of the 2-year study period but were included in the analysis for their time in the study. Eleven patients (two patients in group A; nine patients in group B) discontinued treatment because of progression to FRNS. Nine patients (four patients in group A; five patients in group B) discontinued treatment because of progression to SDNS. Two patients (one patient in group A; one patient in group B) discontinued treatment because of progression to SRNS. One patient (group A) discontinued treatment because of an adverse event, one patient (group A) discontinued treatment because of loss to follow-up, and one patient (group B) discontinued treatment because of noncompliance (Figure 1).

Characteristics of the patients are shown in Table 1. There was no clinically important difference between the two treatment groups.

Table 1. Characteristics of the patients

Variables	Group A (n=43)	Group B (n=42)
Men	32 (74.4)	31 (73.8)
Age at entry (yr)	7.0±4.3	7.1±3.7
1–5	25 (59.5)	19 (45.2)
6–10	8 (19.1)	14 (33.3)
11–13	6 (14.3)	5 (11.9)
14–18	4 (9.3)	4 (9.5)
Minimal change subtype of NS	43 (100.0)	42 (100.0)
Duration of NS (mo)	18.9±35.5	12.7±15.9
History of SDNS	26 (60.5)	26 (61.9)
Previous treatment with immunosuppressive agent(s)	8 (18.6)	10 (23.8)
Mizoribine	6 (14.0)	9 (21.4)
Cyclophosphamide	1 (2.3)	1 (2.4)
Chlorambucil	1 (2.3)	0 (0)
Total protein (g/dl)	5.9±0.6	5.8±0.7
Albumin (g/dl)	3.4±0.7	3.3±0.7
BUN (mg/dl)	11.5±4.0	12.8±3.4
Creatinine (mg/dl)	0.3±0.1	0.4±0.1
Study baseline eGFR (ml/min per 1.73 m^2)	122.3±30.6	116.5±21.4

Values are n (%) or mean±SD. NS, nephrotic syndrome; SDNS, steroid-dependent nephrotic syndrome; eGFR, estimated GFR.

C_2 and AUC_{0-4} Levels of Cyclosporine

The mean C_2 levels during the first 6 months, the mean C_2 levels during the next 18 months, and the AUC_{0-4} levels at 3 and 9 months after randomization were all significantly higher in group A than group B ($P<0.001$ in all cases) (Table 2). The distribution of exact mean C_2 levels and actual doses of mCyA received by patients in the two groups are shown in Supplemental Tables 2 and 3, respectively.

Efficacy

The primary end point, relapse-free survival, is shown in Figure 2. The estimated SRR 24 months after randomization was 64.4% (95% CI, 48.0% to 76.8%) in group A and 50.0% (95% CI, 34.2% to 63.9%) in group B. The SRR in group A was 14.4% higher than the SRR in group B, which was larger than the decision threshold of 8%; 27 of 43 patients in group A and 21 of 42 patients in group B had not experienced any relapse by the end of 24 months after randomization. The hazard ratio for relapse was 0.57 (95% CI, 0.29 to 1.11; P=0.09). The relapse rates in groups A and B were 0.41 and 0.95/person-year, respectively. The ratio of the two relapse rates was 0.43 (95% CI, 0.19 to 0.84; P=0.02) (Table 3).

The estimated progression-free survival rate at 24 months was 88.1% in group A and 68.4% in group B; seven patients in group A showed progression (two patients to FRNS, four patients to SDNS, and one patient to SRNS), whereas 15 patients in group B showed progression (nine patients to FRNS, five patients to SDNS, and one patient to SRNS). The hazard ratio for progression was 0.33 (95% CI, 0.12 to 0.94; P=0.03) (Figure 3).

Safety

The medians (25th and 75th percentiles) of estimated GFRs before mCyA treatment and at month 24 were 119.0 (106.4–130.9) and 116.0 (106.9–129.0) in group A and 114.0 (102.4–125.0) and 121.3 (109.9–134.3) in group B, respectively. There was no difference between the two groups; 61 patients (31 patients in group A; 30 patients in group B) underwent renal biopsies: 60 patients during months 23–27 and one patient at month 31. Two patients in group A (6.5%) and zero patients in group B developed mild to moderate chronic cyclosporine nephrotoxicity (Supplemental Table 4). This difference in the rate of development of chronic cyclosporine nephrotoxicity was not statistically significant.

Table 2. Mean 2-hour postdose cyclosporine levels and areas under the concentration time curve during the first 4 hours after treatment with cyclosporine

Cyclosporine	Group A (Mean±SD)	Group B (Mean±SD)	P Value
C_2 (ng/ml)			
Months 1–6	566.4±86.9 (n=43)	472.7±73.7 (n=42)	<0.001
Months 7–24	489.5±56.4 (n=40)	382.2±86.8 (n=37)	<0.001
AUC_{0-4} (ng·h/ml)			
Month 3	1944.7±487.9 (n=39)	1554.7±462.8 (n=40)	<0.001
Month 9	1704.7±545.2 (n=36)	1316.6±366.0 (n=34)	<0.001

C_2, 2-hour postdose cyclosporine level; AUC_{0-4}, area under the concentration time curve during the first 4 hours after treatment with cyclosporine.

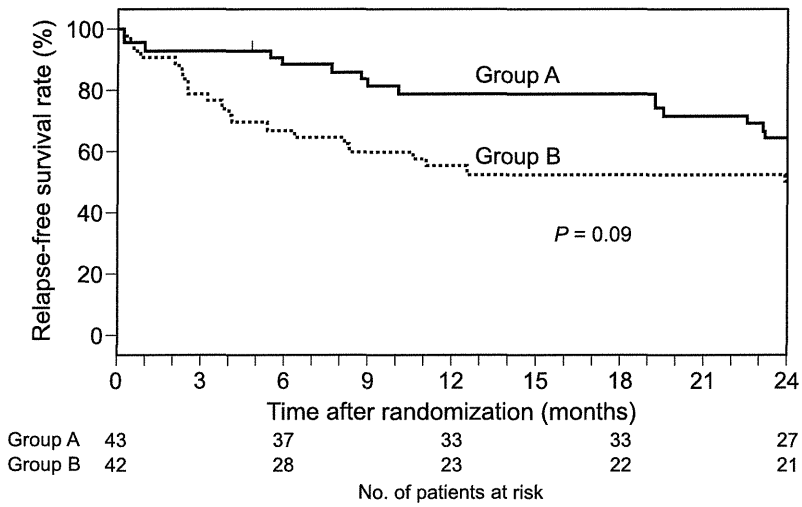

Figure 2. | Relapse-free survival probability (Kaplan–Meier curves).

Table 3. Relapse rates

Treatment Group	Total Number of Relapses	Duration of Observation (d)	Relapse Rate (per person-yr)	Ratio of Relapse Rates (95% Confidence Interval)	*P* Value
Group A	34	30,259	0.41	0.43(0.19 to 0.84)	0.0
Group B	66	25,490	0.95		

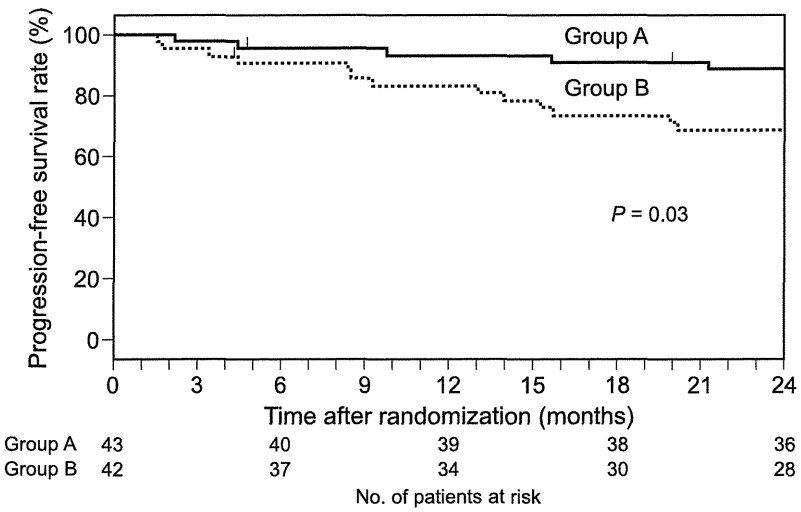

Figure 3. | Progression-free survival probability (Kaplan–Meier curves).

A summary of other adverse events reported during the trial is shown in Table 4. We report cumulative events that occurred within 24 months after randomization, because this time point is when all participants had had an equal opportunity to have an event. The rate and severity of adverse events were similar in both treatment groups. Three patients in group A and two patients in group B had grade III adverse events requiring hospitalization, including one patient in group A who discontinued protocol treatment because of posterior reversible leukoencephalopathy syndrome (25) (month 20), which recovered completely after discontinuation of the protocol treatment. Two of the patients in group A and both of the patients in group B subsequently recovered and restarted protocol treatment as recommended by a physician (Table 4).

