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Protocol Biopsy Findings in Living Donor Kidney Transplant Patients Treated With Once-daily or Twice-daily Tacrolimus Formulation

K. Masutani, A. Tsuchimoto, N. Haruyama, H. Kitada, Y. Okabe, H. Noguchi, M. Tanaka, K. Tsuruya, and T. Kitazono

ABSTRACT

Background. Once-daily extended-release tacrolimus (Tac-QD) has been shown to have equivalent efficacy and safety to the twice-daily formulation (Tac-BID) in kidney transplant patients. However, detailed comparison of allograft pathology found on a protocol biopsy (PB) in Tac-QD– versus Tac-BID–based regimens has not been described.

Methods. We retrospectively investigated 119 de novo living donor kidney transplant patients treated with Tac-QD (n = 90) or Tac-BID (n = 29) and their 3- and 12-month PB results. Other immunosuppressive drugs administered included basiliximab, mycophenolate mofetil, and methylprednisolone. We evaluated daily doses and trough levels of Tac and serum creatinine levels, and compared pathologic findings.

Results. Daily doses were higher in the Tac-QD group, but trough levels and serum creatinine levels were comparable. On 3- and 12-month PB, the frequency of subclinical rejection was similar between the groups, whereas interstitial fibrosis and tubular atrophy (IF/TA) were less common in the Tac-QD group at 12 months (42.2% vs 20.6%, $P = .04$). Univariate and multivariate logistic regression analyses revealed that allograft rejection (borderline changes or higher) was associated with IF/TA (odds ratio 4.09, 95% confidence interval 1.76–10.10, $P = .001$). The Tac-QD–based regimen showed a trend toward the absence of IF/TA but it did not reach statistical significance. Tubular vacuolization and arteriolar hyaline changes were also comparable in the two groups.

Conclusions. We found a trend toward milder IF/TA, but no significant differences in kidney allograft pathology in patients who were administered Tac-QD– versus Tac-BID–based regimens at 12 months. The effects of Tac-QD on chronic allograft injury must be studied by longer observation.

CALCINEURIN inhibitors (CNIs), including cyclosporine and tacrolimus (Tac), have contributed to better graft outcomes in organ transplant patients. Currently, most kidney transplant patients receive antibody induction and triple immunosuppression with CNIs, mycophenolate mofetil (MMF), and corticosteroids. During the maintenance period, treating physicians need to monitor two aspects of CNIs: inhibitory effects on acute rejection and nephrotoxicity. Tac was first developed as a twice-daily formulation (Tac-BID). In 2008, once-daily extended-release Tac (Tac-QD) became available, and its comparative effects on the incidence of rejection, graft survival, and patient survival have already been shown [1,2]. However, detailed allograft pathology, such as differences in subclinical rejection rates and CNI-associated changes on protocol biopsy (PB), has not been well studied. In this study, we reviewed the findings of 3- and 12-month PB in de

From the Departments of Medicine and Clinical Science (K.M., A.T., N.H., H.N., K.T., T.K.), Surgery and Oncology (H.K., Y.O., M.T.), and Integrated Therapy for Chronic Kidney Disease (K.T.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Address reprint requests to Kosuke Masutani, MD, PhD, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812-8582, Japan. E-mail: kosukemasutani@yahoo.co.jp

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novo kidney transplant patients who had been administered Tac-QD or Tac-BID.

PATIENTS AND METHODS

Study Design and Patient Population

We reviewed 119 consecutive de novo living donor kidney transplant patients who had been administered Tac-based immunosuppression who underwent PBs at Kyushu University Hospital from August 2008 through April 2011. In all patients, immunosuppression was induced with basiliximab and maintained with a triple-drug regimen consisting of Tac (Tac-BID in 29 patients and Tac-QD in 90 patients), MMF, and methylprednisolone (mPSL). Recipients of ABO-incompatible transplants (n = 35, 29.4%) and presensitized patients who tested positive for flow cytometric panel reactive antibodies (flow-PRAs; n = 14, 11.8%) were also pretreated with plasmapheresis and rituximab. We investigated clinical information including daily doses and trough levels of Tac, serum creatinine levels, and PB findings as described below. All PBs analyzed were obtained according to our clinical follow-up protocol, and no extra biopsy specimens or urine/blood samples were obtained for the purpose of the study. Informed written consent was obtained from eligible patients, and this study was approved by the Institutional Review Board at Kyushu University Hospital (protocol #24–54).

PB Policy and Pathologic Interpretation

Since August 2008, our hospital has had a fixed PB policy of performing biopsies 3 and 12 months post-transplantation. Our preliminary data revealed the usefulness of this protocol in detecting subclinical acute rejection at 3 months under current immunosuppression [3]. At a 12-month PB, we focus on chronic allograft injuries as well as subclinical rejection. During the study period, all patients were managed with this uniform PB policy. Allograft biopsy was performed under ultrasound guidance using a Bard Magnum device (Bard Biopsy Systems, Tempe, AZ, United States) and 18-gauge needles. For light microscopy, serial tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, methenamine silver, and Masson's trichrome stains. For immunofluorescence study, we examined the biopsy specimens for immunoglobulin (Ig) G, IgA, IgM, complement (C) 3, C1q, fibrinogen, kappa/lambda light chains, and C4d. All biopsy specimens were scored according to the Banff '09 classification [4]. Patients with rejection were classified into those with borderline changes, acute T-cell–mediated rejection (grade Ia or higher), and/or acute antibody-mediated rejection. PBs were defined as procedures performed without an increase in serum creatinine of greater than 10% from baseline levels (determined by average serum creatinine 3 months before the biopsy) and no previous rejection episodes within 1 month.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and number (%). JMP version 9.0.2 (SAS Institute, Cary, NC, United States) was used for all statistical analyses. The Student *t*-test and the Mann-Whitney U test were used to assess differences in numerical variables. The chi-square and Fisher exact probability tests were used for categorical data as appropriate. Daily doses, trough levels of Tac, and serum creatinine levels were compared with repeated-measure analysis of variance (ANOVA). To identify the factors associated with interstitial fibrosis and tubular atrophy (IF/TA) at 12 months, we applied univariate and multivariate logistic regression analyses. Recipient age, gender, and factors selected by the backward stepwise method ($P < .1$) were used for the multivariate analysis. After multivariate analysis, we added multiple comparisons with Bonferroni correction. Results were expressed as odds ratios (ORs) with respective 95% confidence intervals (CIs). A P value less than .05 was considered statistically significant.

Table 1. Demographic and Clinical Characteristics of Tac-BID and Tac-QD Groups

	Tac-BID Group (n = 29)	Tac-QD Group (n = 90)	*P* Value
Recipient age (y)	34 ± 15	42 ± 15	.001
Recipient gender (male/female)	22/7	51/39	.07
Donor age (y)	56 ± 10	53 ± 12	.2
Pre-emptive KT (%)	6 (20.7%)	23 (25.6%)	.6
ABO-incompatible KT (%)	6 (20.7%)	29 (32.2%)	.2
Positive flow-PRA test (%)	3 (10.3%)	11 (12.2%)	.8
HLA mismatch count (A, B, DR)	2.6 ± 1.6	2.8 ± 1.4	.4
Primary disease of ESRD			
Chronic glomerulonephritis	19 (65.5%)	57 (63.3%)	
Diabetes mellitus	5 (17.2%)	15 (16.7%)	.7
Others	5 (17.2%)	18 (20.0%)	
MMF dose at 3 mo	1250 ± 481	1126 ± 267	.09
12 mo	911 ± 238	913 ± 193	.9
mPSL dose at 3 mo	4.2 ± 1.9	3.8 ± 0.7	.08
12 mo	3.1 ± 1.5	3.2 ± 1.2	.9

Abbreviations: Tac, tacrolimus; BID, twice daily; QD, once daily; KT, kidney transplantation; flow-PRA, flow-cytometric panel reactive antibody; HLA, human leukocyte antigen; ESRD, end-stage renal disease; MMF, mycophenolate mofetil; mPSL, methylprednisolone.

RESULTS

Demographic and Clinical Characteristics of the Patients

Demographic characteristics of participating patients are presented in Table 1. Recipient gender, donor age, frequencies of ABO-incompatible transplantation and presensitized recipient, pre-emptive transplantation, HLA-mismatch count, primary diseases, and doses of MMF and mPSL at 12 months were not different between the two groups. The Tac-QD group had a higher mean recipient age ($P = .01$) than the Tac-BID group. There were trends toward lower doses of MMF and mPSL in the Tac-QD group than in the Tac-BID group at 3 months, and the percentage of female recipients in the Tac-QD group was higher, but these differences did not reach statistical significance. All patients were followed in our outpatient clinic for at least 2 years; follow-up periods in the Tac-BID and Tac-QD groups were 1473 ± 170 days and 1018 ± 194 days, respectively.

Daily Doses and Trough Levels of Tac, and Serum Creatinine Levels in Tac-BID and Tac-QD Groups

Daily doses and trough levels of Tac, and serum creatinine levels for the first 24 months after transplantation are shown in Fig 1. Daily doses of Tac were significantly higher in the Tac-QD group than in the Tac-BID group ($P < .01$, repeated-measure ANOVA), as reported in our preliminary

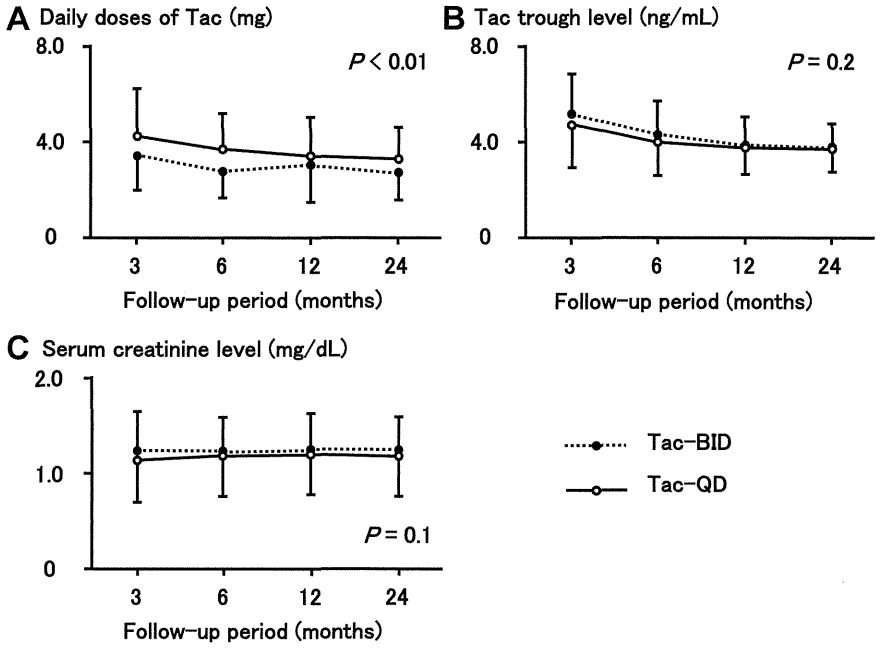

Fig 1. Daily doses and trough levels of tacrolimus (Tac), and serum creatinine levels in twice-daily dosing (Tac-BID) and once-daily dosing (Tac-QD) groups.

investigation [5]. Trough levels of Tac were not different, and serum creatinine levels were also comparable between the two groups.

