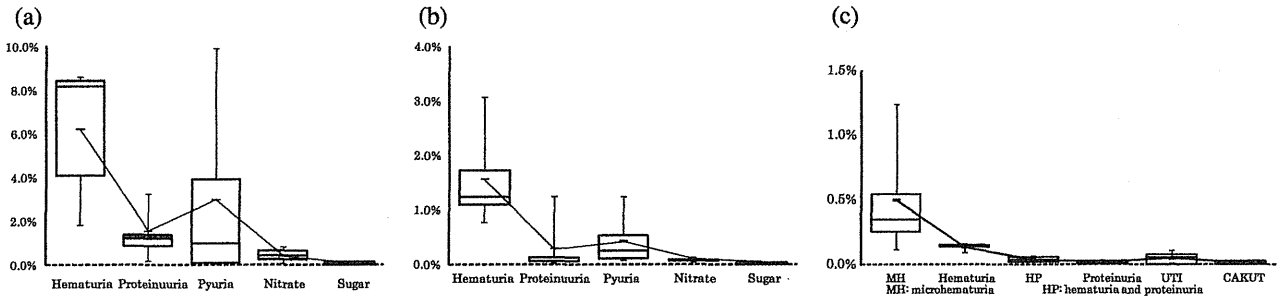

Fig. 1 (a) First, (b) second, and (c) third urinalyses done in Chiba City, Okinawa, Nagasaki, Yokohama, and Kanagawa. (a) Median prevalences of hematuria, proteinuria, and leukocyturia are 8.16%, 1.20%, and 1.01%, respectively. The prevalence of abnormal results was high, especially for hematuria and leukocyturia, the prevalences of which vary in the published literature. (b) The prevalences of hematuria, proteinuria. and leukocyturia decrease to 1.24%, 0.05% and 0.18%, respectively, with decrements of 1/6–1/20. (c) The prevalences of hematuria and proteinuria were 0.48% and 0.02%, respectively, equal to or slightly lower than previously reported results in elementary school children on school urinary screening. In contrast, the prevalence of hematuria and proteinuria (HP) was 0.03%, which was slightly higher than that of elementary school children. Urinary tract infection (UTI) and congenital anomalies of the kidney and urinary tract (CAKUT) were found in 0.05% and 0.01%, respectively. MH, microhematuria. [Correction added on 13 July 2015, after first online publication: The above sentence has been corrected to show the complete sources of Fig. 1 data.]

hematuria and proteinuria was 0.03%, which was slightly higher than that in the elementary school children. UTI and CAKUT were found in 0.05% and in 0.01%, respectively. The prevalences of abnormal results, especially hematuria and leukocyturia, on the first urinalysis of 3-year-old children were much higher than those for elementary school children. On repeat urinalysis, however, the prevalence of abnormal results decreased in 3-year-old children to the level of elementary school children, so that the true prevalence of abnormal results of urinalysis was similar to that of elementary school children. From these data, the importance of repeat urinalysis for making decisions about abnormal urinalysis results in 3-year-old children was confirmed, because a single urinalysis is more likely to provide inaccurate results for such young children.

Diseases identified on urinary screening

Hematuria

It is true that immunoglobulin A (IgA) nephropathy is found in children who test positive for only hematuria, although it has been reported that historical changes in these patients usually show minor glomerular abnormalities. Mori *et al.* reported the histological findings of 16 children who were found to have hematuria, but not proteinuria, on urinary screening for 3-year-old or older preschool children between 1976 and 1988 as an official report for the Ministry of Health, Labour and Welfare study group.[8] In that report, all of the histological diagnoses were of minor glomerular abnormality, including four cases of IgA nephropathy. Furthermore, in other reports, potentially serious illnesses were not found in children who had hematuria alone, other than Alport syndrome. On examination of the Chiba City data, five patients (0.0032%) with Alport syndrome were identified among the 154 456 children screened over 21 years.

Proteinuria

On the third urinalysis and detailed examination, the prevalence of proteinuria was 0.02%, slightly lower than that previously reported for elementary school children in school urinary screening, although nephrotic syndrome including focal segmental glomerulosclerosis was found at a comparatively high rate. Examining the data of Chiba City, eight cases of nephrotic syndrome were identified in 25 children with proteinuria alone. In addition, several CAKUT or idiopathic tubular proteinuria patients were identified among the children with proteinuria. Thus, careful treatment of such children with proteinuria is needed.

Hematuria and proteinuria

In the school urinary screening program, glomerulonephritis was found in 61.2% of children with hematuria and proteinuria, although, in urinary screening for preschool children, glomerulonephritis was found in only 18.5%, and another 81.5% had minor glomerular abnormalities.[9] UTI and CAKUT, however, have been occasionally identified in these children with hematuria and proteinuria. Thus, careful examination of children with hematuria and proteinuria is also needed.

Clinical course of children with urinary abnormalities

Kawakatsu *et al.* reported longitudinal data of 131 patients with abnormal urinalysis results at 3 years of age: 51 patients had normal urinalysis results and stopped follow up, 49 had normal urinalysis results, 22 had microhematuria, four had asymptomatic hematuria, three had asymptomatic proteinuria, and two were diagnosed with non-IgA mesangial proliferative glomerulonephritis at follow up at 6 years of age.[10] Mori *et al.* reported that 8.9% of children who were diagnosed with microhematuria at 3-year-old screening slowly developed nephritis, including suspected nephritis, 9 years later.[8]

Screening for CAKUT via asymptomatic UTI

It is suspected that covert bacteriuria is associated with renal structural abnormalities, including vesicoureteral reflux (VUR), as underlying disorders, and screening for covert bacteriuria in order to identify CAKUT has been considered in several studies. In one of the studies, Matsumura *et al.* performed bacterial culture (replica method) as screening for covert bacteriuria and reported that 39 cases of CAKUT, including 24 associated with VUR, were discovered from among 339 positive children.[11] In contrast, there are several regions that use dipsticks for screening for leukocyturia in Japan. Examining the 21 years of Chiba City data, 154 456 children were screened, and 111 children (0.072%) were ultimately found to have leukocyturia after three screening tests, and 85 of the 111 children could be followed. Finally, 23 (27.1%) of the 85 children were diagnosed as having UTI based on urine culture. Of these 23 children, 11 had VUR, and nine of these 11 patients needed surgery. Righard, however, reported that pyuria occurred in only approximately half of the cases of asymptomatic bacteriuria.[12] Moreover, as mentioned in the first part of this report, the prevalence of leukocyturia is very high and, thus, the dipstick method for screening for leukocyturia is inefficient.