Table 4. Summary of adverse events that occurred within 24 months after randomization

Event	Group A (n=43) n (%)	Group B (n=42) n (%)
Grade 3 adverse events		
Pneumonia[a]	3[b,c,d] (7.0)	1[e] (2.4)
Encephalopathy[a]	1[c] (2.3)	1[f] (2.4)
Posterior reversible encephalopathy syndrome[a]	1[b] (2.3)	0
Pneumomediastinum[g]	1[c] (2.3)	0
Grade 1 or 2 adverse events		
Infection[a]	15 (34.9)	13 (31.0)
Asthma[a]	3 (7.0)	1 (2.4)
Edema[a]	1 (2.3)	2 (4.8)
Moon face[a]	3 (7.0)	4 (9.5)
Centripetal obesity[a]	2 (4.7)	1 (2.4)
Hypertrichosis[a]	23 (53.5)	20 (47.6)
Acne[a]	4 (9.3)	2 (4.8)
Cutaneous striae[a]	0	1 (2.4)
Hypertension[g]	7 (16.3)	5 (11.9)
Gingival hyperplasia[g]	4 (9.3)	7 (16.7)
Gastrointestinal event[g]	2 (4.7)	0
Dermatological event[g]	5 (11.6)	3 (7.1)
Neuropsychiatric event[g]	4 (9.3)	3 (7.1)
Pain[g]	0	3 (7.1)
Cataract[g]	2 (4.7)	0
Glaucoma[g]	1 (2.3)	0
Chronic sinusitis[g]	0	1 (2.4)
Cough[g]	1 (2.3)	0
Hyperglycemia[g]	2 (4.7)	2 (4.8)
Hyperkalemia[g]	1 (2.3)	1 (2.4)
Hyperbilirubinemia[g]	2 (4.7)	3 (7.1)
Hyperuricemia[g]	1 (2.3)	1 (2.4)
High-serum glutamic oxaloacetic transaminase[g]	1 (2.3)	3 (7.1)
High-serum glutamic pyruvic transaminase[g]	2 (4.7)	1 (2.4)
High amylase[g]	1 (2.3)	0
High serum creatinine phosphokinase[g]	1 (2.3)	0
Low GFR[g]	1 (2.3)	0
Others[g]	1 (2.3)	3 (7.1)

[a]Multiple reports were recorded for these adverse events.
[b]One patient in group A had pneumonia at month 11 and recovered after 7 days without discontinuing protocol treatment. The same patient had posterior reversible encephalopathy syndrome at month 20, and protocol treatment was discontinued. He recovered completely after 10 days.
[c]One patient in group A had pneumonia, encephalopathy, and pneumomediastinum after influenza infection at month 5 and recovered after 7 days. Protocol treatment was restarted after the recovery.
[d]One patient in group A had pneumonia at month 21 and recovered after 12 days without discontinuing protocol treatment.
[e]One patient in group B had pneumonia at month 5 and recovered after 7 days without discontinuing protocol treatment.
[f]One patient in group B had encephalopathy after rotavirus infection at month 1 and recovered after 7 days. Protocol treatment was restarted after the recovery.
[g]Only the first occurrence of these adverse events was recorded.

Discussion

This study is the first to attempt to select better C_2 levels of cyclosporine in the form of mCyA for FRNS in children. The SRR in group A was 14.4% higher than the SRR in group B, which was larger than the decision threshold of 8%. Also, there was no difference between the two groups with respect to the frequency and severity of adverse events. Therefore, we considered that the C_2 monitoring regimen for group A, in which the target C_2 level was 600–700 ng/ml for the first 6 months and 450–550 ng/ml for the next 18 months, was better than the regimen for group B, in which the target C_2 level was 450–550 ng/ml for the first 6 months and 300–400 ng/ml for the next 18 months. Referencing the report by Ushijima *et al.* (26) on the pharmacokinetic profile of Japanese nephrotic syndrome children treated with mCyA, the mean C_0 levels for months 7–24 in group A might have ranged from 60 to 80 ng/ml, which was lower than the levels in the previous studies (7).

We found that the rate of relapse of nephrotic syndrome was significantly lower in group A than group B patients. This finding agrees with a previous finding that FRNS patients with higher C_2 levels at month 1 tend to have lower relapse rates during cyclosporine treatment (9).

In the previous studies of mCyA treatment by C_2 monitoring for childhood FRNS, the mean relapse rates varied from 0.2 to 1.5 per year under the mean C_2 levels, which ranged from 497.8 to 729.0 ng/ml (13,16,18,20). The relapse rate in group A in the present study (0.41/person-year) was not inferior to the relapse rates in previous studies. Therefore, we considered that the regimen with C_2 target for group A is acceptable for the treatment for childhood FRNS. However, it remains to be elucidated whether the regimen is also acceptable for other populations, because most of C_2 monitoring studies for childhood FRNS were carried out in Japan.

Several grade III adverse events were reported in both groups in this trial. However, all patients with those severe adverse events recovered completely, and most patients restarted protocol treatment. Therefore, we considered adverse events in this trial acceptable. In the present study, two patients (4.7%) in group A developed mild to moderate chronic cyclosporine nephrotoxicity, and zero patients in group B developed this condition. Although the reason is unclear, the prevalence of chronic cyclosporine nephrotoxicity in the present study was much lower than the prevalence in a previous study (discussed in Supplemental Appendix) (15), suggesting that the regimens used in the present study were safe with respect to the development of this condition. The two patients who developed cyclosporine nephrotoxicity both had 9-month AUC levels that seemed to be notably higher than the mean for group A (Supplemental Table 4). However, it is premature to make a conclusion that the higher 9-month AUC levels were responsible for the nephrotoxicity, because the number of patients who developed chronic cyclosporine nephrotoxicity was very low.

One limitation of our study is that, at one particular center, C_2 levels were not measured in most patients. Because we had defined the full analysis set as registered patients whose treatments were correctly started in the protocol, the steering committee considered that center to be ineligible and decided that all eight patients at the center should be excluded from the full analysis set.

Another limitation is that the mean C_2 levels during the first 6 months in group A did not reach the target range, suggesting that it is difficult to control C_2 levels in children, especially when the C_2 target is relatively high. We speculate that a slight difference in dose of mCyA may induce a relatively large difference in C_2 concentrations in children when the C_2 target is relatively high. Nevertheless, the mean C_2 levels in group A were significantly higher than the mean C_2 levels in group B throughout the trial. In addition, the levels of AUC_{0-4} at months 3 and 9 were significantly higher in group A than group B. We, therefore, conclude that patients in both groups were treated in accordance with the protocol. Additional discussion on the target C_2 levels for phase III trials is in Supplemental Appendix.

It is still controversial whether C_2 or C_0 monitoring is better for renal transplant recipients (10,11,27–34). It is also unclear whether C_2 or C_0 monitoring is better for children with FRNS treated with mCyA. Although our study shows that C_2 monitoring with the target C_2 set for group A is promising, phase III trials are required to compare the efficacy and safety of the regimen with the efficacy and safety of the JSPN-recommended C_0 monitoring protocol.

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Physicians who participated in the Japanese Study Group of Kidney Disease in Children 03 are listed in Supplemental Appendix.

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Original Article

Pre-dialysis chronic kidney disease in children: results of a nationwide survey in Japan

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Keywords: child, creatinine, epidemiology, Japan, kidney diseases

ABSTRACT

Background. Chronic kidney disease (CKD) in children is a progressive and intractable condition that may severely impair the child's growth, development and quality of life. Epidemiological information on pediatric CKD, particularly in Asians, is scant.

Methods. We conducted a nationwide, population-based survey of Japanese children aged 3 months to 15 years with pre-dialysis CKD to examine the prevalence of pediatric CKD in Japan. CKD was classified according to newly established criteria derived from reference serum creatinine levels in Japanese children. Surveys were sent to 1190 institutions across Japan to report on cases of pediatric CKD managed as of 1 April 2010.

Results. A total of 925 institutions (77.7%) responded. Information on 447 children was collected. When subdivided according to our diagnostic criteria, 70.5% of children had stage 3 CKD, 23.9% stage 4 and 5.6% stage 5. The estimated prevalence of Japanese children with CKD was 2.98 cases/100 000 children. Of 407 CKD cases with non-glomerular disease, 278 (68.3%) had congenital anomalies of the kidney and urinary tract (CAKUT). The newly established criteria showed good validity compared with existing criteria, including the abbreviated Schwartz equation.

Conclusions. Findings from the first nationwide survey of pre-dialysis CKD in Asian children indicate that the prevalence of stage 3–5 CKD in children in Japan aged 3 months to 15 years is 2.98 cases/100 000 children. Most children with CKD presented with non-glomerular disease, most frequently CAKUT.

Improved management of CAKUT, including renoprotective treatment and urological intervention, is required.