Protocol Biopsy Findings and the Factors Associated With IF/TA at 12 months

Acute/chronic allograft injury grades are summarized in Fig 2. The incidences of borderline changes and acute rejection (Banff grade Ia or higher) were not different between the groups at a 3- or 12-month PB. With regard to the degree of IF/TA, no difference was found at a 3-month PB, although there was a trend toward lower IF/TA grades in the Tac-QD group, and the absence of IF/TA was more common in the Tac-QD group than in the Tac-BID group (42.2% vs 20.6%, $P = .04$).

We performed univariate and multivariate logistic regression analyses to investigate the factors associated with IF/TA at 12 months (Table 2). On univariate analysis, previous allograft rejection (defined as the sum of borderline changes and acute rejection at a 3-month PB, 12-month PB, or indication biopsy within 1 year) and the Tac-QD–based regimen were significantly associated with IF/TA at 12

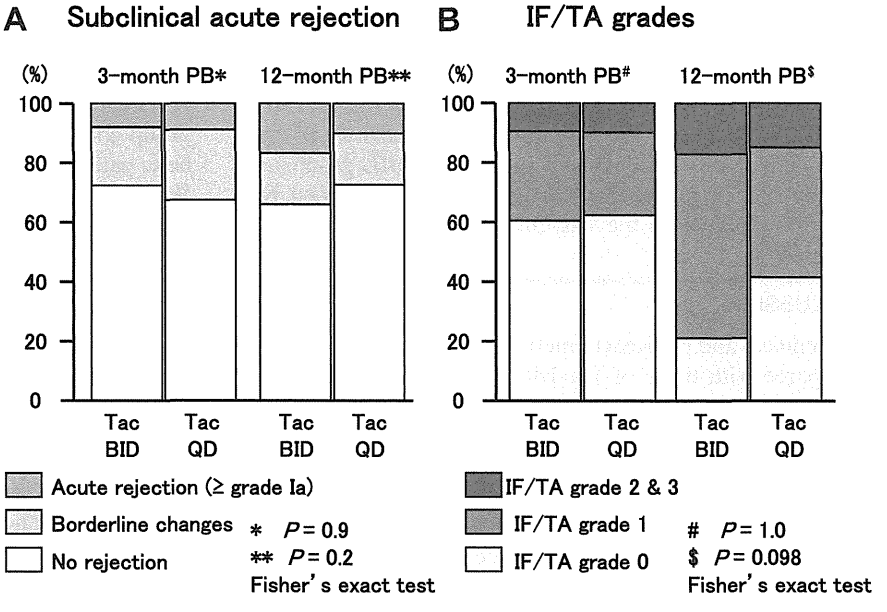

Fig 2. Rates of acute rejection and interstitial fibrosis/tubular atrophy (IF/TA) at 3- and 12-month protocol biopsies (PBs). $^{\$}$Further analysis of presence or absence of IF/TA (2 × 2 chi-square test) revealed that absence of IF/TA was more common in the Tac-QD group than in the Tac-BID group (42.2% vs 20.6%, $P = .04$).

Table 2. Univariate and Multivariate Logistic Regression Analyses for the Factors Associated With IF/TA at 12-month PB

	Univariate Analysis			Multivariate Analysis		
Model	OR	95% CI	*P* value	OR	95% CI	*P* value
Recipient age (>40 y)	0.81	0.38–1.71	.6	0.84	0.37–1.94	.7
Recipient male gender	1.82	0.85–3.94	.1	1.24	0.53–2.84	.6
Donor age (>55 y)	1.79	0.85–3.91	.1			
Donor male gender	1.04	0.48–2.28	.9			
ABO-incompatible transplantation	0.83	0.37–1.90	.7			
Preemptive transplantation	1.74	0.72–4.60	.2			
HLA-mismatch (4–6)	0.69	0.28–1.71	.4			
Positive flow-PRA test	1.54	0.48–5.91	.5			
Primary disease (diabetes mellitus)	1.95	0.69–6.38	.2			
Previous allograft rejection[a]	3.81	1.72–8.97	.001	4.09	1.76–10.10	.001[b]
IF/TA on 3-month PB	2.17	0.98–5.04	.06			
Tac-QD–based regimen	0.36	0.12–0.92	.03	0.33	0.11–0.93	.04

Abbreviations: IF/TA, interstitial fibrosis and tubular atrophy; PB, protocol biopsy; OR, odds ratio; CI, confidence interval; HLA, human leukocyte antigen; flow-PRA, flowcytometric panel reactive antibody; Tac-QD, once-daily tacrolimus.

[a]Borderline changes or higher on 3- and 12-month PBs or on indication biopsy performed within a 12-month period.

[b]Significant after multiple comparisons with Bonnferoni correction ($P < .0083$).

months, and there was a trend toward association between IF/TA at a 3-month PB and IF/TA at 12 months. On multivariate analysis, previous allograft rejection and the Tac-QD–based regimen were selected, and allograft rejection was strongly associated with IF/TA even after multiple comparisons (OR, 4.09; 95% CI, 1.76–10.10; $P = .001$). The Tac-QD–based regimen was inversely associated with IF/TA (OR, 0.33; 95% CI, 0.11–0.93; $P = .04$), but the difference was not significant after Bonferroni correction.

We also investigated other pathologic findings suggestive of CNI-associated changes and found that the incidence of tubular vacuolarization was not significantly different in Tac-BID and Tac-QD groups at 3- and 12-month PBs (15.4% vs 7.0%, $P = .2$ and 6.9% vs 8.0%, $P = .9$, respectively). The degree of arteriolar hyaline change ("ah" score) at 3 and 12 months was also not different between groups ($P = .6$ and $P = .2$, respectively, by the Mann-Whitney U test).

Indication biopsies were performed when patients developed an unexplained increase in serum creatinine or proteinuria levels. We found symptomatic acute rejection in two cases (6.9%) in the Tac-BID group and in five (5.6%) in the Tac-QD group. We also found polyomavirus BK nephropathy in one patient (3.4%) in the Tac-BID group, and in two (2.2%) in the Tac-QD group. We diagnosed two recurrent diseases in the Tac-QD group.

DISCUSSION

The clinical and pharmacokinetic characteristics of Tac-QD compared with those of Tac-BID have already been investigated and include lower peak plasma drug concentration (C_{max}), equivalent 24-hour pharmacokinetic area under the curve (AUC_{0-24}) and trough levels, and small inter- and intra-patient variability in drug concentrations [6,7]. It has also been documented that trough levels tend to be lower in patients who have been administered Tac-QD [5,8]. In this study, higher daily doses of Tac-QD were necessary to adjust the trough level to the target established for Tac-BID. As a result, trough levels of the two groups were well matched. Serum creatinine levels were also comparable. Because current immunosuppressive protocols achieve excellent short-term graft survival, it has become difficult to find differences in hard endpoints, such as graft loss or patient death, among different protocols. Thus, the incidence of acute rejection, changes in glomerular filtration rate, and IF/TA on allograft biopsy are often used as surrogates.

Our hospital has had a standard PB policy since August 2008. In this study, we compared allograft pathology between Tac-BID– and Tac-QD–based regimens, focusing on subclinical changes. The incidence of both symptomatic and subclinical acute rejection was not statistically different between groups, whereas the degree of IF/TA was milder in the Tac-QD group at 12 months. However, it is difficult to confirm that lower levels of IF/TA in the Tac-QD group represents reduced nephrotoxicity. Allograft fibrosis is multifactorial, and there are many causes for IF/TA, including acute or chronic allograft rejection, CNI toxicity, allograft aging, recipients' hypertension, and dyslipidemia. Although there were no statistical differences, the Tac-QD group contained a higher percentage of women, fewer diabetic patients, and younger donors than the Tac-BID group. We were not able to adjust these confounders completely using multivariate analysis. Because other CNI-associated changes such as tubular vacuolization and arteriolar hyaline changes were not different between groups, we concluded that there were almost equivalent pathologic changes in the Tac-BID and Tac-QD groups in the first year of observation. Conversely, we found a strong association between previous allograft rejection and IF/TA at 12 months. This seems reasonable, and we must focus on both symptomatic and subclinical acute rejection as well as borderline changes during the first post-transplantation year.

Data on the long-term effects of Tac-QD are still lacking. Tac-QD has the important advantage of better patient compliance than Tac-BID [9,10] and might prevent

noncompliance-related acute and chronic rejection and graft loss [11]. It has also been shown that high intrapatient variability of Tac concentration is associated with poor graft outcome [12]. In this regard, the lower variability in drug concentration observed with Tac-QD might contribute to better graft outcomes. In addition, there are no supporting data on whether the $AUC_{0\text{-}24}$ or C_{max} has a bigger impact on chronic CNI toxicity. Further investigations focusing on the pharmacokinetics and chronic allograft injuries associated with CNI are necessary.

The strength of this study is that we investigated PB findings, focusing on subclinical changes observed with Tac-BID– and Tac-QD–based regimens under uniform PB policy. Conversely, there are several limitations of this study, including its retrospective nature, small study population, lack of data on $AUC_{0\text{-}24}$ and C_{max}, and short-term follow-up. To confirm the direct and indirect effects of Tac-QD, large-scale, multicenter, prospective controlled studies with long-term observation and well-scheduled PB sampling are necessary.