Discussion

As seen in the present study, urinary screening for 3-year-olds results in very many false-positive results. The number of positive children was reduced by 1/6–1/20 after two consecutive urinalyses. For this reason, urinalysis of young children should not be judged on the basis of one urinalysis in the clinical setting, but on two consecutive urinalyses before proceeding to detailed examination. False-positives are very common for leukocyturia and hematuria, especially in girls. The possibility of minor inflammation including vulvitis is considered, because the genitals are easily contaminated in 3-year-olds.

In screening of 3-year-olds, a relatively large number of children had single-positive results for hematuria, but the frequency of diseases that required urgent treatment was low in these children. Even though the hematuria could be due to chronic nephritis such as IgA nephropathy, because there is no indication for renal biopsy for patients who are positive for hematuria on only one occasion, such patients are only followed up without being given a definitive diagnosis. As for the follow up, if patients also have proteinuria, the patients require renal biopsy for definitive diagnosis and initiation of appropriate treatment. For this reason,

children who are positive for hematuria on one occasion with no family history of disease, such as Alport syndrome, do not need immediate detailed examination, and they should only be followed up. In contrast, children with gross hematuria require close examination by nephrologists, because nephritis, urinary tract stone, tumor, and many other renal disorders are in the differential diagnosis of gross hematuria.

In addition, the prevalence of proteinuria at the final detailed examination was low (0.02%), but, as aforementioned, the prevalence of children who needed some treatment was relatively high. Thus, children who are positive for proteinuria on two consecutive urinalyses should be referred to nephrologists. Children who are twice positive for proteinuria and hematuria are a group at high risk for nephritis other than UTI, and they also should be referred to nephrologists. The urine of 3-year-old children is usually dilute, and we consider that +/− should be judged abnormal, but we will have to re-consider the validity of this in a future analysis.

There is no doubt that CAKUT, including VUR, is found in children with asymptomatic UTI. But asymptomatic bacteriuria has not yet been proven to cause renal damage, although apparent UTI with any symptom may cause renal damages in children. UTI screening on bacterial culture (replica method) reported by Matsumura *et al.*[11] is associated with problems related to cost and difficulty of culture techniques, and these disadvantages do not outweigh the advantages of the replica method. With regard to leukocyte screening, the prevalence of leukocyturia is very high, and screening for leukocyturia is very inefficient.[13] Although the importance of early detection of asymptomatic UTI needs to be surveyed in the future, the value of screening for asymptomatic bacteriuria or leukocyturia is unclear at this time.

There is global controversy about the perceived cost-effectiveness and importance of mass urinary screening for chronic kidney disease (CKD) in children, with differences of opinion between Eastern and Western countries.[14] Sekhar *et al.* reported that urine dipstick screening is a poor test for CKD and not cost-effective.[15] In contrast, they also mentioned that the cost-effectiveness of the dipstick procedure may change if the benefits of early treatment alter outcome, because screening dipstick urinalysis is relatively inexpensive. Recently, several authors have reported the effectiveness of renin–angiotensin system inhibitor treatment for CAKUT.[16,17] The earlier use of RAS inhibitor might be more effective to slow progression of CAKUT, because urinary screening in 3-year-old children seems able to detect CAKUT or other CKD earlier. The present screening system was established based on the results of previous studies, and it will be necessary to re-evaluate the effectiveness of the new screening system for 3-year-olds in the future.

Conclusion

The data of urinary screening of 3-year-old children in Japan were analyzed. The importance of two consecutive urinalyses has already been reported based on school urinary screening data, and this was confirmed by the data from 3-year-old children. Given the high prevalence of hematuria and the severity and urgency of the diseases found in children who were positive for hematuria on one urinalysis, hematuria screening is not necessarily needed as part of urinary screening of 3-year-olds. In contrast, the importance of proteinuria screening was confirmed, because diseases that require immediate intervention, such as CAKUT, were found at a relatively high prevalence on screening. With regard to leukocyturia screening, there was no evidence of the value of early detection of asymptomatic UTI, and it was concluded that the value of screening for leukocyturia is low at this time. The effectiveness of the new screening system for 3-year-olds needs to be evaluated in the future.

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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

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.

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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.

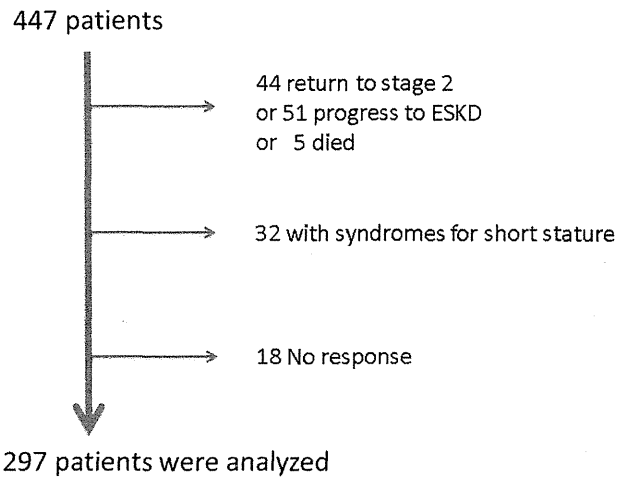

Fig. 1 Patient disposition

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]