INTRODUCTION

Chronic kidney disease (CKD) in children is a progressive and intractable condition, with devastating effects on the patient's growth, development and quality of life. If left untreated, pediatric CKD eventually progresses to end-stage renal disease (ESRD), which requires long-term dialysis or repeated renal transplantation. The mortality rate for children with ESRD on dialysis is estimated to be 30–150 times that of the general pediatric population [1, 2]. Therefore, it is particularly important to detect CKD as early as possible, possibly by applying simple but accurate screening of at-risk children. Early identification of these children can then allow the physician to promptly introduce appropriate therapy that can prevent or slow the progression of CKD to ESRD, reducing the incidence of stage 5 CKD and to control comorbidity.

Epidemiological information on CKD in children is currently limited, but this sort of information is necessary to understand the extent of the problem, to identify populations at risk and to determine the efficacy of current therapeutic interventions. Although several studies have described the epidemiology of pre-dialysis CKD in children in Western countries [3–10], very few have focused on Asian children. It is also important to consider that there may be differences in the epidemiology of CKD among countries that may be due to racial differences, variations in screening methods among medical institutions and differences in in-school screening programs. To address this problem of limited information in Asian children and to assist subsequent population-based surveys, we previously determined reference serum creatinine (SCr) levels in Japanese children [11].

Our first objective in this study was to determine the prevalence of pre-dialysis CKD in a cross-sectional, nationwide survey of Japanese children aged 3 months to 15 years with pre-dialysis CKD. Stage 3–5 CKD was detected and classified using newly established criteria derived from normal SCr levels of age- and sex-matched Japanese children. Because CKD is defined as a glomerular filtration rate (GFR) of <60 mL/min/1.73 m^2 (less than half of normal GFR) in the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines [12, 13] and the Kidney Disease: Improving Global Outcomes (KDIGO) position statement [14] (corresponding to stage 3 or worse), we focused on patients meeting this criterion and who had not yet received dialysis or renal transplantation. Our second objective was to determine the etiology of CKD as well as the method of detection of CKD and the treatment modalities used in routine clinical practice in Japan.

MATERIALS AND METHODS

Establishment of new diagnostic criteria for CKD in children

The new diagnostic criteria for stage 3–5 CKD were based on previously established reference SCr levels of Japanese children [11]. Briefly, in that study, body length and SCr levels were determined in 1151 healthy children aged 1 month to 18 years who presented at the facilities of the Committee of Measures for Pediatric CKD and Tokyo Health Service Association between 2008 and 2009. Reference intervals of SCr against age were calculated in children aged 3 months to 11 years, and those against age and sex were calculated in children aged 12–15 years.

According to the K/DOQI guidelines [12, 13] and KDIGO position statement [14] for CKD, stage 3–5 CKD was classified as GFR 30–59, 15–29 and <15 mL/min/1.73 m^2, respectively (<1/2, <1/4 and <1/8 of normal GFR, respectively), whereas normal GFR was considered to be ~120 mL/min/1.73 m^2. Given that the GFR is inversely proportional to SCr for a given body type and age [15], we classified stage 3–5 CKD as SCr more than twice, four times and eight times, the median normal SCr levels matched for age alone in children aged 3 months to 11 years (Table 1), or matched for age and sex in children aged 12–15 years (Table 2).

Study design and population

This was a cross-sectional, nationwide, population-based survey conducted by the Pediatric CKD Study Group in Japan in conjunction with the Committee of Measures for Pediatric CKD of the Japanese Society for Pediatric Nephrology (JSPN). Two surveys were sent in August 2010 to a total of 1190 institutions in Japan, including all institutions that are members of the JSPN, all university and children's hospitals and all general hospitals with >200 beds, inviting them to report cases of pediatric CKD that were managed as of 1 April 2010. We selected these types of hospitals because children with apparent CKD were usually referred to institutions meeting one of these criteria. The deadlines for the first and second surveys were October 2010 and November 2010, respectively.

The first questionnaire was designed to record the presence and approximate number of children with stage 3–5 CKD in each institution. The second questionnaire recorded data for each case, including age, date of birth, sex, height, SCr level, primary renal diagnosis and associated diseases, method of detection, comorbidities and prescribed treatment. For the purpose of this survey, only data recorded within 6 months of 1 April 2010 were included. The patient's age was calculated from the date of birth and the date of each measurement. This questionnaire also recorded information for each institution, including the SCr assay method used, and prescribed treatment strategies. The respondents were asked to search their medical records for patients with a confirmed diagnosis of CKD or for patients with an abnormal SCr.

The inclusion criteria were as follows: (i) children with CKD aged 3 months to 15 years at the time of 1 April 2010; (ii) stage 3–5 CKD, as determined by the newly established diagnostic criteria and (iii) no prior treatment with dialysis or renal transplantation. Only cases with kidney dysfunction that had lasted for >3 months were included and cases with transient increases in creatinine were excluded.

The study was conducted in accordance with the ethical principles set out in the Declaration of Helsinki, and with the

Table 1. Diagnostic criteria for stage 3–5 chronic kidney disease based on reference serum creatinine levels (mg/dL) of Japanese children aged 3 months to 11 years

Age	2.5th percentile	50th percentile	97.5th percentile	CKD stage 3	CKD stage 4	CKD stage 5
<2 years						
3–5 months	0.14	0.20	0.26	0.41–0.80	0.81–1.60	≥1.61
6–8 months	0.14	0.22	0.31	0.45–0.88	0.89–1.76	≥1.77
9–11 months	0.14	0.22	0.34	0.45–0.88	0.89–1.76	≥1.77
1 year	0.16	0.23	0.32	0.47–0.92	0.93–1.84	≥1.85
2–11 (years)						
2	0.17	0.24	0.37	0.49–0.96	0.97–1.92	≥1.93
3	0.21	0.27	0.37	0.55–1.08	1.09–2.16	≥2.17
4	0.20	0.30	0.40	0.61–1.20	1.21–2.40	≥2.41
5	0.25	0.34	0.45	0.69–1.36	1.37–2.72	≥2.73
6	0.25	0.34	0.48	0.69–1.36	1.37–2.72	≥2.73
7	0.28	0.37	0.49	0.75–1.48	1.49–2.96	≥2.97
8	0.29	0.40	0.53	0.81–1.60	1.61–3.20	≥3.21
9	0.34	0.41	0.51	0.83–1.64	1.65–3.28	≥3.29
10	0.30	0.41	0.57	0.83–1.64	1.65–3.28	≥3.29
11	0.35	0.45	0.58	0.91–1.80	1.81–3.60	≥3.61

Values were matched for age alone. Values for the 2.5, 50 and 97.5th percentiles are as presented in Uemura *et al.* [11]. Table reproduced with the permission of the Japanese Society of Nephrology.

Table 2. Diagnostic criteria for stage 3–5 chronic kidney disease based on reference serum creatinine levels (mg/dL) of Japanese male and female children aged 12–15 years

Age	2.5th percentile	50th percentile	97.5th percentile	CKD stage 3	CKD stage 4	CKD stage 5
Males						
(years)						
12	0.40	0.53	0.61	1.07–2.12	2.13–4.24	≥4.25
13	0.42	0.59	0.80	1.19–2.36	2.37–4.72	≥4.73
14	0.54	0.65	0.96	1.31–2.60	2.61–5.20	≥5.21
15	0.48	0.68	0.93	1.37–2.72	2.73–5.44	≥5.45
Females						
(years)						
12	0.40	0.52	0.66	1.05–2.08	2.09–4.16	≥4.17
13	0.41	0.53	0.69	1.07–2.12	2.13–4.24	≥4.25
14	0.46	0.58	0.71	1.17–2.32	2.33–4.64	≥4.65
15	0.47	0.56	0.72	1.13–2.24	2.25–4.48	≥4.49

Values were matched for age and sex. Values for the 2.5, 50 and 97.5th percentiles are as presented in Uemura *et al.* [11]. Table reproduced with the permission of the Japanese Society of Nephrology

ethical guidelines for epidemiological studies issued by the Ministry of Health, Labour and Welfare in Japan. The study was approved by the JSPN ethics board and a central ethics board (the institution of the Principal Investigator, KI) before study commencement. Because, data were reported retrospectively using patient charts, informed consent was not obtained in accordance with the above guidelines.