In summary, we found a trend toward milder IF/TA, but no significant differences in kidney allograft pathology in patients who were administered Tac-QD versus Tac-BID. The effects of Tac-QD on chronic allograft injury must be studied by long-term observation.

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Early Disappearance of Urinary Decoy Cells in Successfully Treated Polyomavirus BK Nephropathy

Y. Matsukuma, K. Masutani, A. Tsuchimoto, Y. Okabe, H. Kitada, H. Noguchi, M. Tanaka, K. Tsuruya, and T. Kitazono

ABSTRACT

Background. Polyomavirus BK nephropathy (BKVN) is an important infectious complication in kidney transplant patients. Regular screening using polymerase chain reaction for BK virus DNA in plasma and urinary cytology is effective for early diagnosis of BKVN. However, methods of follow-up and therapeutic targets are not well described.

Methods. Ten patients with BKVN who received biweekly urinary cytology and repeat biopsies after diagnosis were retrospectively studied. Histological remission of BKVN was determined when biopsy revealed negative SV40 large T-antigen (TAg) staining. Results of urinary cytology and repeat biopsy findings were compared.

Results. Urinary decoy cells disappeared in 8 of 10 patients 55 ± 25 (range 13–79) days after index biopsies. In those cases, allograft function was preserved and the final serum creatinine level was 2.14 ± 1.19 (0.80–4.55) mg/dL after 962 ± 393 (325–1563) days of follow-up. Two cases with persistent urinary decoy cells shedding lost their graft 195 and 362 days later. Amongst 29 repeat biopsies, there were 13 TAg-positive and 16 negative biopsies. In 12 of 13 TAg-positive biopsies (92%), urinary decoy cells were still positive, whereas at the same time in 15 TAg-negative biopsies, decoy cells had already disappeared (94%).

Conclusions. Cytology testing is advantageous because of its cost effectiveness. Clearance of decoy cells from urine was closely related to histological remission of BKVN, and may possibly be a therapeutic target in BKVN.

POLYOMAVIRUS BK nephropathy (BKVN) is one of the important infectious complications in kidney transplant patients. According to Kidney Disease Improving Global Outcome Clinical Practice Guidelines for the Care of Kidney Transplant Recipients [1], and the guidelines of the American Society for Transplantation Infectious Disease Community of Practice [2], polymerase chain reaction (PCR) for BK virus (BKV) DNA in plasma is recommended for screening and diagnosis of BKVN. PCR of urine, urinary cytology, and electron microscopy of urine are also described as optional examinations [2]. However, methods of follow-up, therapeutic targets, and the definition of remission of BKVN are not described. Although PCR has high sensitivity and specificity and is essential to confirm diagnosis, it is expensive to perform frequently, and allograft biopsy is an invasive examination. This study reviewed biopsy-proven BKVN with repeated biopsy procedures and repeated urinary cytology, and details the predictive value of cytology on remission of BKVN.

PATIENTS AND METHODS

Between January 2009 and June 2013, 351 patients underwent kidney transplantation at Kyushu University Hospital (living donor kidney transplants for 331 patients and deceased donor kidney transplants for 20 patients). Among them, 13 patients (3.7%)

From the Department of Medicine and Clinical Science (Y.M., K.M., A.T., H.N., K.T., T.K.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, the Department of Surgery and Oncology (Y.O., H.K., M.T.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, and the Department of Integrated Therapy for Chronic Kidney Disease (K.T.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Address reprint requests to Kazuhiko Tsuruya, MD, PhD, Department of Integrated Therapy for Chronic Kidney Disease, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: tsuruya@intmed2.med.kyushu-u.ac.jp

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developed biopsy-proven BKVN. Ten of the 13 patients received both follow-up biopsies (29 biopsies in total, 1 to 4 per patient) and biweekly urinary cytology testing after an index biopsy.

BKV screening, allograft biopsy, and the diagnostic procedures of BKVN were uniform throughout the study period. After a kidney transplant, monthly screening for BKV infection using urinary cytology testing was performed until at least 1 year after kidney transplantation. Urinary cytology smears were stained using the Papanicolaou method and were evaluated for the presence or absence of cells with intranuclear viral inclusions (decoy cells). Patients also received protocol biopsy procedures at 3 and 12 months, and if a patient developed an unexpected increase in serum creatinine, indication biopsy procedures were performed. When persistent positive decoy cells in the urine were observed, PCR for BKV DNA in plasma was performed. Allograft biopsy samples were evaluated based on the Banff 2009 classification for the diagnosis of acute/chronic active T-cell or antibody-mediated rejection [3]. Diagnosis of BKVN was confirmed by viral cytopathic effects using conventional light microscopy, and positive immunohistochemical staining for SV40 large T antigen (TAg). All 13 patients showed positive urinary decoy cells and positive BK viremia determined by PCR at the time of diagnosis.

Once diagnosis of BKVN was confirmed, the basic strategy was first to reduce the dose of mycophenolate mofetil (MMF) by 50%, then to reduce the dose of the calcineurin inhibitor by 25% to 50%, and then discontinue MMF, according to American Society for Transplantation recommendations [2]. MMF was changed to everolimus in 1 patient, cidofovir was administered in 4 patients, and ciprofloxacin was administered in 1 patient.

To evaluate whether the disease was resolved, urinary cytology was repeated biweekly until at least 3 negative tests for decoy cells were recorded. The follow-up biopsy schedule was not uniform, and a repeat biopsy was done when a persistent increase in serum creatinine was observed and it was considered whether immunosuppression should be reduced, or subsequent acute rejection was suspected. For repeat biopsy specimens, histological remission of BKVN was determined when negative SV40 TAg staining was observed. All urinary cytology findings were investigated to identify the timing of the disappearance of decoy cells. Follow-up biopsies were reviewed to confirm histological remission of BKVN, and comparisons were made to evaluate the predictive value of urinary cytology for histological resolution of BKVN.

Excel 2007 (Microsoft, Redmond, WA, United States) was used for statistical analyses. Data are expressed as mean ± SD and range. Fisher exact probability tests were used for categorical data of the presence/absence of urinary decoy cells vs. positive/negative SV40 TAg staining of biopsy samples. A P value of $<.05$ was considered statistically significant. Informed consent was obtained from eligible patients, which was approved by the institutional review board in Kyushu University Hospital (protocol #24-54).

RESULTS

Demographic and clinical characteristics of the 10 patients are shown in Table 1. The mean age of patients was 49 ± 13 (31–64) years at diagnosis of BKVN. There were 8 male and 2 female patients. Living donor kidney transplantations were performed for 8 patients and deceased donor kidney transplantations for 2 patients. All patients received antibody induction with basiliximab followed by triple immunosuppression with a calcineurin inhibitor (tacrolimus for 9 patients, cyclosporine for 1 patient), MMF, and corticosteroids. Four patients experienced acute T-cell-mediated rejection episodes before development of BKVN. Diagnosis of BKVN was confirmed 153 ± 74 (87–322) days after kidney transplantations, and baseline serum creatinine, defined as the lowest value in the 3 months prior to diagnosis, was 1.21 ± 0.46 (0.75–2.34) mg/dL, and the creatinine level at diagnosis of BKVN was 2.06 ± 0.67 (0.98–3.15) mg/dL.

Table 1. Demographic and Clinical Characteristics of 10 Studied Cases of BKVN with Follow-up Biopsies

Age (y)	49 ± 13
Gender (Male/Female)	8/2
Primary disease (CGN/DM/HT)	8/2/0
Living/Deceased donor	8/2
HLA mismatch counts	2.5 ± 1.3
Immunosuppressive regimen (Tacrolimus/Cyclosporine)*	9/1
Previous acute rejection episodes	4 (40%)
Time after the transplant (d)	156.1 ± 70.4
Baseline serum creatinine (mg/dL)**	1.21 ± 0.46
Serum creatinine at the time of index biopsy	2.06 ± 0.67

Abbreviations: BKVN, polyomavirus BK nephropathy; CGN, chronic glomerulonephritis; DM, diabetes mellitus; HLA, human leukocyte antigen; HT, hypertensive nephrosclerosis.

*All patients were received antibody induction with basiliximab followed by triple immunosuppression including calcineurin inhibitor, mycophenolate mofetil and steroid.

**The best serum creatinine level within 4 months prior to the diagnosis of BKVN.

The time course of urinary cytology and repeat biopsy findings for the 10 patients are shown in Fig 1. Urinary decoy cells disappeared in 8 of 10 patients (cases 1 and 4–10) after 55 ± 25 (13–79) days of index biopsies, and never showed recurrence thereafter. In those patients, 29 repeat biopsies were performed, and 12 of 13 biopsy specimens (92%) before clearance of urinary decoy cells still showed positive SV40 TAg staining, whereas 15 of 16 biopsy specimens (94%) after the clearance of decoy cells showed negative TAg staining. One biopsy specimens in case 10 showed negative SV40 TAg staining during the period of positive decoy cell shedding. However, focal nuclear inclusion in tubular cells was evident in that biopsy specimen, and the histological diagnosis was tubulointerstitial nephritis with focal tubular cell atypia, suggestive of false-negative TAg staining. Clearance of decoy cells and histological remission of BKVN were well synchronized ($P < .0001$, Fisher exact test).

Repeat biopsies in 8 cases showed tubulointerstitial inflammation that fulfilled the criteria for Banff Ia rejection in 1 case, Ib in 4 cases, IIb in 2 cases, and chronic active T-cell-mediated rejection in 1 case. Despite these findings, allograft function was preserved and final serum creatinine was 2.14 ± 1.19 (0.80–4.55) mg/dL after 962 ± 393 (325–1563) days of follow-up. Cases 2 and 3 showed persistent positive decoy cell shedding in urine. They received 8 repeat biopsies, and all of them revealed persistent BKVN with positive TAg staining. Eventually, both patients lost their grafts at 195 and 369 days after index biopsies.