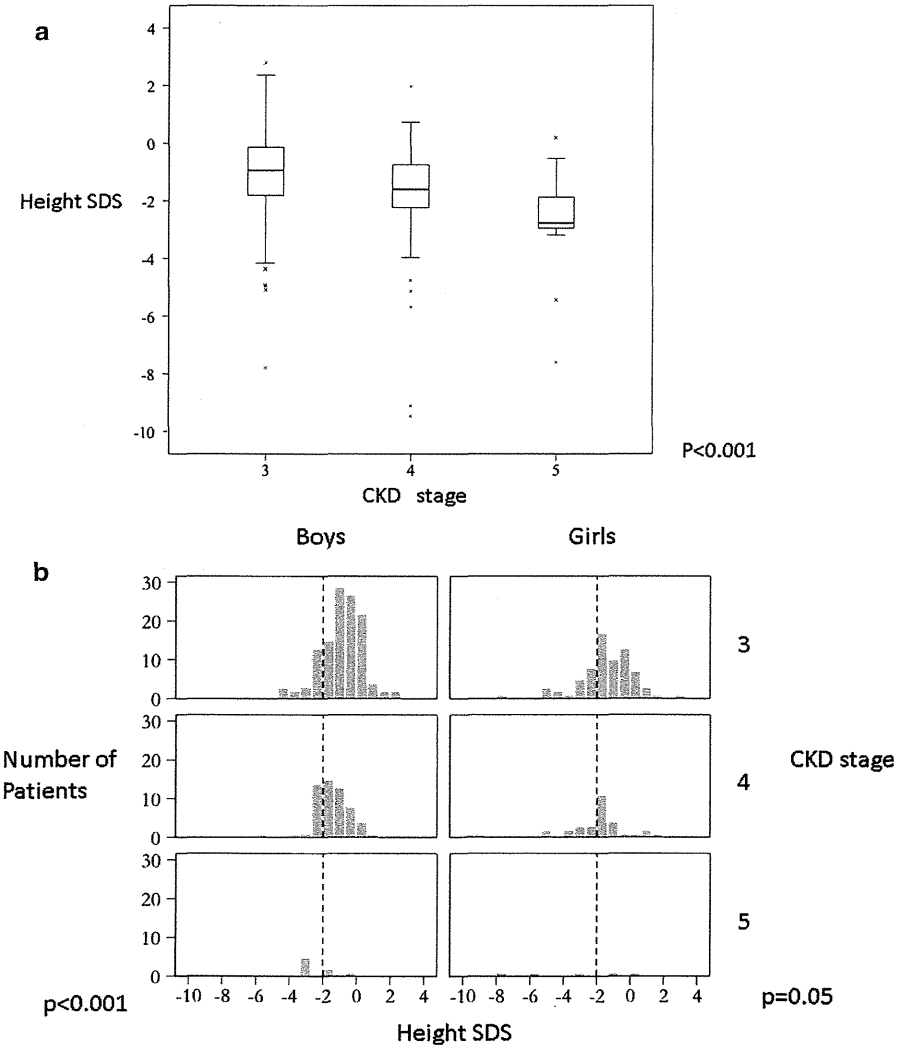

Fig. 2 **a** Height SDS according to CKD stage. The *bottom*, *middle*, and *top lines* of each box represent the 25th, 50th (median), and 75th percentiles of height SDS, respectively. The ends of the whiskers represent the range from 1.5 times the interquartile range (IQR) added to the 75th percentile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

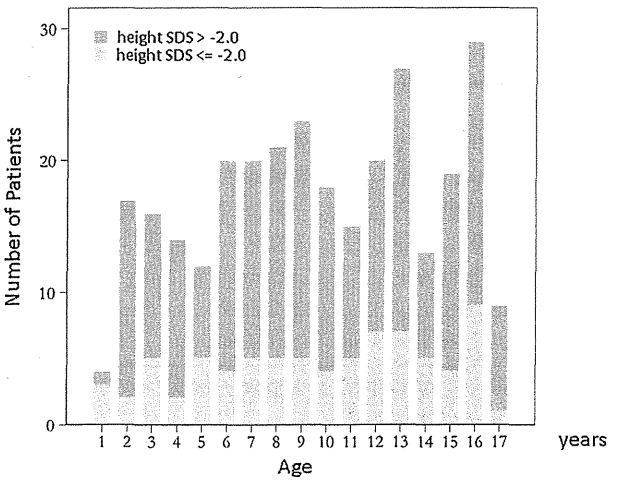

Fig. 3 Age distribution of patients by gender. The numbers of patients are plotted against age in years. *Blue bars* height SDS > -2, *Pink bars* height SDS ≤ -2

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

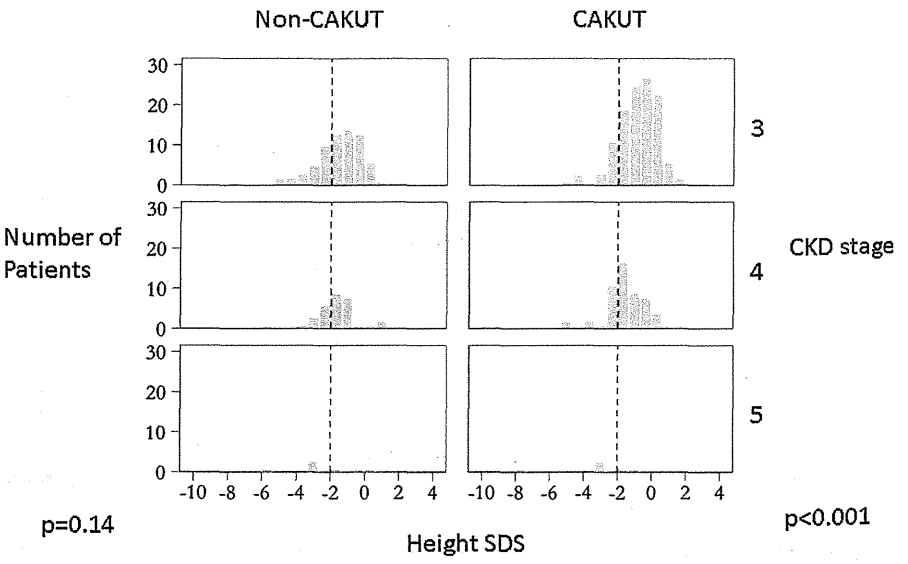

Fig. 4 Distribution of height SDS according to CKD stage and primary disease (CAKUT/non-CAKUT). *SDS* standard deviation score, *CKD* chronic kidney disease, *CAKUT* congenital anomalies of the kidney and urinary tract

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

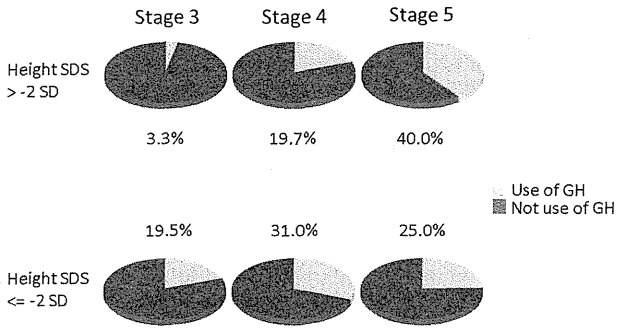

Fig. 5 Use of growth hormone according to CKD stage and height SDS. *SDS* standard deviation score, *CKD* chronic kidney disease

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 $\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.