Statistical analyses

Estimation of the number of patients with stage 3–5 CKD in Japan from the reported number of patients in our survey was conducted as follows. The estimates were derived as the reported number divided by the response rate. Because the response rate tends to be lower in institutions with fewer patients, simple estimates can overestimate the true prevalence. Therefore, the reported patients were stratified according to institution type (i.e. university hospital, children's hospital and general hospital) and the number of beds (<200, 200–500 and ≥500), based on the assumption that the response rate is independent of the number of patients in each stratified category [16]. Then, the number of reported patients in each category was divided by the response rate and summed to calculate the total estimated number of patients in Japan. The total estimated number of patients was divided by the size of the population at risk in Japan reported by the Statistics Bureau of the Ministry of Internal Affairs and Communications of Japan (http://www.stat.go.jp/english/index.htm) to calculate the prevalence as of 1 April 2010. Weighted κ with 95% confidence interval (CI) was calculated to compare the CKD classification used here with the abbreviated Schwartz equation. All statistical analyses were carried out using SAS system version 9 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Subject characteristics

A total of 925 of 1190 institutions (77.7%) responded to the first questionnaire. A total of 479 children were identified in the second questionnaire. Of these, 447 children (272 males and 175 females) with stage 3–5 CKD who had not been treated with dialysis/renal transplantation fulfilled the eligibility criteria and were included in this study. Their characteristics are summarized in Table 3. Most of the children (315; 70.5%) had stage 3 CKD, whereas 107 (23.9%) had stage 4 and 25 (5.6%) had stage 5. The number of Japanese children with stage 3–5 CKD was estimated to be 542.5 (95% CI: 497.5–587.5) as of 1 April 2010. On the basis of this, the prevalence of stage 3–5 CKD was calculated to be 2.98 cases/100 000 Japanese children aged 3 months to 15 years. Figure 1 shows the SCr values for males and females according to CKD stage. All of the responding institutions used enzyme immunoassays to determine SCr levels for the assessment of CKD stage; none used other methods, such as the Jaffe method.

Figure 2 shows the frequencies of CKD stage according to the estimated GFR (eGFR) of 412 children in whom height was measured. Stage 3–5 CKD was classified using our diagnostic criteria derived from SCr levels of age- and sex-matched Japanese children, while the eGFR was determined using the abbreviated Schwartz equation, which was recently revised from the original Schwartz equation [17]. This figure also shows the distribution of children classified in each CKD stage determined using both methods. These data indicate that the distribution of CKD stages determined using population-based reference values is comparable with the distribution derived using a method based on the abbreviated Schwartz

Table 3. Patient characteristics according to chronic kidney disease stage

	All subjects	Stage 3	Stage 4	Stage 5
n	447	315	107	25
Age (years)	8.7 ± 4.5	8.7 ± 4.6	8.5 ± 4.3	10.0 ± 4.5
Serum creatinine (mg/dL)	1.6 ± 1.2	1.1 ± 0.4	2.2 ± 0.8	5.3 ± 2.0
Height (cm)	119.8 ± 28.9	121.1 ± 28.7	118.8 ± 27.4	107.8 ± 35.6
Height SDS[a]	−1.6 ± 1.8	−1.3 ± 1.5	−2.2 ± 2	−3.5 ± 3
BUN (mg/dL)	35.6 ± 18.8	28.4 ± 9.8	48.6 ± 18.2	74.9 ± 31.5
CysC (mg/L)	2.1 ± 0.8	1.9 ± 0.5	3.1 ± 1.0	4.1 ± 0.9
eGFR–abbreviated (mL/min/1.73 m^2)[b]	39.5 ± 16	47.2 ± 11.2	22.6 ± 5.5	9.6 ± 3.2
eGFR–complete (mL/min/1.73 m^2)[c]	39.6 ± 12.3	43.7 ± 9.7	24.9 ± 5.3	11.6 ± 4.1

Values are means ± standard deviation.
SDS, standard deviation score; BUN, blood urea nitrogen; CysC, cystatin C.
[a]Height SDS was calculated using data recorded by the Japanese Society for Pediatric Endocrinology in 2000 (http://jspe.umin.jp/ipp_taikaku.htm).
[b]Determined using the abbreviated Schwartz equation.
[c]Determined using the complete Schwartz equation.

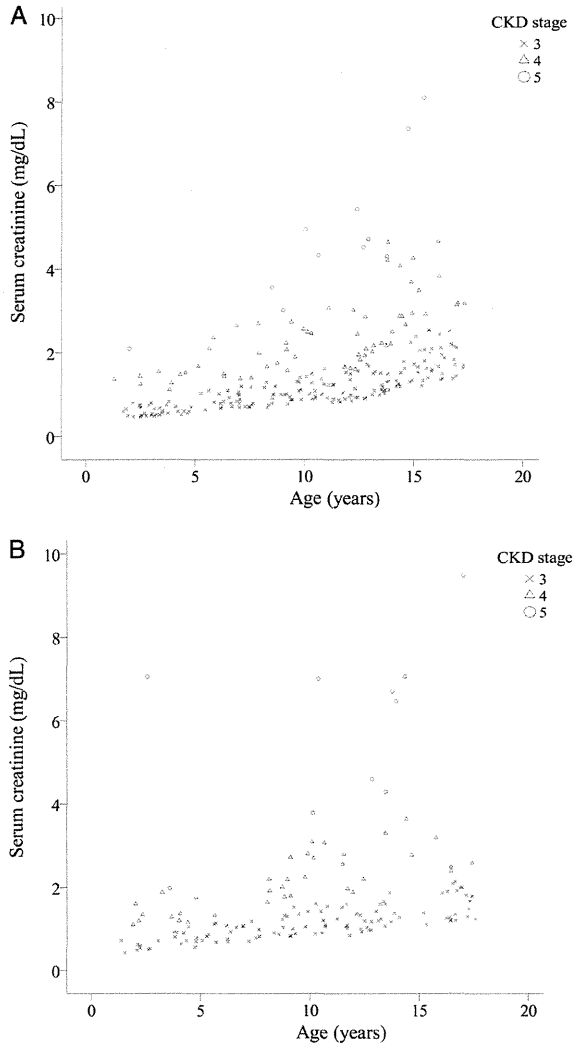


FIGURE 1. Serum creatinine levels according to age and CKD stage. Actual serum creatinine levels according to age and CKD stage are plotted separately for males (**A**) and females (**B**).

equation. The weighted κ-value for this comparison was 0.71 (95% CI: 0.65–0.77). For 198 children with cystatin C data, similar distributions were obtained when we compared our new classification with the complete Schwartz equation, which requires cystatin C-values [17] (data not shown).

Primary etiologies of pediatric CKD in Japan

The primary etiologies of CKD in the study population are presented in Table 4. Non-glomerular disease (407/447; 91.1%) was the most common primary cause of CKD, whereas glomerular disease accounted for 7.8% (35/447) of all cases.

Among those with non-glomerular diseases, 278 (68.3%) children had congenital anomalies of the kidney and urinary tract (CAKUT), of which 60 (21.6% of those with CAKUT) had obstructive urological malformations comprising posterior urethral valve, stricture of the urethra, hydronephrosis, hydroureter and cloacal anomaly (Table 4). The three most common causes of glomerular diseases were Alport's syndrome, focal segmental glomerulosclerosis and chronic glomerulonephritis ($n = 8$ each). No children presented with definitively diagnosed IgA nephropathy. Figure 3 shows the distribution of CAKUT and non-CAKUT diseases by age.

The diseases included recognizable syndrome [$n = 46$ (10.3%)] as follows: Down syndrome (OMIN, #190685, $n = 6$); VATER association (#192350, $n = 4$); Kabuki syndrome (#147920); Wolf–Hirschhorn syndrome (#194190) and Townes–Brocks syndrome (#107480, $n = 3$ each); prune belly syndrome (#100100) and branchio-oto-renal syndrome (#113650, 2 each) and others.

Methods of detecting Stage 3–5 CKD

Table 5 summarizes the methods and reasons for the detection of children with stage 3–5 CKD. Table 5 also presents the age at diagnosis for each of the methods. Fetal and perinatal ultrasonography was the most common method, followed by analysis by chance and urinary tract infection. As might be expected, CKD was generally detected at an earlier age in children with CAKUT than in children with other forms of CKD, particularly for analysis by chance (3.9 versus 5.8 years), urinary tract infection (0.7 versus 1.8 years) and failure to thrive (0.3 versus 2.2 years). Annual urinalysis at school detected CKD in 27 children (9.7%; median age, 8.9 years) with CAKUT and 12 children (7.1%; median age, 8.3 years) with other forms of CKD.