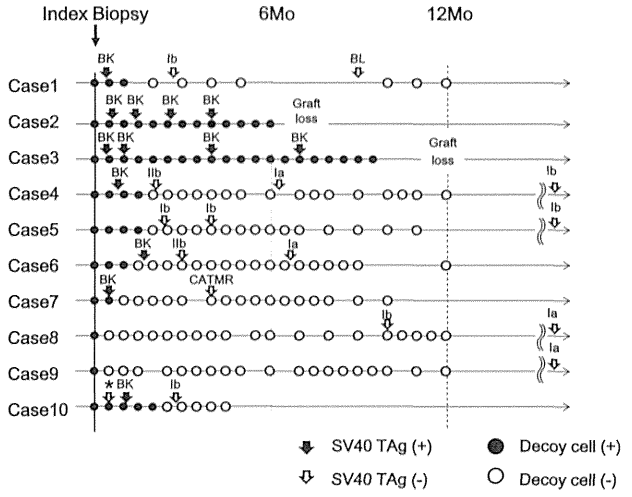

Fig 1. Relationship between the disappearance of urinary decoy cells and histopathological clearance of BKVN. Cases 2 and 3 showed persistent decoy cells and findings of BKVN until graft loss. Cases 1 and 4 to 10 showed both clearance of decoy cells and SV40 TAg staining in repeat biopsies. The timings of those clearances were well synchronized, but most of the follow-up biopsies showed overt tubulointerstitial inflammation fulfilling the criteria of T-cell-mediated acute rejection (Banff Ia, Ib, and IIa). *Biopsy showed overt inclusion in tubular cells but negative staining for SV40 TAg. The diagnosis was tubulointerstitial nephritis with focal tubular cell atypia, suggestive of BKVN. BKVN, polyomavirus BK nephropathy; CATMR, chronic active T-cell-mediated rejection; TAg, large T antigen.

DISCUSSION

BKVN has a strong impact on kidney allograft dysfunction or graft loss. Unfortunately, it is still difficult to estimate global immunosuppression and to estimate patients at high risk for BKVN [4]. Effective and safe antiviral treatment has not yet been developed [5]. Current strategy focuses on prevention by early detection of viremia and pre-emptive reduction of immunosuppression [1,2]. Regular PCR of plasma or the combination of PCR and urine cytology are established screening methods. SV40 TAg staining or in situ hybridization of BKV DNA are also commonly performed in kidney allograft pathology. Currently, confirmation of diagnosis of BKVN is not very difficult at experienced transplant centers.

There are limited data about the detailed clinical course of BKVN, for example, the frequency and timing of viral clearance, follow-up biopsy findings, and histological remission of BKVN. There are several PCR-guided studies about viral clearance. Menter et al [6] surveyed 35 patients with presumptive and diagnostic BKVN and reported that positive viremia was observed at a median of 3 months, peak viral load at 4 months, and clearance of viremia at 9 months posttransplantation. In that study, clearance of viremia was observed in 97% of patients. Masutani et al [7] reported that clearance of viremia was observed in 81% (26 of 32) of biopsy-proven BKVN at a median of 3 months after index biopsy, whereas the clearance of viruria (in addition to viremia) was observed only in 16% (5 of 32) of patients. Those findings suggest that clearance of viremia frequently occurs, and the time course is fast, but clearance of viruria is rare in BKVN. A detailed comparison between urine cytology and BK viral loads in urine and plasma was performed by Drachenberg et al [8]. In that study of 940 sets of blood and urine samples, there were 364 sets with no decoy cells (38.7%). Among those sets, only 2 sets (0.005%) showed low levels of viremia and viruria, 313 sets (86.0%) showed low levels of viruria (<1000 copies/mL), and 49 sets (13.5%) showed moderate to high levels of viruria (median: 1.6 × 10e4, range 1350–1 × 10e9 copies/mL). Conversely, in 576 decoy-cell-positive sets, 411 sets (71.3%) showed moderate to high titers of viruria (median 32000, range 1540–3.97 × 10e7 copies/mL), and 165 sets (28.7%) showed viremia (median 7.8 × 10e4 copies/mL) accompanied with viruria (median 6.78 × 10e5, range 7800–7.16 × 10e10 copies/mL). Thus, no decoy cells strongly indicated no viremia and lower viral load of viruria, and if a positive urinary cytology test returned negative in BKVN, viremia would be cleared, and the urinary viral load markedly decreased. It was also reported that BKVN without clearance of viremia showed poorer graft outcomes [7]. Because sustained viruria is reported not to affect graft outcome after a medium-term follow-up [9], the goal can be a clearance of viremia. The disappearance of urinary decoy cells also means a clearance of viremia, and can be a good therapeutic target for remission in BKVN.

Histological remission of BKVN is another difficult issue because there are no systematic data about repeat biopsies. In the current study, histological remission was defined as being when negative SV40 TAg staining from repeat biopsies was confirmed. However, this definition is problematic because the focality of BKV-infected tubules might cause a sampling error with any biopsy. SV40 TAg staining only detects infected cells in the early viral replicating phase [10], and it is possible to recognize only about half the infected cells using this stain [11]. However, persistent TAg positivity was associated with early graft loss in the current study. TAg positivity from repeat biopsies might be a poor prognostic factor, as well as high peak viral loads in plasma, tacrolimus-based immunosuppression, delayed diagnosis, and prolonged viral reduction time [12].

The strengths of this study were the focus on urine cytology to evaluate resolution of BKV infection, the comparison between clearance of decoy cells and the disappearance of SV40 TAg-positive cells, and the observation that negative urinary decoy cells may be a potential therapeutic target. This study also had some limitations. First, this study is a retrospective series of a small number of cases from a single center. Second, although cytology testing was performed regularly, the timing of repeat biopsies was not uniform. Favorable methods of follow-up, frequency of testing, repeat biopsy schedule, and histological evaluation of repeat biopsies should be confirmed with a multicenter study and a larger number of patients.

In summary, urinary decoy cells rapidly disappeared after successful treatment of BKVN, and the result of cytology was well synchronized with the histological remission in this current series. Cytology testing is advantageous because of its cost effectiveness, and the clearance of decoy cells may be a possible therapeutic target of BKVN.

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ORIGINAL ARTICLE
Renal Disease

Serum 1,25-Dihydroxyvitamin D and the Development of Kidney Dysfunction in a Japanese Community

– The Hisayama Study –

Kensuke Izumaru, MD; Toshiharu Ninomiya, MD, PhD; Masaharu Nagata, MD, PhD; Tomoko Usui, MD, PhD; Daigo Yoshida, PhD; Koji Yonemoto, PhD; Masayo Fukuhara, MD, PhD; Kazuhiko Tsuruya, MD, PhD; Takanari Kitazono, MD, PhD; Yutaka Kiyohara, MD, PhD

Background: Recent evidence indicates that vitamin D deficiency is associated with an increased risk of renal impairment, but studies addressing the influence of vitamin D deficiency on the development of chronic kidney disease (CKD) in the general Asian population have been few.

Methods and Results: A total of 2,417 community-dwelling individuals without CKD stage 3–5 aged ≥40 years were followed for 5 years (mean age, 60 years; women, 59.1%). The cumulative incidence of CKD stage 3–5, defined as estimated glomerular filtration rate (eGFR) <60 ml·min^{-1}·1.73 m^{-2}, and the rate of decline in eGFR according to quartile of serum 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), were estimated. During follow-up, 378 subjects experienced CKD stage 3–5. The age- and sex-adjusted incidence of CKD stage 3–5 increased significantly with decreasing serum 1,25(OH)$_2$D (P for trend <0.001). Compared with the highest quartile, the multivariate-adjusted odds ratio for the development of CKD stage 3–5 was 1.90 in the lowest quartile and 1.74 in the second lowest quartile, after adjusting for confounding factors. Additionally, lower serum 1,25(OH)$_2$D was significantly associated with a greater change in eGFR (–0.10 ml·min^{-1}·1.73 m^{-2}·year^{-1} per 10-pg/ml decrement in serum 1,25(OH)$_2$D).

Conclusions: Lower serum 1,25(OH)$_2$D is a significant risk factor for the development of CKD stage 3–5 in the general Asian population. (*Circ J* 2014; **78:** 732–737)

Key Words: 1,25-dihydroxyvitamin D; Chronic kidney disease; Epidemiology; Glomerular filtration rate

The burden generated by the expense of dialysis has been overwhelming in several countries in which the number of dialysis patients has increased continuously over the past few decades.[1] The early stages of chronic kidney disease (CKD) are likely to progress to end-stage kidney disease requiring costly dialysis or transplantation.[2] It is also increasingly apparent that individuals with CKD are more likely to develop cardiovascular disease.[3–8] These comorbidities related to CKD produce significant socioeconomic burden for patients, families, society, and the health-care system.[9,10] Thus, the identification and treatment of risk factors for the early stages of CKD will help prevent the progression of advanced kidney disease and reduce the risk of cardiovascular events.[11]

Editorial p599

Vitamin D has been recognized for decades as a key player in the control of bone metabolism through the regulation of calcium and phosphate homeostasis.[12] Vitamin D can be obtained from the diet and by the action of sunlight on the skin. The liver and kidney are the two primary sites for producing the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), which is activated mainly in the kidney after vitamin D is hydroxylated in the liver at the 25-carbon atom (25(OH)D).[13] Growing evidence suggests that 1,25(OH)$_2$D is involved in cardiovascular disease, malignant disease, infectious disease, autoimmune disease, and more.[14–17] Additionally, several prospective studies have shown that vitamin D deficiency is as-

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Department of Environmental Medicine (K.I., T.N., M.N., T.U., D.Y., M.F., Y.K.), Department of Medicine and Clinical Science (K.I., T.N., M.N., T.U., M.F., K.T., T.K.), Graduate School of Medical Sciences, Kyushu University, Fukuoka; and Biostatistics Center, Kurume University, Kurume (K.Y.), Japan

Mailing address: Toshiharu Ninomiya, MD, PhD, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: nino@intmed2.med.kyushu-u.ac.jp

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Table 1. Baseline Subject Characteristics vs. 1,25(OH)2D in Hisayama Subjects (n=2,417)

Variables	Serum 1,25(OH)2D level (pg/ml)				P for trend
	≥79.3 (n=610)	66.8–79.2 (n=602)	56.5–66.7 (n=603)	<56.5 (n=602)	
Age (years)	61±11	60±11	60±11	59±12	0.01
Women	51.0	53.8	65.0	66.8	<0.001
Serum creatinine (mg/dl)	0.66±0.14	0.66±0.14	0.65±0.13	0.66±0.13	0.21
eGFR (ml·min^{-1}·1.73 m^{-2})	85±18	83±16	83±16	82±16	0.01
Proteinuria	7.1	4.7	5.1	6.8	0.96
SBP (mmHg)	134±21	131±21	130±21	129±21	<0.001
DBP (mmHg)	80±12	79±11	78±11	77±12	<0.001
Antihypertensive medication use	25.9	20.9	18.7	20.1	0.01
Hemoglobin A1c (%)	5.5±0.8	5.5±0.7	5.4±0.8	5.5±0.9	0.98
Serum TC (mg/dl)	204±32	205±36	206±36	203±35	0.64
Serum HDL-C (mg/dl)	64±16	62±16	64±17	62±15	0.04
BMI (kg/m^2)	23.0±3.1	23.2±3.4	23.2±3.3	23.6±3.7	0.03
Smoking habits	20.7	23.4	20.7	21.1	0.86
Alcohol intake	54.4	49.2	39.8	39.2	<0.001
Regular exercise	12.5	12.0	9.8	9.6	0.06

Data given as mean±SD or %.
1,25(OH)2D, 1,25-dihydroxyvitamin D; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol.

sociated with an increased risk of deterioration in kidney function or the development of end-stage kidney disease.[18–21]

These findings raise the possibility that 1,25(OH)2D level has an impact on the progression of kidney disease, but most of these studies were conducted in high-risk subjects, and there are limited longitudinal studies evaluating this issue in general populations, especially in Asia. In the present study, we investigated the association between serum 1,25(OH)2D level and the development of CKD stage 3–5 in the general Japanese population.