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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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ORIGINAL ARTICLE

Reference glomerular filtration rate levels in Japanese children: using the creatinine and cystatin C based estimated glomerular filtration rate

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Abstract
Background The present study was performed to determine the reference values of glomerular filtration rate (GFR) in children by age using the new eGFR equations derived from serum creatinine (Cr) and cystatine C (cysC).
Methods A total of 1137 children (509 males and 628 females) between the ages of 3 months and 16 years presenting at our facilities between 2008 and 2009 without diseases affecting the renal function were included in this study as in our previous reports for reference values of serum Cr and cysC. We calculated eGFR with the Cr based equation in children aged 2–16 years, and with the cysC based equation in those aged 3–23 months, and determined the reference values of GFR in Japanese children by each age group.
Results We reviewed the median, 2.5 and 97.5 percentile of GFR reference value in each age group. The medians of reference GFRs are 91.7, 98.5, 106.3, and 113.1 mL/min/1.73 m^2 in children aged 3–5, 6–11, 12–17, and 18 months–16 years, respectively.
Conclusion We determined the normal reference values of GFR in children. It is important for pediatricians who examine pediatric chronic kidney disease patients to know the values of normal renal function.

Keywords Children · Creatinine · Cystatin C · Glomerular filtration rate · Reference levels

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Introduction

Renal inulin clearance (Cin) to measure glomerular filtration rate (GFR) directly is compromised by problems of collecting urine samples in children. Therefore, Schwartz et al. expressed the serum creatinine (Cr) based estimated GFR (eGFR; ml/min/1.73 m^2) by k × body length (cm)/serum Cr level (mg/dL) by the Jaffe method Cr determination in 1987 [1], and by enzymatic Cr determination in children 1–16 years old in 2009 [2]. We doubt whether the new Schwartz equations can be used to estimate the GFR in Japanese children with chronic kidney disease (CKD), because there are differences in renal function and muscle mass between Japanese and American individuals and only one common "bedside" linear equation is used in children aged from 1 to 16 years, including the period of adolescence [3]. Therefore, we determined the reference values of serum creatine (Cr) and cystatin C (cysC) [4, 5] and derived formulas to estimate glomerular filtration rate based on enzymatic Cr and cysC determination in Japanese children [6, 7].

However, we have no reference value of GFR in Japanese children, and there are few reports on detailed reference values of GFR in children by age. The present study was performed to determine the reference values of GFR in children by age group using the new eGFR equations derived from serum Cr and cysC.

Materials and methods

Study population

A total of 1137 children (509 males and 628 females) between the ages of 3 months and 16 years presenting at

the facilities of the members for the Committee of Measures for Pediatric Chronic Kidney Disease and Tokyo Health Service Association between 2008 and 2009 without kidney disease, urogenital disease, infectious disease, inflammatory disease, dehydration, muscular disease, anomaly syndrome, malignant disease, hypertension, cardiovascular disease, liver or pancreas disease, or pregnancy were included in this study as in past reports of reference values of serum Cr [4] and cysC [5]. Of the total study population shown in Table 1, 45 % were male, and 58 % were enrolled on preoperative examination. The median age was 6.9 years, median height was 117.5 cm, and median weight was 21.7 kg. The median values of serum Cr and cysC were 0.36 mg/dL and 0.78 mg/L, respectively. The study was approved by the local ethics boards, and written informed consent was obtained from the parents of each subject. The ethics committee approval number in Aichi Children's Health and Medical Center is 200706.

Serum Cr and cysC measurements, and Cr-based and cysC-based eGFR calculations

The serum Cr level was determined by an enzymatic method as reported previously [4]. The serum cysC level was determined using the cysC assay: Nescaute GC cystatin C (Alfresa Pharma Corporation) as reported previously [5]. In Japan, the standardized method of cystatin C measurement traceable to ERM-DA471/IFCC became available in 2011. In the present study, serum cysC values of 174 samples were measured by the colloidal gold immunoassay before 2011 and were calibrated to a standardized value using the correction factor 0.96 which showed calculated cysC reflected the standardized cysC actually measured [7].

Table 1 Characteristics of 1137 children included in this study

Characteristics	Median (IQR)[a]	*n*
Total		1137
Age (year)	6.9 (3.9–12.2)	
<2 years		132
≥2 years		1005
Gender		
Male		509
Female		628
Height (cm)	117.5 (99.1–150.0)	
Weight (kg)	21.7 (15.0–41.2)	
Serum creatinine (mg/dL)	0.36 (0.28–0.48)	
Serum cystatin C (mg/L)	0.78 (0.72–0.84)	
Reasons for blood tests		
Preoperative evaluation		655
Strabismus		292
Inguinal hernia		270
Blepharoptosis		42
Congenital hip dislocation		10
Cataract		9
Entropion of lids		8
Others		24
Health examination		356
Allergen examination		38
Miscellaneous		88

[a] *IQR* interquartile range

The pediatric Cr based equation [6] was calculated as shown below;

eGFR (mL/min/1.73 m^2) = 110.2 × (reference serum Cr (mg/dL)/patient's serum Cr (mg/dL)) + 2.93.

Reference serum Cr levels (y) are shown by the following two equations of body length (x):

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

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

The pediatric cysC based equation [7] was calculated as shown below;

eGFR (mL/min/1.73 m^2) = 104.1/serum cysC (mg/L) − 7.80

Cr based eGFR is not suitable for children aged under 2 years. The Flanders metadata equation based by Cr and age developed by Pottel et al. [8] is proposed for children between 1 month and 14 years of age. However, the valuables of our formulas do not include age. Therefore, we calculated eGFR with a Cr based equation in children aged 2–16 years, and with a cysC based equation in those aged 3–23 months.

Results

We examined the correlations between eGFR and age in all subjects by gender (Fig. 1). Scattergrams showed that reference eGFR slightly increases with age only in infants. We reviewed the median, 2.5 and 97.5 percentile of GFR reference value in each age group, regardless of gender between age 3 and 23 months, and by gender between 2 and 16 years (Table 2). We determined a reference value of eGFR for males and females aged between 18 months and 16 years (Table 3), because in these age and gender groups the distributions of the reference values seemed to differ only slightly and we thought that accuracy increased with increased number of cases. The medians of reference GFRs are 91.7, 98.5, 106.3, and 113.1 mL/min/1.73 m^2 in children aged 3–5, 6–11, 12–17, and 18 months–16 years, respectively. In addition, it is simple and easy to use at the bedside.