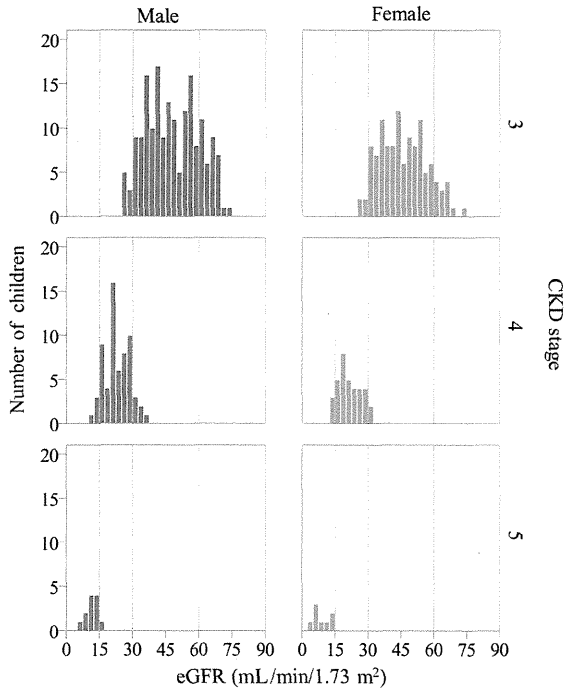
Treatment modalities for pediatric CKD

The treatment modalities for all patients included in this survey, and for patients with CAKUT and those with other forms of CKD, are summarized in Table 6. The most common treatments for CAKUT were angiotensin II receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEIs) which were used in approximately one-quarter of the patients. Both ARBs and ACEIs together were used in 11 and 23 patients with CAKUT and other forms of CKD, respectively (data not shown). Carbon adsorbents (e.g. AST-120), which are approved as renoprotective agents adsorbing uremic toxins in the gastrointestinal tract [18] and calcium antagonists, were used in 13.0 and 7.2% of patients, respectively.

DISCUSSION

Our findings revealed that the prevalence of stage 3–5 CKD in children in Japan aged 3 months to 15 years is 2.98 cases/100 000 children. Out of 447 CKD cases surveyed, 407 (91.1%) had non-glomerular disease; among them, 278 (68.3%) had CAKUT. To our knowledge, this is the first cross-sectional, nationwide, population-based survey of children with pre-dialysis CKD in Asia. Several reports to date have described the epidemiology of pre-dialysis CKD in children; however, these studies were restricted to Western countries [3–10].

SCr levels were frequently used to estimate the GFR and screen for CKD. The original Schwartz equation has been used extensively in clinical practice for estimating the GFR in children, where GFR (mL/min/1.73 m^2) = age-dependent



Our classification	CKD classification determined using the abbreviated Schwartz equation				
	2	3	4	5	Total
Males					
3	35 (19.7%)	135 (75.8%)	8 (4.5%)	0 (0.0%)	178 (100.0%)
4	0 (0.0%)	6 (9.5%)	53 (84.1%)	4 (6.3%)	63 (100.0%)
5	0 (0.0%)	0 (0.0%)	1 (8.3%)	11 (91.7%)	12 (100.0%)
Females					
3	13 (11.2%)	99 (85.3%)	4 (3.4%)	0 (0.0%)	116 (100.0%)
4	0 (0.0%)	2 (5.7%)	30 (85.7%)	3 (8.6%)	35 (100.0%)
5	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (100.0%)	8 (100.0%)

FIGURE 2. Distribution of CKD stage in males and females. Stage 3–5 CKD was classified using our newly established diagnostic criteria derived from normal SCr levels of age- and sex-matched Japanese children. The eGFR was determined using the abbreviated Schwartz equation [17]. Stage 3–5 CKD was classified as GFR 30–59, 15–29 and <15 mL/min/1.73 m^2, respectively (<1/2, <1/4 and <1/8 of normal GFR, respectively). Only subjects in whom height was measured were included in this analysis. Values in the table are *n* (%).

coefficient $\kappa \times$ height (cm)/SCr (mg/dL) [15]. This equation was recently modified because of the increasing use of enzymatic methods to determine SCr levels, replacing the Jaffe method [17]. However, there are some possible limitations of the original Schwartz equation. First, it requires the patient's height, which is not always measured in routine clinical practice. Secondly, the GFR was reported to be lower in Asian adults than in Caucasians [19], which may have led us to overestimate the GFR when using the Schwartz equation in Asian children. To overcome these perceived limitations, several research groups have sought to establish reference levels in large populations of children [11, 20], which may be more practical and relevant for screening purposes in a specific country. Accordingly, in our present study, we evaluated renal function by comparison with established reference values [11]. In this way, CKD was determined based on SCr, rather than relying on equations adjusted for height and mathematical constants. As a result, children aged <2 years, to whom the normal CKD classification could not be applied, could be included. Similarly, Pottel *et al.* [20] proposed and validated a height-independent, population-normalized equation derived from the patient's SCr and the median SCr for age-matched healthy children. Based on their results, population-based reference levels for renal function and CKD may provide a valid approach to determine CKD stage for screening purposes, as in the present study. Indeed, our newly established CKD classification showed good validity compared with the abbreviated and complete Schwartz equations.

To classify stage 3–5 CKD, we used new diagnostic criteria based on previously determined SCr reference levels in age- and sex-matched Japanese children [11]. In that study, SCr was determined using enzymatic methods; in our current study, the participating institutes only used the enzymatic method to determine SCr. Therefore, our current results are

Table 4. Primary etiologies of stage 3–5 chronic kidney disease in Japanese children aged 3 months to 15 years

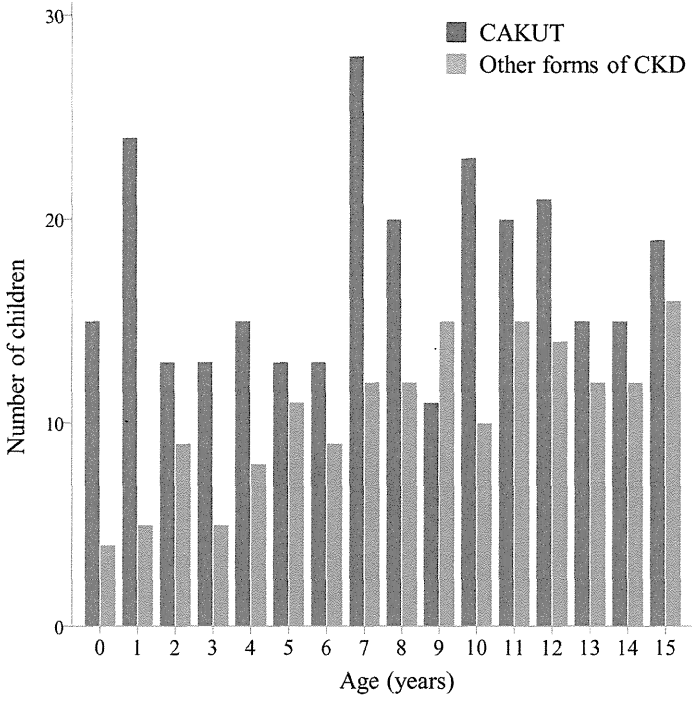
Primary disease	Non-glomerular kidney disease (n = 407, 91.1%)	Glomerular kidney disease (n = 35, 7.8%)	Unclassified (n = 5, 1.1%)
	n (%)	n (%)	n (%)
CAKUT	278 (68.3)	0 (0.0)	0 (0.0)
CAKUT with obstructive urological malformations[a]	60 (21.6)	0 (0.0)	0 (0.0)
CAKUT without obstructive urological malformations	218 (78.4)	0 (0.0)	0 (0.0)
Cortical necrosis (perinatal period)	40 (9.8)	0 (0.0)	0 (0.0)
Polycystic kidney disease	20 (4.9)	0 (0.0)	0 (0.0)
Nephronophthisis	19 (4.7)	0 (0.0)	0 (0.0)
Drug induced	17 (4.2)	0 (0.0)	1 (20.0)
Other inherited kidney damage	10 (2.5)	1 (2.9)	0 (0.0)
Acute kidney injury	10 (2.5)	0 (0.0)	0 (0.0)
Neurogenic bladder	6 (1.5)	0 (0.0)	0 (0.0)
Other non-inheritable character	4 (1.0)	2 (5.7)	0 (0.0)
Alport's syndrome	0 (0)	8 (22.9)	0 (0.0)
Cystinosis	1 (0.2)	0 (0.0)	0 (0.0)
Wilms tumor	1 (0.2)	0 (0.0)	0 (0.0)
Chronic tubulointerstitial nephritis	1 (0.2)	0 (0.0)	0 (0.0)
Focal segmental glomerulosclerosis	0 (0.0)	8 (22.9)	0 (0.0)
Chronic glomerulonephritis	0 (0.0)	8 (22.9)	0 (0.0)
Congenital nephrotic syndrome	0 (0.0)	3 (8.6)	0 (0.0)
Hemolytic uremic syndrome	0 (0.0)	3 (8.6)	0 (0.0)
Systemic lupus erythematosus	0 (0.0)	2 (5.7)	0 (0.0)
Unknown	0 (0.0)	0 (0.0)	4 (80.0)

[a]Posterior urethral valve, stricture of the urethra, hydronephrosis, hydroureter, and cloacal anomaly.

not subject to confounding because of the use of multiple assay types.