Methods

Subjects

The Hisayama Study is an ongoing population-based prospective cohort study of cardiovascular disease and its risk factors in the town of Hisayama, which is located in a suburb of the Fukuoka metropolitan area on Kyushu Island, Japan. The population of the town is approximately 8,000, and full community surveys of the residents have been repeated since 1961.[22,23] In 2002 and 2003, a screening survey for the present study was performed in the town. A detailed description of this survey was published.[24] Briefly, a total of 3,328 residents aged ≥40 years (77.6% of the total population of this age group) underwent examination. After we excluded 30 subjects who did not consent to participate in the study, one subject for whom no blood sample was obtained, five subjects with frozen blood samples inadequate for measuring serum 1,25(OH)2D, and 473 subjects with an estimated glomerular filtration rate (eGFR) <60 ml·min^{-1}·1.73 m^{-2}, the remaining 2,819 participants (1,195 men and 1,624 women) were enrolled in the study. Of those, 400 subjects did not undergo the examination in 2007 and two subjects had no available eGFR data in 2007. The final subject group enrolled was 2,417 subjects (988 men and 1,429 women).

Follow-up

The subjects were followed up prospectively via annual repeated health examinations. Their health status was checked yearly by mail or telephone for any subjects who did not undergo the annual examination in that year, or who moved out of town. We also established a daily monitoring system among the study team, local physicians, and the members of the town's health and welfare office.

Risk Factor Measurements

A self-administered questionnaire concerning the current use of anti-hypertensive agents, smoking habit, and alcohol intake was checked by trained interviewers at the screening. These variables were classified as being either habitual or not. The subjects engaging in sports or other forms of exercise ≥3 times a week during their leisure time constituted the regular exercise group. Blood pressure was measured three times using an automated sphygmomanometer with subjects in the sitting position after at least 5 min rest. The mean of the three measurements was used for the present analysis. Body height and weight were measured in light clothing without shoes, and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Blood samples were collected from an antecubital vein after overnight fast. Part of the serum was stored at −80°C until measurement of 1,25(OH)2D. Fresh voided urine samples were collected at the examination, and proteinuria was defined as 1+ or more using a reagent strip. Serum creatinine concentration was measured using the enzymatic method. Hemoglobin A1c was measured on high-performance liquid chromatography. Total cholesterol and high-density lipoprotein cholesterol (HDL-C) was determined enzymatically. Frozen serum samples were thawed in 2010 and assayed for serum 1,25(OH)2D level with a radioimmunoassay kit (TFB, Tokyo, Japan).

Definition of CKD and Decline in eGFR

eGFR was calculated using the following new Japanese equation: eGFR (ml·min^{-1}·1.73 m^{-2})=194×[serum creatinine (mg/dl)]$^{-1.094}$×[age (years)]$^{-0.287}$×[0.739 if female].[25] CKD stage 3–5 was defined as reduced eGFR (eGFR <60 ml·min^{-1}·1.73 m^{-2}) according to the National Kidney Foundation Kidney

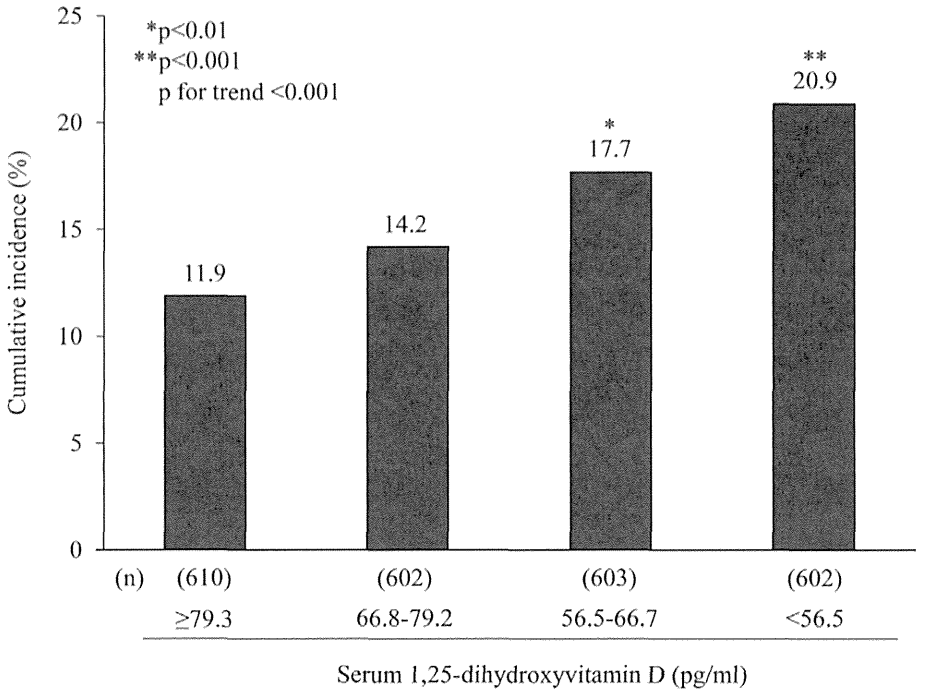

Figure 1. Age- and sex-adjusted 5-year cumulative incidence of chronic kidney disease stage 3–5 according to serum 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) quartile. *P<0.01, **P<0.001 vs. serum 1,25(OH)$_2$D ≥79.3 pg/ml.

Disease Outcomes Quality Initiative clinical practice guidelines Improving Global Outcomes definition and classification of CKD.[26,27] The rate of change in eGFR was calculated as the decline in eGFR using the following equation: change in eGFR (ml·min^{-1}·1.73 m^{-2}·year^{-1})=[eGFR in 2007 (ml·min^{-1}·1.73 m^{-2})–eGFR in 2002 (ml·min^{-1}·1.73 m^{-2})]/follow-up period (years).

The urine albumin-creatinine ratio (ACR, in mg/g) was calculated by dividing urinary albumin by urinary creatinine concentration. Albuminuria was defined as ACR ≥30.0 mg/g, and all CKD was defined as the presence of albuminuria and/or reduced eGFR.

Statistical Analysis

Serum 1,25(OH)$_2$D level was divided into quartiles: ≥79.3, 66.8–79.2, 56.5–66.7, and <56.5 pg/ml. The linear trends in the means and the frequencies of risk factors across serum 1,25(OH)$_2$D level were tested using a linear regression analysis and a logistic regression analysis, respectively. The age- and sex-adjusted cumulative incidence of CKD stage 3–5 was calculated using the direct method with the age and sex distribution of the overall study group.

Logistic regression analysis was also used to estimate odds ratios (ORs) with 95% confidence intervals (CIs) of CKD stage 3–5, all CKD, and albuminuria according to serum 1,25(OH)$_2$D level. The relationships between serum 1,25(OH)$_2$D level and eGFR slope were tested using multiple regression analysis. We calculated the multivariate-adjusted mean of the eGFR slope according to serum 1,25(OH)$_2$D level with analysis of covariance and compared these values with a Dunnett t-test. SAS (SAS Institute, Cary, NC, USA) was used to perform all statistical analysis. Two-sided P<0.05 was considered significant in all analyses.

Ethics Considerations

This study was conducted with the approval of the Kyushu University Institutional Review Board for Clinical Research. Written informed consent was obtained from all participants.

Results

Baseline subject characteristics according to serum 1,25(OH)$_2$D concentration quartile are listed in **Table 1**. Subjects with lower serum 1,25(OH)$_2$D were younger and were likely to be female. Mean eGFR, systolic and diastolic blood pressure (SBP and DBP), and the frequency of anti-hypertensive medication use were significantly decreased with lower serum 1,25(OH)$_2$D. Lower serum 1,25(OH)$_2$D level was also associated with lower mean serum HDL-C and higher mean BMI. The frequency of alcohol intake decreased significantly with lower serum 1,25(OH)$_2$D.