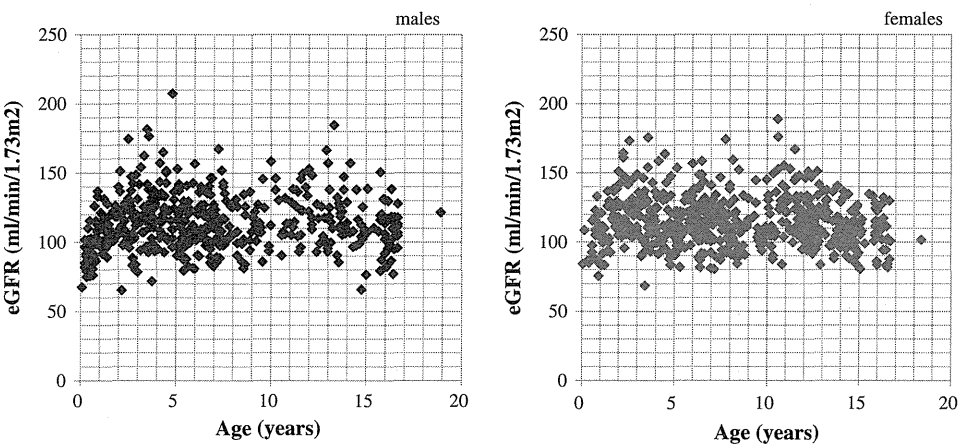

Fig. 1 Correlations between eGFR and age in subjects by gender. Scattergrams showing reference eGFR slightly increase with age only in infants

Discussion

Cin is the gold standard for evaluations of kidney functions, but cannot be measured easily. Therefore, the Schwartz formulas by the Jaffe method Cr determination [1] have been widely used to estimate GFR in children worldwide. Recently, however, enzymatic methods have been used to measure Cr rather than the Jaffe method, and the original Schwartz equations are not accurate using creatinine measured enzymatically. Schwartz et al. reported the updated formula, the so-called "bedside" version, as eGFR = 0.413 × body length (cm)/serum Cr level (mg/dL) by the enzymatic method showing a 25 % reduction in value of k from the previous value of 0.55 generated from Jaffe-based serum Cr measurements [2]. They concluded that the formula can be used in American children aged 1–16 years. We have reported that the new Schwartz bedside formula cannot be used to estimate GFR in Japanese children with normal renal function between 1 and 16 years, including the adolescent period [3]. There seems to be a large problem in that the ranges of the reference value for boys >12 years old and girls >14 years old overlap with the range for CKD stage 2. We doubted whether the new "bedside" Schwartz formula could be used to estimate GFR in Japanese pediatric CKD patients as well as in children with normal renal function.

Therefore, we have established two estimated creatinine-based GFR equations for use in Japanese children aged between 2 and 11 years [9] and in Japanese children and adolescents aged between 2 and 18 years [6]. In addition, we have derived a cystatin C based estimated GFR equations in Japanese children and adolescents aged between 1 month and 18 years, including infants [7]. In the present study, we presented the reference GFR levels in Japanese children using the Cr and cysC based eGFR, in children aged 2–16 years and aged 3–23 months, respectively.

The rise in GFR from birth to adulthood is well known. In brief, GFR rises to around 100 mL/min/1.73 m^2 by about 1 year, and mature values of GFR range from 100 to 120 mL/min/1.73 m^2 [10]. However, a few reports give detailed reference values of GFR in children by age. The present study was performed to determine the reference values of GFR in children by each group using the new eGFR equations derived from serum Cr [6] and cysC [7], and reference values of serum Cr [4] and cysC [5] in Japanese children aged between 3 months and 16 years. The medians of reference GFRs were 91.7, 98.5, 106.3, and 113.1 mL/min/1.73 m^2 in 3–5, 6–11, 12–17, and 18 months–16 years old, respectively, and showed a gradual increase with age, but were approximately constant between 18 months and 16 years old.

Brodehl et al. reported that GFRs derived from Cin approached adult levels within 2 years and were approximately constant between 3 and 15 years, showing values of 111.2 and 117.2 mL/min/1.73 m^2 at 3–4 years and 13–15 years, respectively [11]. Piepsz et al. reported that there was a progressive increase in chromium-51 ethylene diamine tetra-acetic acid clearance from the first weeks of life with a plateau at around 18 months, and between 2 and 17 years of age, the clearance values remained constant, with a mean value of 114 ml/min/1.73 m^2 (SD: 24 ml/min) [12]. Despite the racial difference, our report and theirs considerably agree.

The present study has some limitations. First, we calculated eGFR with Cr based equation in children aged 2–16 years, and with cysC based equation aged 3–23 months, respectively. This is because Cr based eGFR is not suitable for children under 2 years of age. Second, in this study we did not undergo Cin, but utilized eGFR equations derived from serum Cr or cysC in children without diseases affecting renal function. Therefore, we should call these the reference value of eGFR rather than GFR. Third, we have not validated our equations, including

Table 2 The median, 2.5 and 97.5 percentile of GFR reference value in each age group, regardless of gender between 3 and 23 months, and by gender between 2 and 16 years

Age	Gender	*n*	2.5 % tile	50 % tile	97.5 % tile
3–5 months	Male and female	17	76.6	91.7	106.7
6–11 months	Male and female	47	75.7	98.5	133.0
12–17 months	Male and female	31	83.3	106.3	132.6
18–23 months	Male and female	37	97.9	115.4	138.7
2 years	Male	41	79.5	115.3	151.2
	Female	34	102.3	118.7	166.0
3 years	Male	48	81.0	119.2	174.0
	Female	41	89.8	116.8	154.0
4 years	Male	43	95.1	120.0	164.5
	Female	37	87.6	111.9	159.3
5 years	Male	47	81.5	109.8	142.6
	Female	48	90.9	109.6	133.5
6 years	Male	44	83.9	109.8	146.6
	Female	57	87.9	116.5	153.6
7 years	Male	37	85.2	108.0	153.4
	Female	47	81.8	113.6	145.9
8 years	Male	18	82.5	103.7	135.7
	Female	38	88.8	109.7	152.8
9 years	Male	18	89.1	109.9	137.7
	Female	17	95.3	108.0	139.4
10 years	Male	11	94.0	113.4	155.2
	Female	32	86.8	113.1	178.9
11 years	Male	19	94.5	122.9	147.7
	Female	39	99.7	123.1	153.8
12 years	Male	15	95.1	117.5	161.6
	Female	54	92.2	110.4	149.9
13 years	Male	30	89.2	115.8	164.7
	Female	38	90.2	115.6	137.8
14 years	Male	17	78.2	108.1	149.4
	Female	40	88.2	106.6	134.5
15 years	Male	15	77.5	116.1	146.5
	Female	22	80.6	112.2	134.0
16 years	Male	30	81.0	106.5	132.8
	Female	28	82.7	108.4	130.7

in general children. The equation may lose its accuracy at different GFR levels [13]. However, we had determined s-Cr and cysC based eGFR equations in cases with GFR < 150 mL/min/1.73 m^2 [6, 7]; therefore, our equations may be reliable in general population same as in CKD patients.