The prevalence of pre-dialysis stage 3–5 CKD was estimated to be 2.98 cases/100 000 Japanese children, which was lower than that reported in the ItalKid and REPIR II Projects (7.47 and 7.106 cases/100 000 children, respectively). The reason for this lower prevalence of CKD in Japan in comparison with Western countries is unclear, but differences in the age of the cohort and the method of case definition may account for some of the difference. For example, the ItalKid Project [3] included children aged <20 years, used the original Schwartz equation to determine GFR and included children with eGFR <75 mL/min/1.73 m^2. Similarly, the REPIR II study [4] included children aged <19 years with stage 2 CKD, which accounted for 42% of their cases. Nevertheless, the estimated prevalence of stage 3–5 CKD in Spain, based on data from the REPIR II study, is 4.12 cases per 100 000 children (7.106 × 58%), which is slightly higher than that estimated in our study. The low frequency of pre-dialysis CKD in our study is consistent with the low frequency of children with ESRD in Japan [7].

A number of factors, such as differences in racial and ethnic distributions, primary cause of CKD and quality of medical care, may contribute to the difference in reported prevalence estimates between Japan and Western countries. Additionally, the prevalence of obstructive uropathy is low in Japan, being detected in just 21.6% of patients with CAKUT; by contrast, in Western countries, obstructive uropathy accounts for many cases of non-glomerular disease in children with CKD [21, 22]. Several factors may explain the differences in the prevalence of CAKUT with obstructive uropathy, including (i) genetic differences that affect the distribution of



Age	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total
Population (thousands)[a]	1,078	1,092	1,084	1,072	1,050	1,088	1,111	1,145	1,160	1,180	1,179	1,193	1,188	1,183	1,206	1,208	18,217
CAKUT[b]	15	24	13	13	15	13	13	28	20	11	23	20	21	15	15	19	278
Other forms of CKD[b]	4	5	9	5	8	11	9	12	12	15	10	15	14	12	12	16	169

FIGURE 3. Age distribution of children with stage 3–5 CKD in Japan. Children with CAKUT are shown in dark gray bars, while those with other forms of CKD are shown in light gray bars. [a]Total numbers of children of each age in Japan derived from national census data (1 April 2010) published by the Statistics Bureau of Ministry of Internal Affairs and Communications in Japan (http://www.stat.go.jp/english/index.htm). [b]Number of children with CAKUT or other forms of CKD reported in the survey. CKD, chronic kidney disease; CAKUT, congenital anomalies of the kidney and urinary tract.

obstructive diseases (e.g. prune-belly syndrome) and (ii) the diagnosis of these congenital diseases may be difficult, resulting in underestimation of obstructive uropathies. However, despite the lower frequency of obstructive uropathy in Japan, appropriate urological interventions are still an indispensable part of the management of children with CKD, because they are one of very few treatments that can change the outcome of CKD [23].

Despite the lower prevalence of CKD in our study compared with European cohorts, we believe that our data accurately represent the current situation in Japan because 1190 institutes, including all institutes belonging to the JSPN, were included in the survey and there was a very high response rate (77.7%). We also stratified institutions by hospital type and the number of beds to improve the accuracy of the estimated prevalence. Because the response rate tended to be lower for institutions with fewer patients, estimates of CKD prevalence that do not take strata (hospital size and type) into account are possibly overestimates. For example, a simple estimate without stratification in the present study would have been 599.0 children rather than the 542.5 estimated with strata taken into account. Thus, the stratified estimation method should correct for a bias between response rates and hospital type/size. Nevertheless, it is possible that some patients with stage 3–5 CKD were treated at other types of institutions not included in this survey.

The majority of Japanese children with CKD presented with non-glomerular disease. CAKUT was the primary cause of CKD (i.e. 62.2% of all CKD cases). This observation was expected. Unlike in adults, in whom diabetes and hypertension are the primary cause of CKD, congenital causes are responsible for majority of pediatric CKD cases [1, 7]. The prevalence of CAKUT in our study is also consistent with that reported in the ItalKid and REPIR II studies (67.5 and 59%, respectively) [3, 4].

Interestingly, there were very few cases of glomerular disease, such as focal segmental glomerulosclerosis, and no confirmed cases of IgA nephropathy (one case was suspected, but diagnosis was not confirmed). In a Japanese registry of pediatric ESRD patients conducted in 1998, 19% of patients had focal segmental glomerulosclerosis and 3% had IgA nephropathy [24]. The present analysis is likely to have underestimated the prevalence of these diseases for several reasons. First, these diseases progress more rapidly than non-

Table 5. Method of detection of Stage 3–5 CKD

Screening method	CAKUT (n = 278)	Age at which CKD was detected (years)		Other formsof CKD (n = 169)	Age at whichCKD was detected (years)	
	n (%)	Median	IQR	n (%)	Median	IQR
Fetal ultrasonography/ultrasonography in the neonatal period	88 (31.7)	0.0	0.0–0.0	19 (11.2)	0.0	0.0–0.0
Analysis by chance	38 (13.7)	3.9	1.2–6.1	32 (18.9)	5.8	1.7–9.4
Urinary tract infection	38 (13.7)	0.7	0.3–2.0	4 (2.4)	1.8	0.3–3.6
Annual urinalysis at school	27 (9.7)	8.9	7.0–10.3	12 (7.1)	8.3	7.1–10.9
Blood analysis in the neonatal period, asphyxia, neonatal shock and other events	25 (9.0)	0.0	0.0–0.1	31 (18.3)	0.0	0.0–0.0
Failure to thrive, weight loss and general fatigue	25 (9.0)	0.3	0.1–1.0	7 (4.1)	2.2	0.2–12.3
Urinalysis at 3 years	9 (3.2)	3.2	3.0–3.4	7 (4.1)	3.1	3.0–3.6
Routine health check (infants/toddlers)	7 (2.5)	0.3	0.1–1.7	4 (2.4)	2.8	0.4–5.1
Symptoms of glomerulonephritis (edema, oliguria or gross hematuria)	5 (1.8)	3.8	1.0–5.0	13 (7.7)	5.3	2.7–8.7
Analysis because of anomalies and syndromal stigmata	3 (1.1)	0.0	0.0–0.1	1 (0.6)	1.7	1.7–1.7
Detected during the management of other diseases(e.g. heart disease and malignancy)	2 (0.7)	5.3	5.3–5.3	18 (10.7)	3.2	0.2–8.2
Dysuria, including neurogenic bladder and nocturia	2 (0.7)	4.9	4.9–4.9	4 (2.4)	5.7	1.2–9.5
Analysis because of family history	0 (0.0)	—	—	3 (1.8)	6.2	4.5–9.7
Sepsis	0 (0.0)	—	—	3 (1.8)	0.0	0.0–0.1
Others	0 (0.0)	—	—	2 (1.2)	2.2	0.8–3.7
Unknown (not available)	9 (3.2)	—	—	9 (5.3)	—	—

CKD, chronic kidney disease; CAKUT, congenital anomalies of the kidney and urinary tract.

glomerular diseases and could have been missed in the survey. Secondly, we restricted our analysis to those aged <16 years, but chronic glomerulonephritis frequently affects patients aged 16–20 years. Furthermore, these diseases respond well to novel treatment regimens that are well established in Japan, including combination therapy for IgA nephropathy [25] and cyclosporine in combination with steroids for steroid-resistant nephrotic syndrome, including focal segmental glomerulosclerosis [26].

Fetal/neonatal ultrasonography was the most frequently used method to detect CAKUT, followed by blood analyses by chance and urinary tract infection. Only 27 children with CAKUT and 12 with other forms of CKD were detected following annual urinalysis at school. Patients with CKD, particularly children with CAKUT, do not necessarily show abnormal urinalysis, and are missed by the screening. It is also possible that CKD (particularly non-CAKUT forms of CKD) could be detected in the earlier stages (earlier than stage 3) and patients could then receive appropriate intervention to treat the underlying disease. The treatment strategies for CAKUT and other forms of CKD in each institution were generally similar, although the responding institutions more often reported using carbon absorbents for CAKUT and ACEIs in other forms of CKD (data not shown).