During the average 5-year follow-up period, 378 subjects experienced CKD stage 3–5 events. The age- and sex-adjusted cumulative incidence of CKD stage 3–5 is shown according to serum 1,25(OH)$_2$D level in **Figure 1**. The age- and sex-adjusted cumulative incidence of CKD stage 3–5 was significantly higher in the third and fourth quartile groups than in the highest quartile group. The age- and sex-adjusted ORs increased gradually with lower serum 1,25(OH)$_2$D level (P for trend <0.001; **Table 2**). This association remained unchanged after adjusting for potential confounding factors, namely age, sex, SBP, anti-hypertensive medication use, hemoglobin A1c, serum total cholesterol and HDL-C, proteinuria, BMI, smoking habit, alcohol intake, and regular exercise. There was no evidence of heterogeneity in the association between the sexes (P for heterogeneity =0.88). The upward trend in the risk of CKD stage 3–5 with lower serum 1,25(OH)$_2$D was observed

Table 2. Development of CKD Stage 3–5 vs. 1,25(OH)2D Quartile (n=2,417)†

Serum 1,25(OH)2D (pg/ml)	No. events	No. subjects	Age- and sex-adjusted			Multivariate-adjusted‡			Multivariate-adjusted§		
			OR (95% CI)	P-value	P-value for trend	OR (95% CI)	P-value	P-value for trend	OR (95% CI)	P-value	P-value for trend
≥79.3	75	610	1.00 (reference)		<0.001	1.00 (reference)		<0.001	1.00 (reference)		0.008
66.8–79.2	84	602	1.20 (0.84–1.70)	0.32		1.27 (0.89–1.82)	0.19		1.31 (0.88–1.93)	0.18	
56.5–66.7	107	603	1.68 (1.20–2.37)	0.003		1.74 (1.23–2.47)	0.002		1.71 (1.17–2.50)	0.006	
<56.5	112	602	1.87 (1.33–2.62)	<0.001		1.90 (1.34–2.70)	<0.001		1.59 (1.08–2.34)	0.02	

†Five-year follow-up in Hisayama subjects. ‡Adjusted for age, sex, SBP, anti-hypertensive medication, HbA1c, serum total cholesterol, serum HDL-C, proteinuria, BMI, smoking habits, alcohol intake, and regular exercise. §Adjusted for age, sex, SBP, anti-hypertensive medication, HbA1c, serum total cholesterol, serum HDL-C, proteinuria, BMI, smoking habits, alcohol intake, regular exercise, and baseline eGFR. CI, confidence interval; CKD, chronic kidney disease; OR, odds ratio. Other abbreviations as in Table 1.

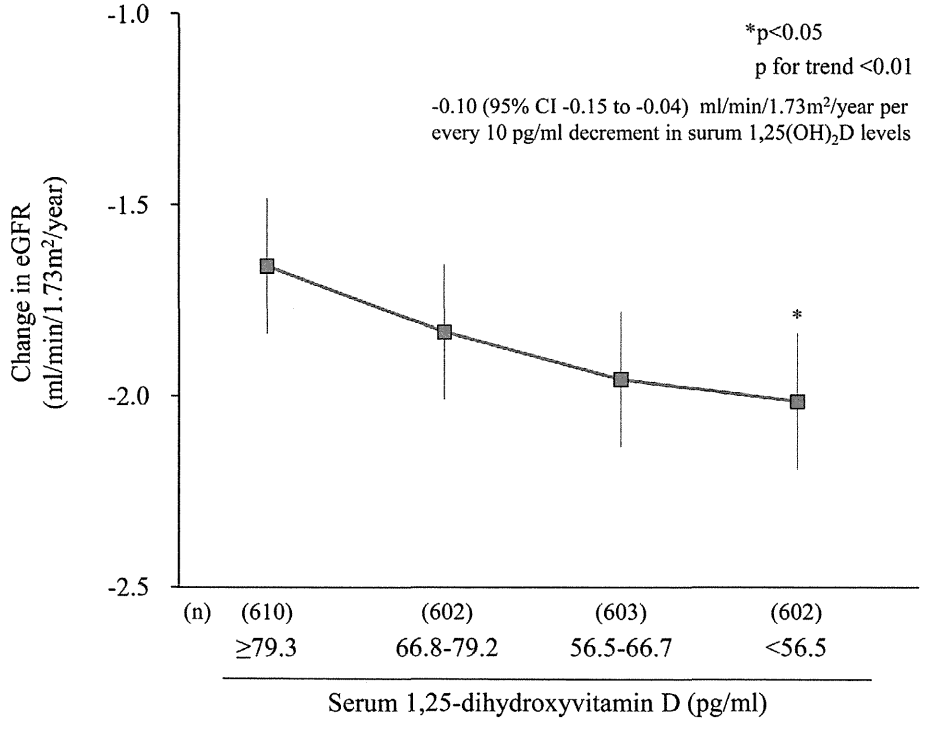

Figure 2. Multivariate-adjusted mean change in estimated glomerular filtration rate (eGFR) during a 5-year follow-up period according to serum 1,25-dihydroxyvitamin D (1,25(OH)2D) quartile. *P<0.05 vs. serum 1,25(OH)2D ≥79.3 pg/ml. Adjusted for age, sex, systolic blood pressure, anti-hypertensive medication, hemoglobin A1c, serum total cholesterol, serum high-density lipoprotein cholesterol, proteinuria, body mass index, smoking habit, alcohol intake, and regular exercise. CI, confidence interval.

after adjusting for the aforementioned covariates plus baseline eGFR, although the lowest quartile had a lower OR than the second lowest quartile.

To analyze sensitivity, we assessed the association between serum 1,25(OH)2D level and the likelihood of albuminuria and all CKD in 1,945 participants without all CKD for 5 years. The multivariate-adjusted OR for the development of all CKD was 1.38 (95% CI: 1.00–1.90) in the lowest quartile and 1.41 (95% CI: 1.03–1.93) in the second-lowest quartile compared to the highest quartile, after adjusting for potential confounding factors. In contrast, no significant association was found between serum 1,25(OH)2D level and the multivariate-adjusted OR of albuminuria (**Table S1**).

We also carried out slope analysis, in which the relationship between serum 1,25(OH)2D level and the decline in eGFR was examined using a multiple regression model after adjusting for the aforementioned potential confounding factors (**Figure 2**). We found that lower serum 1,25(OH)2D was significantly associated with a greater decline in eGFR (P for trend <0.01): the difference in the multivariate-adjusted mean of the decline in eGFR was significant in the subjects with serum 1,25(OH)2D <56.5 pg/ml compared to those with serum 1,25(OH)2D ≥79.3 pg/ml.

The amount of change in eGFR was $-0.10\,ml \cdot min^{-1} \cdot 1.73\,m^{-2} \cdot year^{-1}$ (95% CI: −0.15 to −0.04) per 10 pg/ml decrement in serum 1,25(OH)2D level. This relationship was not altered substantially after adjusting for eGFR at baseline in addition to the covariates included in the model for **Figure 2**

(Figure S1). The sensitivity analyses using the Chronic Kidney Disease Epidemiology Collaboration equation for eGFR calculation instead of the Japanese equation also identified a significant relationship between eGFR level and mean change in eGFR: ≥79.3 pg/ml, −1.1 (95% CI: −1.2 to −1.0); 66.8–79.2 pg/ml, −1.2 (95% CI: −1.3 to −1.1); 56.5–66.7 pg/ml, −1.3 (95% CI: −1.4 to −1.2); and <56.5 pg/ml, −1.4 (95% CI: −1.5 to −1.3): P for trend <0.001.

In the subgroup analyses stratified by sex, age, diabetes, and hypertension, there was no evidence of a significant difference in the association of serum 1,25(OH)$_2$D level with the risk of the development of CKD stage 3–5 or the eGFR slope between the subgroups (all P for heterogeneity >0.18; Tables S2,S3).

Discussion

The present results clearly show that lower serum 1,25(OH)$_2$D is significantly associated with an increased risk of development of CKD stage 3–5. Additionally, the subjects with lower serum 1,25(OH)$_2$D had a greater decline in eGFR. These relationships remained significant after adjusting for potential confounding factors. To the best of our knowledge, this is the first study to investigate the relationship between serum 1,25(OH)$_2$D level and the incidence of CKD stage 3–5 prospectively in a community-based Asian population.

Several epidemiological studies examined the association of serum 25(OH)D or 1,25(OH)$_2$D level with the development of CKD. In the Cardiovascular Health Study, lower serum 25(OH)D level was associated with a greater decline in eGFR among 1,705 community-dwelling older adults in the USA.[18] The Third National Health and Nutrition Examination Survey in the USA also found that lower serum 25(OH)D level was a significant risk factor for development of end-stage kidney disease.[19] A hospital-based prospective study conducted in 168 subjects in Italy found that lower serum 1,25(OH)$_2$D level, as well as serum 25(OH)D level, was significantly linked to an increased risk of progression from moderate–severe CKD to end-stage kidney disease.[20] In the Homocysteinemia in Kidney and End Stage Renal Disease Study of 1,099 US patients with advanced CKD, lower serum 1,25(OH)$_2$D level was associated with an increased risk of end-stage kidney disease.[21] These findings are in accord with the present results.

Several possible explanations have been proposed for the mechanism underlying the association between serum 1,25(OH)$_2$D level and the risk of CKD. Experimental studies have suggested that 1,25(OH)$_2$D can regulate the proliferation of vascular smooth muscle cells and vascular inflammation, which may promote systemic arteriosclerosis.[28,29] Several epidemiological studies reported that decreased eGFR is closely correlated with systemic atherosclerosis.[30,31] These properties may play a key role in the pathogenesis of CKD by promoting arteriosclerosis within the kidney. 1,25(OH)$_2$D also promotes the survival of podocytes by inducing differentiation and preventing apoptosis, and reduces glomerulosclerosis, intestinal fibrosis, and albuminuria in animal models.[32–36] Through these mechanisms, lower serum 1,25(OH)$_2$D level may cause arteriosclerosis, glomerulosclerosis, and intestinal fibrosis, resulting in CKD.

It has been recognized that subjects with lower eGFR at baseline are likely to have lower serum 1,25(OH)$_2$D, raising the possibility that the present findings merely reflect that subjects with lower eGFR at baseline develop CKD more quickly.[37,38] To avoid this possibility, we excluded the subjects with eGFR <60 ml·min^{-1}·1.73 m^{-2}, because serum 1,25(OH)$_2$D decreases progressively when eGFR falls below 60 ml·min^{-1}·1.73 m^{-2}, and we adjusted the risk estimates for eGFR level at baseline.[37,38]

We also compared decline in eGFR across serum 1,25(OH)$_2$D level. Even after taking these precautions into consideration, we found that lower serum 1,25(OH)$_2$D level was a significant risk factor for the development of CKD stage 3–5. Nevertheless, confounding may still exist in the association between serum 1,25(OH)$_2$D level and CKD stage 3–5. Further intervention studies addressing whether treatment to combat lower 1,25(OH)$_2$D level reduces the risk of CKD are necessary to clarify this issue.