Table 3 The median, 2.5 percentile, and 97.5 percentile of GFR reference value in each age group regardless of gender between 3 months and 16 years old

Age	*n*	2.5 % tile	50 % tile	97.5 % tile
3–5 months	17	76.6	91.7	106.7
6–11 months	47	75.7	98.5	133.0
12–17 months	31	83.3	106.3	132.6
18 months–16 years	1042	83.5	113.1	156.7

However, we consider that these reference GFR values are sufficient to evaluate the quality of renal function in children. It is important for pediatricians who examine pediatric CKD patients to know the normal renal function.

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

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Novel Splice Site Mutation in *MAMLD1* in a Patient with Hypospadias

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Key Words
Hypospadias · *MAMLD1* · Mutation · Protein · Splicing · Translation

Abstract
MAMLD1 is a causative gene for disorders of sex development. Several *MAMLD1* mutations have been shown to cause hypospadias by generating dysfunctional proteins and/or unstable mRNAs. Here, we identified an intronic mutation of *MAMLD1* (g.IVS4−2A>G) in 1 of 180 hypospadias patients. RT-PCR of the patient's skin sample showed normal expression of full-length *MAMLD1* and markedly reduced expression of a known splice variant lacking exon 4. A hitherto unreported splice variant that lacks exon 5 was similarly identified in samples of the patient and control individuals. The full-length transcript of the patient contained mutant mRNA lacking the first 10 nucleotides of exon 5 (c.1822_1831delACTCATGTAG, p.K609fsX1070). In vitro assays using cells expressing the full-length wild-type and mutant proteins revealed reduced expression of the mutant. The expression of the wild-type and mutant MAMLD1 showed parallel changes upon treatment with a proteasome inhibitor and a translation inhibitor. The mutant-expressing cells exerted low transactivation activity for the *Hes3* promoter, which reflected limited expression of the mutant protein. These results imply that the pathogenic events resulting from *MAMLD1* mutations include splice errors. Furthermore, this study raises the possibility of translation failure of MAMLD1 mutants, which deserves further investigation.

MAMLD1 (NM_001177465) on Xq28 is a causative gene for 46,XY disorders of sex development (DSD) [Fukami et al., 2006]. The major clinical feature of patients with *MAMLD1* mutations is hypospadias [Fukami et al., 2006]. Previous studies have shown that MAMLD1 transactivates the promoter of the non-canonical Notch target *Hes3* and enhances expression of multiple genes in murine fetal Leydig cells [Fukami et al., 2008; Miyado et al., 2012]. Human *MAMLD1* comprises at least 7 exons, of which exons 3–6 correspond to the coding region of a 701-amino-acid protein [Laporte et al., 1997]. RT-PCR analysis of human cDNA samples detected the expression

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of full-length *MAMLD1* and its in-frame splice variant lacking exon 4 (Δexon 4) in all tissues examined [Fukami et al., 2006]. All essential domains of the MAMLD1 protein appear to be encoded by the sequences in exon 3, because the Δexon 4 variant retains normal transactivation activity for the *Hes3* promoter [Fukami et al., 2008], and a boy carrying a microdeletion involving exons 5–7 of *MAMLD1* had normal external genitalia [Tsai et al., 2005].

To date, several nucleotide alterations in *MAMLD1* have been identified in patients with hypospadias as well as in unaffected individuals [Fukami et al., 2006, 2008; Kalfa et al., 2008, 2011, 2012; Chen et al., 2010; Metwalley and Farghaly, 2012]. Of these, 4 nonsense [p.S70X (formerly p.S143X), p.E124X, p.Q197X, and p.R653X], 1 frameshift [p.E109fsX121 (formerly c.325delG)], and 2 missense substitutions [p.P311L (formerly p.P384L) and p.Q529K] have been identified exclusively in DSD patients and are therefore regarded as pathogenic mutations. In vitro assays using a luciferase reporter vector containing the *Hes3* promoter (pHes3-luc) indicated that p.S70X, p.E124X, p.Q197X, and p.P311L encode amorphic or hypomorphic proteins [Fukami et al., 2008; Kalfa et al., 2012]. The transactivation activities of the proteins encoded by p.E109fsX121 and p.Q529K have yet to be examined. Although p.R653X encodes a protein with normal transactivation activity, the mRNA transcript carrying this mutation is degraded through nonsense-mediated mRNA decay (NMD) [Fukami et al., 2008]. Similarly, p.S70X, p.E124X, p.Q197X, and p.E109fsX121 satisfy the condition for NMD [Kuzmiak and Maquat, 2006]. Taken together, all hypospadias-associated *MAMLD1* mutations reported to date are likely to generate dysfunctional proteins and/or unstable mRNAs.

Mutations in human genes exert pathogenic effects not only through generation of amorphic/hypomorphic proteins or unstable mRNAs, but also through several other mechanisms such as splicing defects, early protein degradation, and translation failure [Kuzmiak and Maquat, 2006; Bartoszewski et al., 2010; Ward and Cooper, 2010; Lee et al., 2011; Strachan and Read, 2011]. In particular, early protein degradation mediated by the proteasome or autophagy has been implicated in several genetic disorders [Kuroha et al., 2009; Strachan and Read, 2011; Ihara et al., 2012]. In the present study, we investigated the disease-causing mechanism associated with a novel mutation in *MAMLD1*.

Materials and Methods

Primers and Plasmid Vectors

Primers used in this study are shown in online supplementary table 1 (see www.karger.com/doi/10.1159/000380842 for all online suppl. material). The expression vector for the myc-tagged full-length wild-type MAMLD1 was created by inserting a cDNA fragment comprising 2,103 nucleotides of the coding region and 1,132 nucleotides of the 3′ untranslated region into a pCMV-Myc vector (Takara Bio, Ohtsu, Japan). Expression vectors for the mutant/variant MAMLD1 were created by site-directed mutagenesis (Takara Bio). The pHes3-luc reporter vector was kindly provided by Professor Kageyama.