Some limitations of the study merit consideration. First, only 77.7% of the surveyed institutions responded to the questionnaire, which may limit the accuracy of the estimate. Secondly, although the classification system used for CKD staging in the present study was based on reference SCr levels determined via enzymatic methods from Japanese children, these diagnostic criteria have not been validated globally and other reference values would be needed for other populations. Height could have also been determined to estimate GFR via the Schwartz equation; however, because the GFR is inversely

Table 6. Treatment strategies for CAKUT and other forms of CKD for individual patients

	CAKUT ($n = 278$)	Other forms of CKD ($n = 169$)	All patients ($n = 447$)
	n (%)	*n* (%)	*n* (%)
ARBs			
No	201 (72.3)	115 (68.0)	316 (70.7)
Yes	74 (26.6)	53 (31.4)	127 (28.4)
NA	3 (1.1)	1 (0.6)	4 (0.9)
ACEIs			
No	209 (75.2)	108 (63.9)	317 (70.9)
Yes	66 (23.7)	60 (35.5)	126 (28.2)
NA	3 (1.1)	1 (0.6)	4 (0.9)
Carbon absorbents			
No	237 (85.3)	144 (85.2)	381 (85.2)
Yes	34 (12.2)	24 (14.2)	58 (13.0)
NA	7 (2.5)	1 (0.6)	8 (1.8)
Calcium antagonists			
No	264 (94.9)	147 (87.0)	411 (91.9)
Yes	11 (4.0)	21 (12.4)	32 (7.2)
NA	3 (1.1)	1 (0.6)	4 (0.9)

CAKUT, congenital anomalies of the kidney and urinary tract; CKD, chronic kidney disease; ARB, angiotensin II receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; NA, not available.

proportional to SCr in age- and sex-matched individuals, and because we used age- and sex-matched reference SCr levels established in a previous study with 1151 children, our measurements should be accurate enough and more practical for screening purposes. Indeed, our CKD staging showed good agreement with CKD staging based on the abbreviated Schwartz equation (Figure 2). Because, our CKD staging method is based on the SCr level, CKD may be missed in children with small muscle mass, such as those with spina bifida, neuromuscular disease and short stature.

To our knowledge, this is the first nationwide, population-based survey of children with pre-dialysis CKD in Asia and applied reference levels for CKD derived from a large cohort of Japanese children. This method showed good agreement with the abbreviated Schwartz equation and is practical for screening purposes, including children aged <2 years, as current methods are not appropriate for estimating CKD in this age group. The estimated prevalence of stage 3–5 CKD in Japan was 2.98 cases/100 000 children, which is lower than that in Western countries. Most cases presented with non-glomerular disease, and CAKUT was the most common cause of CKD. Improved management of CAKUT in children with CKD, including renoprotective treatment and urological interventions, is required. We are planning randomized and longitudinal studies to improve the management of pediatric CKD, and better understand its long-term prognosis.

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CONFLICT OF INTEREST STATEMENT

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ORIGINAL ARTICLE

Endoplasmic reticulum stress with low-dose cyclosporine in frequently relapsing nephrotic syndrome

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Abstract
Background A possible mechanism of cyclosporine (CsA) nephrotoxicity is tubular apoptosis. Endoplasmic reticulum (ER) stress has been shown to be an apoptosis activator. Glucose-regulated proteins 78 and 94 (GRP78, GRP94, respectively) are ER stress-induced chaperones. Eukaryotic translation initiation factor 2α (EIF2α) attenuates protein synthesis. If stress is prolonged, cells undergo apoptosis, inducing the production of GADD153, a transcription factor, which in turn downregulates anti-apoptotic protein B-cell lymphoma 2 (Bcl-2).
Methods Endoplasmic reticulum stress-related molecules were evaluated by real-time polymerase chain reaction (PCR) using renal biopsy tissues from 17 children with frequently relapsing nephrotic syndrome before and after 2 years of CsA therapy.
Results GRP78, GRP94, eIF2α, and Bcl-2 were significantly upregulated in renal biopsy tissues from children 2 years post-CsA treatment. However, there was almost no change in GADD153. Mean ratios of post- to pre-CsA expression of GRP78, GRP94, eIF2α and Bcl-2 were 2.53, 1.80, 2.38 and 1.92, respectively. Post-CsA administration, GRP78 and eIF2α were upregulated by up to sixfold, and GRP94 and Bcl-2 were upregulated by up to fourfold compared with the respective pre-CsA levels. There were significant correlations between GRP78, GRP94, eIF2α, and Bcl-2 levels. These findings suggest that CsA induced an unfolded protein response due to ER stress, but did not cause apoptosis.
Conclusions An unfolded protein response due to ER stress induced by CsA may function in a defensive manner, with less apoptosis occurring under low-dose conditions. This finding is important for the rationale for CsA administration.

Keywords Cyclosporine A · Endoplasmic reticulum stress · Frequently relapsing nephrotic syndrome · Nephrotoxicity · Unfolded protein response

Introduction

Idiopathic nephrotic syndrome (NS) is a common disease in children. More than 80 % of children with idiopathic NS have steroid-sensitive NS (SSNS) [1]. However, approximately 60 % of patients with SSNS experience relapses, and a considerable number of these show frequently relapsing NS (FRNS) and/or steroid-dependent NS (SDNS) and subsequently develop severe corticosteroid toxicity, including hypertension, obesity, growth suppression, diabetes, and glaucoma, after repeated treatment with corticosteroid for the relapses [1]. Therefore, in FRNS/SDNS cases, immunosuppressive drugs are often used to avoid the severe side effects of corticosteroids. Calcineurin inhibitors, especially cyclosporine A

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(CsA), are often used to treat FRNS/SDNS. However, nephrotoxicity due to tubular injury is the main adverse effect of CsA [1].

The mechanisms of chronic CsA nephrotoxicity appear to be multifactorial [2]. One of the possible mechanisms of CsA nephrotoxicity is tubular cell apoptosis. An increased rate of tubular cell apoptosis has been observed in human renal biopsy specimens obtained from patients with CsA nephrotoxicity [3]. Endoplasmic reticulum (ER) stress has been shown to be an activator of apoptosis [4]. ER stress is defined as an accumulation of unfolded or misfolded proteins in the ER, both physiologically and pathologically. In response to this accumulation of unfolded/misfolded proteins, cells adapt to the stressful conditions via an unfolded protein response (UPR) [5]. The UPR involves transient attenuation of new protein synthesis, induction of ER chaperones, such as glucose-regulated proteins 78 and 94 (GRP78 and GRP94), and activation of ER-associated degradation to eliminate immature proteins. However, if the stress is prolonged or if the adaptive response fails, cells undergo apoptosis, which involves the induction of genes such as growth arrest and DNA damage-inducible gene 153 (GADD153) [5–8]. Therefore, apoptosis induced by ER stress is characterized by the induction of the transcription factor GADD153 [4, 9]. Elevated GADD153 expression has been shown to downregulate the expression of anti-apoptotic protein, B-cell lymphoma 2 (Bcl-2) [6]. Eukaryotic translation initiation factor 2α (eIF2α) is one of the key molecules for attenuation of new protein synthesis in the UPR [10, 11]. Systemic administration of CsA in mice causes rapid, significant induction of ER stress in the kidney [12]. It is also thought that CsA causes upregulation of indicators for ER stress in renal tubular cells in humans [5, 8].

However, to date, ER stress in the human kidney due to CsA in clinical practice has not been fully examined. Therefore, we evaluated ER stress and related molecules (GRP78, GRP94, eIF2α, GADD153 and Bcl-2) in renal biopsy tissues obtained from children before and after 2 years of CsA therapy [13, 14].

Methods

Patients

The study was performed in accordance with the Declaration of Helsinki and was approved by the regional research ethics vetting boards (Wakayama Medical University #571). The study group comprised children with FRNS who had idiopathic NS. Patients showing steroid-resistant NS (SRNS) and SDNS were excluded. The criteria for and definitions of NS, remission, and relapse were in accordance with the International Study of Kidney Disease in Children [15]. FRNS was defined as two or more relapses of NS within 6 months after the initial episode or four or more relapses within any 12-month period. Patients received treatment with CsA from January 2006 to December 2011. Only first CsA administrations were included in the study, and no patient had been previously treated with cyclophosphamide. The total duration of CsA treatment was 2 years. For the first 6 months, all patients received microemulsified CsA (Neoral; Novartis, Basel, Switzerland) in a dose that maintained a whole-blood trough (C_0) level between 80 and 100 ng/mL or a 2-h post-dose (C_2) level between 450 and 700 ng/mL of CsA. For the next 18 months, the dose was adjusted to maintain a C_0 level between 60 and 80 ng/mL or a C_2 level between 300 and 550 ng/mL [14]. CsA levels were measured monthly by monoclonal radioimmunoassay. Maintenance prednisolone was not prescribed. Before and after the 2-year treatment, all patients were scheduled to undergo renal biopsies, and the dose of CsA was tapered after the second biopsy. All renal biopsies were performed when patients with FRNS showed no proteinuria. There was no concomitant use of other immunosuppressants among the patients, except for corticosteroids for treatment relapses. Each patient's family gave written informed consent for the renal biopsies and studies. We attempted to include all patients with similar treatment conditions during the study period.

Renal tissues were obtained by needle biopsy under ultrasound guidance, and the biopsy tissues thus obtained were investigated by routine light, immunofluorescence, and electron microscopy. All biopsy specimens were examined and diagnosed by one of the study investigators (NY). Typical CsA-induced chronic nephrotoxicity was defined as arteriolopathy and striped interstitial fibrosis with tubular atrophy [16, 17].