Several limitations of the present study should be noted. First, it is possible that the present results are biased by the exclusion of the 402 subjects who did not return to the follow-up examination; of these, 148 subjects died during the follow-up period. The excluded subjects had lower serum 1,25(OH)$_2$D and eGFR, suggesting that a high-risk group for the development of CKD stage 3–5 was excluded in this study. Second, serum 1,25(OH)$_2$D level was based on a single measurement at baseline, as was the case in most of the prior epidemiologic studies. This may cause a misclassification of serum 1,25(OH)$_2$D level. These limitations could have weakened the association found in this study, biasing the results toward the null hypothesis. We therefore believe these biases would not alter the conclusion. Last, we have no information about the type of underlying renal disease. Such information could be obtained by detailed clinical examination, including renal biopsy and ultrasonography, but these diagnostic procedures are not considered feasible for a cohort study recruited from a general population, such as the present one.

Conclusions

Lower serum 1,25(OH)$_2$D level is a significant risk factor for the development of CKD stage 3–5 in the general Asian population. At present, the extent to which raising serum 1,25(OH)$_2$D level can attenuate the risk of CKD stage 3–5 is not known. A clinical trial raising serum 1,25(OH)$_2$D level is needed to clarify whether higher serum 1,25(OH)$_2$D level will result in an improved renal prognosis.

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Supplementary Files

Supplementary File 1

Table S1. Development of all CKD and albuminuria vs. serum 1,25$(OH)_2$D during 5-year follow-up

Table S2. Multivariate-adjusted OR and 95% CI for development of CKD stage 3–5 vs. 1,25$(OH)_2$D

Table S3. Multivariate-adjusted mean change in eGFR (95% CI) vs. 1,25$(OH)_2$D in 5-year follow-up

Figure S1. Multivariate-adjusted mean change in estimated glomerular filtration rate (eGFR) during a 5-year follow-up period according to serum 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) quartile after adjusting for eGFR at baseline in addition to the covariates included in the model for **Figure 2.**

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ORIGINAL ARTICLE

Subclinical nephrosclerosis is linked to left ventricular hypertrophy independent of classical atherogenic factors

Naoki Haruyama[1], Akihiro Tsuchimoto[1], Kosuke Masutani[1], Masaharu Nagata[1], Hidehisa Kitada[2], Masao Tanaka[2], Kazuhiko Tsuruya[1,3] and Takanari Kitazono[1]

Recently, cardio–renal interactions have been considered to be important and it has been demonstrated that mild renal dysfunction is associated with left ventricular hypertrophy (LVH). However, the correlation between LVH and subclinical renal damage is unclear. We investigated this association by assessing pretransplant biopsies from living kidney donors with normal renal function. We retrospectively categorized 238 living kidney donors into tertiles according to the percentage of global glomerulosclerosis (%GGS) observed in pretransplant biopsies (low, 0–3.45% ($n=80$); moderate, 3.46–11.76% ($n=78$); high, $\geqslant 11.77\%$ ($n=80$)) to analyze trends in their left ventricular mass index (LVMI) measured by echocardiography and baseline factors. LVH was defined as LVMI $>110\,g\,m^{-2}$ in female and $>125\,g\,m^{-2}$ in male subjects. We used a logistic regression model to evaluate any correlations between %GGS and LVH. LVMI increased significantly with increasing tertiles of %GGS, as did the prevalence of left ventricular remodeling and LVH. According to multivariate logistic regression analysis, subjects with high %GGS tertiles had a sevenfold greater risk of LVH than did those with low tertiles, even after adjusting for age, sex, systolic blood pressure, history of diabetes mellitus, total serum cholesterol and glomerular filtration rate (GFR) measured by a radioisotopic technique. There is an association between GGS and LVH in subjects with normal renal function. This association is significant after adjustment for age, sex, blood pressure, GFR and other atherogenic factors.
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Keywords: cardio–renal interaction; global glomerulosclerosis; nephrosclerosis

INTRODUCTION

Recently, basic and clinical research have improved our understanding of cardio–renal damage and suggested that an interaction between pathophysiological (that is, hemodynamic, inflammatory and neurohormonal) mechanisms amplifies structural and functional cardio–renal derangement.[1] The presence of both renal dysfunction and left ventricular hypertrophy (LVH) indicates cardiovascular risk and a poorer prognosis compare with the isolated cardiac or renal damage. This interaction is evident even in patients with mild renal insufficiency.[2] According to the Multi-Ethnic Study of Atherosclerosis, concentrations of cystatin C, a superior marker of renal function, were inversely associated with left ventricular end-diastolic volumes and directly associated with concentricity independent of traditional cardiovascular risk factors in 4910 subjects with normal to mild renal dysfunction.[3] In addition, Fesler *et al.*[4] reported that increased left ventricular mass predicts subsequent renal dysfunction in both chronic kidney disease (CKD) patients and healthy subjects. However, it remains unclear whether subclinical renal damage, such as that detectable only by histological examination, is associated with altered left ventricular geometry in healthy subjects.

Although in general living kidney donors are healthy individuals, their pretransplant biopsy (0-h biopsy) specimens, obtained immediately after nephrectomy, often reveal varying degrees of global glomerulosclerosis (GGS) and arteriolosclerosis. In the general population, GGS is reportedly present in 1–3% of total glomeruli in adult subjects older than 50 years of age: the number of subjects with GGS increases with increasing age, reaching up to 30% by 80 years of age.[5,6] However, the precise causes are still unknown. A study analyzing biopsy specimens from 1208 living kidney donors reported that GGS is not completely attributable to age-related decline in glomerular filtration rate (GFR).[7] On the other hand, we have noted that preoperative echocardiography in these subjects occasionally reveals LVH or left ventricular remodeling. LVH and

[1]Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and [3]Department of Integrated Therapy for Chronic Kidney Disease, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
Correspondence: Dr K Tsuruya, Department of Integrated Therapy for Chronic Kidney Disease, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: tsuruya@intmed2.med.kyushu-u.ac.jp
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left ventricular remodeling are common in hypertensive subjects and the elderly;[8] however, some of our subjects with LVH have been normotensive and not elderly.

Therefore, we examined the cardio–renal interaction relationship between GGS at 0-h biopsies and LVH in near healthy subjects.

MATERIALS AND METHODS

Patients

Three hundred and nine consecutive living kidney donors from March 2006 to October 2012 were initially included. Twenty-two of these donors were excluded because a 0-h biopsy or preoperative echocardiography was not performed. Thirty-nine donors were excluded because of inadequate biopsy specimens that contained fewer than 10 glomeruli or included simple cysts. Another 10 donors were excluded because some relevant laboratory findings were missing. The remaining 238 subjects were enrolled in this study. No subject had a history of stroke, coronary heart disease or heart failure. This study was part of a retrospective cohort study conducted by our department, which was approved by the Clinical Research Ethical Committee of the Kyushu University of Japan (Practice #24–54). Written informed consent was obtained from each subject.

Evaluation of echocardiography

Echocardiographic studies had been performed within the 6 months before donor nephrectomy using a iE33 echography system (Philips Health Care, Andover, MA, USA), Vivid 7 Dimension cardiovascular ultrasound system (GE Healthcare, Chalfont St Giles, Bucks, UK) or Artida 4D (Toshiba Medical Systems, Tochigi, Japan). All tracings were evaluated by several laboratory technicians and cardiologists. Overall, one-dimensional left ventricular measurements and two-dimensional (apical 4- and 2-chamber) views were obtained, in accordance with the recommendations of the American Society of Echocardiography. Relative wall thickness at end diastole was calculated as the ratio of the sum of left ventricular septal and posterior wall thickness to left ventricular internal end-diastolic diameter. Left ventricular mass was calculated using the Penn-cube method as described by Devereux *et al.*[9] LVH was defined as left ventricular mass index greater than $110\,g\,m^{-2}$ in female and $125\,g\,m^{-2}$ in male subjects.[10] Left ventricular remodeling was defined as increased relative wall thickness (more than 0.45) without hypertrophy. Concentric and eccentric LVH were defined as the presence or absence of increased relative wall thickness (more than 0.45), respectively.

Definitions of renal function and other relevant clinical variables before surgery

Hypertension was considered to be present if at least two consecutive resting blood pressure measurements before surgery met the criteria of the Seventh Joint National Committee Guidelines on Prevention, Detection, Evaluation and Treatment of High Blood Pressure[11] or the subject had an established history of hypertension that had necessitated medical treatment. GFR was determined based on urinary clearance of ^{99m}technetium-labeled diethylene triamine pentaacetic acid using the constant infusion technique with urine collection and normalized according to body surface area. Diabetes mellitus was defined as a fasting blood glucose concentration of more than $126\,mg\,dl^{-1}$ and hemoglobin A1c more than 6.5% during preoperative evaluation. Hemoglobin A1c was measured according to Japanese Diabetes Society/Japanese Society of Clinical Chemistry guidelines, which were standardized by adding 0.4% to the estimate as the National Glycohemoglobin Standardization Program equivalent value.[12] All subjects who were defined as having impaired glucose tolerance or diabetes mellitus received strict dietary therapy or hypoglycemic agents or both until normal glucose tolerance had been achieved. By the time of kidney donation, glucose tolerance had normalized in all donors. Obesity was defined as body mass index $\geqslant 25\,kg\,m^{-2}$. Dyslipidemia was defined as fasting total cholesterol concentration $\geqslant 220\,mg\,dl^{-1}$, triglyceride concentration $\geqslant 150\,mg\,dl^{-1}$ or use of lipid-modifying agents.

Data collection

Patient history, physical examination findings and laboratory values were collected. The assessed clinical variables were age, sex, comorbid medical conditions, use of medication (for example, antihypertensive drugs), body mass index and systolic and diastolic blood pressure. Body surface area was calculated by the du Bois method.[13] Baseline laboratory data, such as blood hemoglobin and urea nitrogen, and serum creatinine, uric acid, total cholesterol, triglyceride and glucose concentrations, were obtained within a few days before surgery, along with the evaluation of a spot urine sample to assess the urinary protein/creatinine ratio and a dipstick urinary test.

Histological evaluation of 0-h renal biopsy

A wedge biopsy had been obtained from the outer cortex of each donor kidney immediately before transplantation. The sample was fixed in a paraformaldehyde–glutaraldehyde solution and then embedded in paraffin. Next, the sample was stained with hematoxylin-eosin, periodic acid-Schiff, methenamine silver and Masson trichrome stains. %GGS was determined using light microscopy. To quantify other characteristics of nephrosclerosis, such as renal arteriosclerosis, arteriolar hyalinosis and interstitial fibrosis, a semi-quantitative grading score based on the Banff criteria was used.[14] Arteriolosclerosis was defined as follows: +1, fibro-intimal thickening < intimal diameter; +2, fibro-intimal thickening = intimal diameter; and +3, fibro-intimal thickening > intimal diameter. Each biopsy specimen was evaluated by at least two specialists in renal pathology (AT and KM) who were blinded to the clinical data.