Mutation Analysis of MAMLD1

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent. Mutation analysis of *MAMLD1* was performed for 180 patients with hypospadias. All patients had a 46,XY karyotype. Genomic DNA was extracted from peripheral leukocytes and amplified by PCR for coding exons 3–6 and their flanking splice sites of *MAMLD1*. The PCR products were subjected to direct sequencing.

mRNA and Protein Expression Analyses of Genital Skin Samples

A genital skin sample was obtained from a patient with a *MAMLD1* mutation during surgery for hypospadias. Control skin samples were obtained from 3 individuals with normal *MAMLD1* who underwent surgery for buried penis. Total RNAs and protein extracts were obtained from homogenized tissue samples. RT-PCR was performed using primers on exons 3 and 6 of *MAMLD1*. The PCR products were subcloned into the TOPO TA cloning vector (Life Technologies, Carlsbad, Calif., USA) and subjected to direct sequencing. PCR was also performed using primers specific for each splice variant. The products were analyzed by polyacrylamide gel electrophoresis using TBE-PAGE mini kit (TEFCO, Tokyo, Japan).

Western blot was carried out using anti-MAMLD1 antibodies (ab49150, Abcam, Cambridge, Mass., USA; and sc-131477, Santa Cruz Biotechnology, Calif., USA). Protein extracts were subjected to standard SDS-PAGE. The signals were visualized using BCIP/NBT Color Development Substrate (Promega, Madison, Wis., USA) or ECL Prime Western Blot Detection kit (GE Healthcare, Buckinghamshire, UK).

In vitro Protein Expression Assays

HEK293 cells were seeded in 10-cm plates (3.0×10^6 cells/plate) and transiently transfected using Lipofectamine 2000 (Life Technologies) with 10 μg of the expression vectors for the full-length wild-type or mutant/variant MAMLD1. The cells were harvested 48 h after transfection and the lysates were subjected to standard SDS-PAGE. The signals for the MAMLD1 proteins and the internal controls were probed with an anti-myc-tag antibody (Takara Bio) and an anti-β-actin antibody (Abcam), respectively. The relative expression levels of MAMLD1 proteins were calculated by dividing the signal intensities of the myc-tag by those of the internal control. Each experiment was performed in triplicate and repeated 3 times.

To clarify whether mutant MAMLD1 protein underwent early degradation, we performed further experiments using HEK293 cells seeded in 6-cm plates (1.0×10^6 cells/plate) and transfected

with 5 μg expression vectors. The transfected cells were treated with MG132, which inhibits proteasome-mediated degradation [Zhang et al., 2013]. In this experiment, MG132 (Peptide Institute, Osaka, Japan; final concentration, 50 μmol/l) was added to the medium 48 h after transfection, and the cells were cultured for further 1, 2, or 4 h. The transfected cells were also treated with cycloheximide (CHX), which inhibits both NMD and translation [Freddi et al., 2000]. CHX (Sigma-Aldrich, St. Louis, Mo., USA; final concentration, 100 μg/ml) was added to the medium 24 h after transfection, and the cells were cultured for further 24 or 48 h. The cells were harvested, and the lysates were subjected to Western blot analysis as mentioned above. Each experiment was repeated at least 3 times.

In vitro Transactivation Analysis

Transactivation activities of the full-length wild-type and mutant/variant MAMLD1 proteins were examined by luciferase reporter assays. HEK293 cells were seeded in 6-well plates (1.5×10^5 cells/well) and transiently transfected with 0.2 μg of the expression vectors and 0.2 μg of the pHes3-luc vector, together with 3 ng of the internal control vector. Relative luciferase activity was measured 48 h after transfection by the Dual Luciferase Reporter Assay System (Promega), using a pRL-CMV vector (Takara Bio) as an internal control. In addition, the same assays were carried out using different quantities of the expression vectors; we adjusted the amount of each plasmid such that all MAMLD1 proteins showed similar expression levels relative to that of β-actin. In these experiments, protein expression levels were quantified by ImageJ (imagej.nih.gov/ij/). The relative expression levels were calculated by dividing the signal intensities of MAMLD1 by those of β-actin (online suppl. fig. 1). Specifically, 8, 200, and 100 ng of the vectors for the full-length wild-type, mutant, and variant proteins, respectively, were used for transfection. Each experiment was performed in triplicate and repeated 3 times.

Statistical Analysis

The results are expressed as the mean ± SD, and statistical significance was determined using the t test. p values <0.05 were considered significant.

Results

Mutation Analysis of MAMLD1

A hemizygous nucleotide substitution (g.IVS4−2A>G) was identified in a patient with hypospadias (fig. 1A). The g.IVS4−2A>G mutation affected the consensus sequence at the splice acceptor site in intron 4. No pathogenic mutations were identified in the remaining 179 patients. The mutation-positive patient presented with penoscrotal hypospadias (fig. 1B). He had no family history of hypospadias. Ultrasonography at 1 month of age delineated 2 testes, each 10 × 10 × 14 mm, in the scrotum. Endocrine evaluation at 2 years and 11 months of age showed age-appropriate levels of gonadotropins and testosterone (LH <0.5 IU/l; FSH 1.6 IU/l, and testosterone <0.17 nmol/l).

mRNA and Protein Expression Analyses of Genital Skin Samples

Representative results are shown in figure 1C. RT-PCR of the control samples yielded bands of 3 different sizes, which corresponded to the full-length transcript, the Δexon 4 variant, and a hitherto unreported variant lacking exon 5 (Δexon 5). The Δexon 5 variant was predicted to encode a protein of 991 amino acids (c.1822_2065del, p.Q608fsX992). The majority of PCR products of the patient's sample were found to be the full-length transcript or the Δexon 5 variant. Furthermore, 4 of 17 clones of the full-length transcript lacked the first 10 nucleotides of exon 5, while the remaining clones had wild-type sequences. The full-length transcript with the 10-bp deletion was predicted to encode an elongated protein of 1,069 amino acids (c.1822_1831delACTCATGTAG, p.K609fsX1070). RT-PCR using specific primers for each splice variant yielded almost similar amounts of the full-length transcript and the Δexon 5 variant for all samples, whereas the PCR product for the Δexon 4 variant was barely detectable in the patient's sample.

In the Western blot analysis, no clear signals of MAMLD1 protein were detected in the genital skin samples of the patient or control individuals (data not shown).

In vitro Protein Expression Assays

Western blot analysis of cells transiently transfected with expression vectors for full-length wild-type MAMLD1, the p.K609fsX1070 mutant and the Δexon 5 variant detected proteins of expected masses (fig. 2A). However, the relative amounts of the mutant and Δexon 5 variant proteins were significantly lower than that of the wild-type protein (21.0% and 59.4% of the amount of wild-type protein, respectively). The expression levels of the wild-type and mutant proteins showed parallel increases upon MG132 treatment, indicating that proteasome-mediated degradation was unlikely in this case (fig. 2B). Likewise, the expression of the wild-type and mutant proteins decreased at a similar rate upon CHX treatment, indicating normal stability of the mutant MAMLD1 protein (fig. 2C).