Table 1 Patient characteristics

Characteristics	Study population (N=17)
Sex (F/M)	6/11
Age at onset (years)	3.7±1.7
Age at initial biopsy (years)	5.2±4.1
CsA monitoring (C_0/C_2)	9/8
Mean of the mean C_0 of each patient	78.6±4.4 (N=9)
Mean of the mean C_2 of each patient	462.5±62.6 (N=8)
Relapse during CsA administration (yes/no)	4/13

Values are presented as the mean ± standard deviation (SD) where appropriate

CsA, Cyclosporine A; C_0, whole-blood trough; C_2, 2 h post-dose; F, female; M, male

In our institution, routine renal biopsies are performed in children with persistent (at least >3 months) proteinuria (urine proteinuria/ creatinine >0.2 g/g) with or without hematuria in the first urine specimen. Particular care is taken to exclude transient or orthostatic proteinuria. Following biopsy, some patients show a clinical course with recurrent intermittent proteinuria during their observation period. As controls, we selected samples from five of these patients with intermittent isolated proteinuria who showed minor glomerular abnormalities in their renal biopsy tissues.

C_2 monitoring level

A Japanese study group that included the authors of the present study recently reported an effective and safe treatment protocol for CsA titrated by monitoring C_0 levels in children with FRNS [14]. In that study, the mean (± standard deviation, SD) C_2 level at 1 month was 486.0± 203.9 ng/mL, and there was a tendency for patients with higher C_2 levels at month 1 to have lower relapse rates during the treatment, but the difference was not statistically significant [14]. An international consensus statement on patient management using CsA C_2 monitoring described that the C_2 target used for maintenance phase adult kidney transplantation was 800 ng/mL [18]. Based on these previous results, we considered that 24 months of treatment for children with FRNS by CsA C_2 monitoring with a C_2 target of between 300 and 700 ng/mL should be effective and safe.

Sample collection

Renal specimens were frozen with dry ice and refrigerated immediately at −80 °C until processing. We sliced frozen renal biopsy tissues for routine immunofluorescence and at the same time collected some 5-μm slices which were processed using the RNeasy Mini kit (Qiagen, Hilden, Germany) to isolate total RNA. Using microscopy, we confirmed that slices were from the renal cortex, including glomeruli.

Real-time PCR

We performed real-time PCR assays with a Takara Thermal Cycler Dice ®TP800 system (Takara Bio, Otsu, Japan) to evaluate the gene expressions of GRP78, GRP94, Bcl-2, eIF2α, and GADD153. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Primer sequences were provided by Takara Bio. First-strand cDNA was produced from total RNA using a PrimeScript RT Reagent kit (Takara Bio), and a SYBR Premix Ex Taq kit (Takara Bio) was used to perform quantitative PCR. Collected data were analyzed with the Thermal Cycler Dice Real-time PCR system and its software (Takara Bio). The ΔΔCT method

Table 2 Changes in endoplasmic reticulum stress-related molecule expression between post- and pre-CsA administration in 17 children with frequently relapsing nephrotic syndrome

	GRP78		GRP94		eIF2α		Bcl-2		GADD153	
	Pre-CsA administration	Post-CsA administration	Pre-CsA administration	Post-CsA administration	Pre-CsA administration	Post-CsA administration	Pre-CsA administration	Post-CsA administration	Pre-CsA administration	Post-CsA administration
Mean (SD)	0.76 (0.33)	1.64 (0.99)	1.19 (0.44)	1.78 (0.51)	0.44 (0.17)	0.94 (0.74)	1.16 (0.39)	1.95 (0.98)	1.35 (0.31)	1.51 (0.29)
P	<0.001		0.0067		0.0046		0.0093		0.13	
Mean ratio of post- to pre-CsA administration (SD)	2.53 (1.57)		1.80 (0.99)		2.38 (1.46)		1.92 (1.11)		1.17 (0.33)	

GRP78, Glucose-regulated protein 78; GRP94, glucose-regulated protein 94; eIF2α, eukaryotic translation initiation factor 2α; Bcl-2, B-cell lymphoma 2; GADD153, growth arrest and DNA damage-inducible gene 153

was used to analyze the data. All data are shown as a ratio to the control.

Indirect immunofluorescence

Cryosections (thickness 3 μm) from the renal biopsy tissue were placed on slides and dried at room temperature. Sections were immersed in phosphate-buffered saline (PBS) for 15 min at 37 °C, fixed in 4 % paraformaldehyde for 20 min at 37 °C, and washed in PBS. Sections were then permeabilized in 0.2 % Triton X-100 in PBS for 15 min at room temperature and washed in PBS. To block nonspecific binding, we used Image-iT FX signal enhancer (Life Technologies, Grand Island, NY) for 30 min at room temperature and washed the sections. Primary antibodies [anti-GRP78 goat polyclonal immunoglobulin G (IgG), Santa Cruz Biotechnology, Santa Cruz, CA; anti-GRP94 rabbit polyclonal IgG, Abcam, Cambridge, UK] were used at a dilution of 1:150 in 1 % bovine serum albumin/PBS. Incubations were carried out overnight at 4 °C. After washing in PBS, the primary antibodies were detected using Alexa Fluor 555 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-rabbit IgG (A21432 and A21206; Life Technologies) at a dilution of 1:150 for 120 min at room temperature. After washing in PBS, the sections were examined under a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

Statistical analysis

The results were analyzed using JMP ver. 9 software (SAS Institute Japan Ltd., Tokyo, Japan). The Wilcoxon signed rank test was used for paired comparisons of data. Correlations between gene expression ratios of post- to pre-CsA administration were evaluated by Spearman rank correlation coefficient analysis. A P value of <0.05 was taken as the level of significance.

Results

A total of 17 children with FRNS were included in the study. Characteristics of the participants are summarized in Table 1. All 17 patients had minor glomerular abnormalities in the first and second renal biopsy. None of patients showed CsA nephrotoxicity in renal histological evaluation performed after the 2-year treatment. There was no remarkable change in renal function within the normal range in each patient during the study period.

The coefficient of variation of gene expression level in five controls for the five molecules under study ranged from 0.12 to 0.31. These findings appear to demonstrate the validity of these controls. Changes in the expression of ER stress-related molecules between pre- and post-CsA administration in the 17 children with FRNS are shown in Table 2. The expressions of GRP78, GRP94, eIF2α, and Bcl-2 were significantly upregulated post-CsA administration compared with pre-CsA administration (Wilcoxon signed rank test for paired comparisons). However, there was almost no change in GADD153 expression between pre- and post-CsA administration. The mean (SD) gene expression ratios of post- to pre-CsA administration for GRP78, GRP94, eIF2α, and Bcl-2 were 2.53 (1.57), 1.80 (0.99), 2.38 (1.46), and 1.92 (1.11), respectively. The distribution of gene expression ratios of post- to pre-CsA administration for five genes in individual patients is depicted in Fig. 1. Post-CsA administration, the genes encoding GRP78 and eIF2α were upregulated by up to approximately sixfold, and the genes encoding GRP94 and Bcl-2 were upregulated by up to approximately fourfold compared with pre-CsA administration.

The correlations between each pair of ratios of post- to pre-CsA administration in five genes are shown in Table 3. There were significant correlations between the expression of genes encoding GRP78, GRP94, eIF2α, and Bcl-2. However, there was no significant correlation between the expression of the gene encoding GADD153 and the other genes.

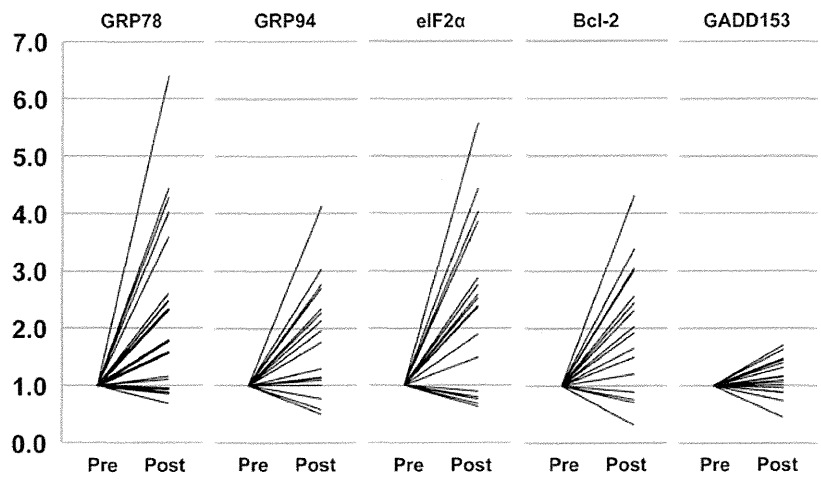

Fig. 1 Ratios of gene expression at post- to pre-cyclosporine A (CsA) administration for endoplasmic reticulum stress-related molecules in 17 children with frequently relapsing nephrotic syndrome. *GRP78* Glucose-regulated protein 78, *GRP94* glucose-regulated protein 94, *eIF2α* eukaryotic translation initiation factor 2α, *Bcl2* B-cell lymphoma 2, *GADD153* growth arrest and DNA damage-inducible gene 153