In vitro Transactivation Analysis

The cells expressing the mutant and the Δexon 5 variant proteins showed lower luciferase activities than the cells expressing the wild-type protein (fig. 3A). The mutant and Δexon 5 variant proteins exerted apparently normal transactivating function when their expression was adjusted to a level similar to that of the wild-type protein (fig. 3B).

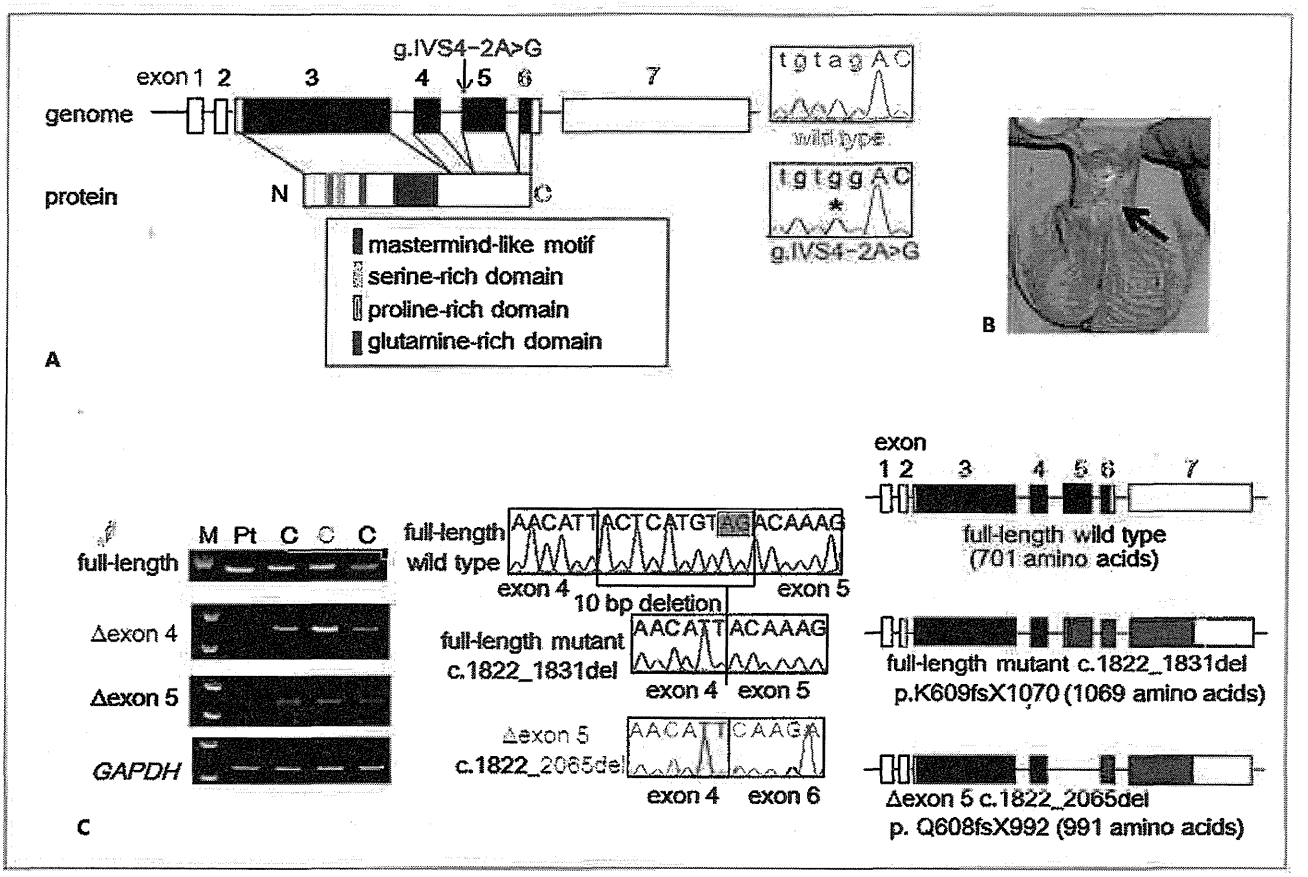

Fig. 1. *MAMLD1* mutation identified in the present study. **A** Position and sequence of the g.IVS4–2A>G mutation. The black and white boxes indicate the coding and non-coding regions, respectively. The asterisk depicts the mutated nucleotide. **B** Genital appearance of the mutation-positive patient. The arrow indicates the urethral meatus. **C** Representative results of the RT-PCR analysis of genital skin fibroblasts. Left panel: specific transcripts of *MAMLD1* splice variants. As an internal control, a house-keeping gene *(GAPDH)* was amplified. M = Molecular weight marker; C = control individuals; Pt = patient. Middle panel: the full-length transcript of the patient included the c.1822_1831del mutant. The 10-bp deletion can be ascribed to the activation of a cryptic splice acceptor site in exon 5 (shaded in red). Right panel: predicted protein structure of the transcripts. The black and white boxes indicate the coding and non-coding regions, respectively. The red box depicts the deleted region, and the blue boxes indicate aberrant amino acids encoded by frameshift mutation/variation.

Discussion

We identified a hemizygous intronic mutation in *MAMLD1* in 1 of 180 patients with hypospadias. These results support the previously proposed notion that *MAMLD1* mutations account for a small fraction of the etiology of hypospadias [Fukami et al., 2006]. RT-PCR of the patient's skin sample showed markedly reduced expression of the Δexon 4 variant and apparently normal expression of the full-length and Δexon 5 transcripts. Although the cause of the reduced expression of the Δexon 4 variant remains to be clarified, there are several examples of point mutations in human genes that alter the balance of splice variants [Sterne-Weiler et al., 2011; Ward and Cooper, 2010]. Disruption of a *cis*-acting splice enhancer or suppressor may underlie such a phenomenon [Sterne-Weiler et al., 2011]. Since the Δexon 4 variant of MAMLD1 is known to retain normal transactivation activity [Fukami et al., 2008], reduced expression of this variant may have played a role in the development of hypospadias in the patient.

Furthermore, the g.IVS4–2A>G mutation generated an mRNA lacking the first 10 nucleotides of exon 5. The 10-bp deletion can be explained by disruption of the