Statistical analysis

Values are expressed as mean (s.d.) or number (%). The subjects were divided into tertiles according to %GGS (low, 0–3.45%; moderate, 3.46–11.76%; and high, $\geqslant$11.77%). Trends in continuous and categorical values across the tertiles were examined by the Jonckheere–Terpstra and Cochran–Armitage tests, respectively.[15,16] The prevalence of left ventricular morphological changes and the various renal histology grades in each tertile of %GGS were evaluated using the linear regression analysis.[17] After adjusting for the potential confounding risk factors (except triglyceride) that had been found to be statistically significant by the trend analyses (two-tailed P-value < 0.05), the odds ratios and 95% confidence intervals for LVH were calculated by logistic regression. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using JMP version 9.0.2, or SAS version 9.2 (SAS Institute, Cary, NC, USA) for trend testing of continuous values.

RESULTS

Trend analysis of baseline characteristics

Table 1 shows the characteristics of all subjects according to each tertile of %GGS. Overall subjects, the mean number of glomeruli per biopsy was 26 ± 13. Trace proteinuria ($\pm$) was detected by the dipstick urinary test in only two subjects; however, their urinary protein/creatinine ratios were less than 0.10 g per gCr. The other 236 subjects had negative dipstick urinary tests and urinary protein/creatinine ratios. The mean GFR was $117 \pm 27\,ml\,min^{-1}$ per $1.73\,m^2$. On admission, 28 subjects were taking antihypertensive drugs, which were angiotensin-converting enzyme inhibitors or angiotensin receptor blockers in 17 cases. Eighteen subjects with dyslipidemia were receiving statins, bezafibrate or ezetimibe. Sixteen subjects had been diagnosed with diabetes mellitus during preoperative evaluation. Some of them had been receiving diet therapy and short-term antidiabetic medication or both to normalize their fasting blood glucose concentrations by the time of kidney donation. There were significant trends toward correlations between %GGS tertiles and age, hypertension, history of diabetes mellitus, dyslipidemia, systolic/diastolic blood pressure, pulse pressure, GFR, total serum cholesterol and triglyceride.

Table 1 Mean values or frequencies of potential risk factors and laboratory variables for overall baseline characteristics and tertiles according to %GGS

		Low (n = 80)	*Moderate (n = 78)*	*High (n = 80)*	
Percentage of GGS	*Whole study population (n = 238)*	*0–3.45%*	*3. 46–11.76%*	*⩾11.77%*	P *for trend*
Age, years	54 (12)	47 (13)	54 (11)	60 (9)	<0.01
Men, %	42	48	38	40	0.33
Obesity, %	23	21	23	25	0.57
Hypertension, %	23	9	28	33	<0.01
ACEi/ARB, %	7	4	8	10	0.12
Smoking habits, %	34	35	32	34	0.87
History of diabetes mellitus, %	7	1	9	10	<0.01
Dyslipidemia, %	36	26	38	43	<0.01
Systolic blood pressure, mm Hg	121 (16)	117 (13)	122 (15)	126 (17)	<0.01
Diastolic blood pressure, mm Hg	75 (11)	72 (10)	76 (10)	77 (13)	<0.05
Pulse pressure, mm Hg	47 (12)	45 (10)	46 (12)	49 (12)	0.01
Glomerular filtration rate, ml min^{-1} per 1.73 m^2	117 (27)	122 (27)	119 (26)	110 (28)	<0.01
Proteinuria (−)/(±), %	99.2/0.8	100/0	98.7/1.3	98.7/1.3	0.39
Hemoglobin, g dl^{-1}	13.4 (1.5)	13.6 (1.4)	13.4 (1.5)	13.2 (1.5)	0.05
Fasting glucose, mg dl^{-1}	97 (13)	95 (12)	96 (14)	99 (13)	0.05
Total cholesterol, mg dl^{-1}	207 (35)	195 (31)	212 (37)	213 (35)	<0.01
Triglycerides, mg dl^{-1}	116 (56)	108 (55)	116 (53)	126 (59)	<0.05
Uric acid, mg dl^{-1}	5.1 (1.4)	5.1 (1.2)	5.1 (1.5)	5.0 (1.3)	0.66

Abbreviations: %GGS, percentage of global glomerulosclerosis; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; Up/Ucr, urinary protein/urinary creatinine ratio. Statistical significance was tested by the Jonckheere–Terpstra trend test for continuous variables and the Cochran–Armitage test for categorized variables. Values are expressed as average (s.d.) or percentage.

Distribution of left ventricular geometric changes according to tertiles of %GGS

Figure 1 shows the left ventricular morphological changes for each tertile of %GGS. The number of patients with left ventricular morphological changes was proportional to these tertiles ($P<0.01$). In addition, a substantial trend was observed between tertiles of %GGS and left ventricular mass index (P for this trend <0.01).

Histological analysis of the relationship between %GGS and other nephrosclerotic changes

Table 2 shows proportion of other histological findings characteristic of nephrosclerosis categorized according to Banff's criteria. Individuals in the higher two categories of tertiles had a significantly higher proportion of advanced lesions than those in the lowest tertile.

Multivariate logistic regression models for LVH

The results of multivariate logistic regression analyses are shown in Table 3. In Model 1, unadjusted analysis showed that the OR for the highest tertile of %GGS was significantly greater than that for the lowest tertile. In Model 2, the OR for the highest tertile of %GGS remained significantly increased after adjusting for age and sex. Finally, even after adjusting for age, sex, systolic blood pressure, history of diabetes mellitus, total serum cholesterol and GFR, the subjects in the highest tertile of %GGS had a sevenfold greater risk of LVH than did those in the lowest tertile of %GGS (in Model 3, $P<0.05$).

DISCUSSION

In the present study, we identified a significant association between GGS at 0-h biopsies and LVH as evaluated by preoperative echocardiography. This is the first study that has demonstrated a subclinical cardio–renal interaction in a near healthy population.

Nephrosclerosis is defined by the presence of glomerulosclerosis, arteriosclerosis, tubular atrophy and interstitial fibrosis on renal biopsy. Age or variations in blood pressure cannot completely explain the prevalence of GGS at 0-h biopsies from living kidney donors.[5] In experimental models of nephrosclerosis, the renin–angiotensin–aldosterone system, sympathetic nerve overactivity, inflammatory reactions induced by reactive oxygen species and impaired bioavailability of nitric oxygen are considered to have important roles in the development of nephrosclerosis.[18–21]

On the other hand, LVH is an adaptive response to increased cardiac work, which is the product of left ventricular pressure and stroke volume.[22] The causes for this compensatory mechanism are the same as those listed above for nephrosclerosis.[23–25]

In patients with essential hypertension, LVH is more frequent in those with mild to moderate CKD than in those with normal renal function. Nardi *et al.*[26] reported an increasingly higher prevalence of LVH with declining renal function in their study of 293 CKD patients. Moreover, after adjusting for age, sex, diastolic blood pressure and hemoglobin, they found that CKD is an independent predictor of left ventricular mass index. In the present study, we also determined that even in patients with subclinical kidney dysfunction, the relationship between LVH and renal histological damage is independent of other established factors for atherosclerosis. These findings suggest that nonclassical atherogenic factors such as the renin–angiotensin–aldosterone system in the peripheral organs, sympathetic nervous system overactivity, local inflammation and oxidative stress contribute to both left ventricular remodeling and chronic renal injury, even in subjects without CKD.[27]

Recently, an *in vivo* study showed that a decrease in nephron numbers is a significant determinant of increased blood pressure and

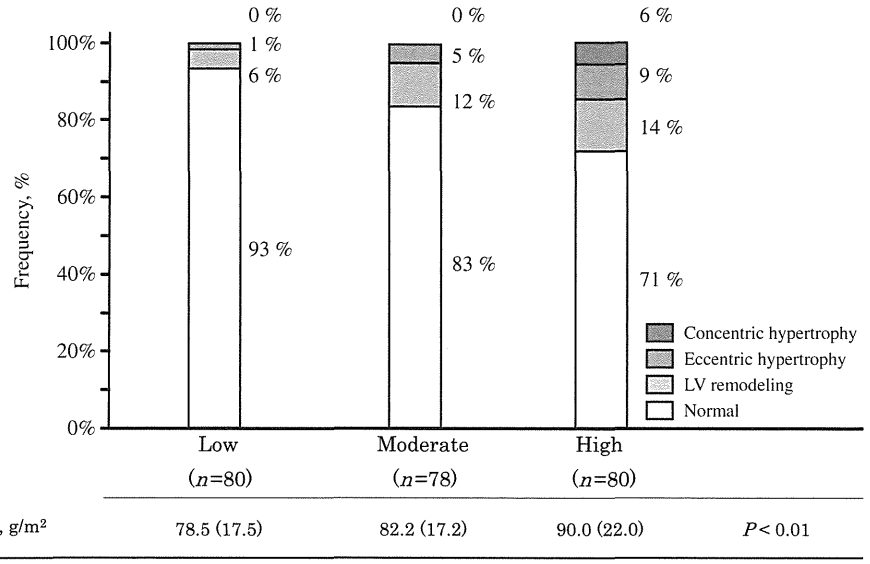

Figure 1 The distribution of left ventricular geometric changes and left ventricular mass index (LVMI). The overall prevalence of left ventricular remodeling and left ventricular hypertrophy (concentric and eccentric) is increased along with the tertiles of %GGS ($P<0.01$). A substantial trend is also observed between the tertiles of %GGS and LVMI (P for trend <0.01). %GGS; percentage of global glomerulosclerosis, LVMI; left ventricular mass index.

Table 2 Analysis of relationship between tertiles according to %GGS and various histological changes of nephrosclerosis

Tertiles of the %GGS	*Low (n = 80)*	*Moderate (n = 78)*	*High (n = 80)*	
Percentage of GGS (%)	*0–3.45*	*3.46–11.76*	*⩾11.77*	P *for trend*[a]
Renal arteriolosclerosis, %				
(−)	74	60	49	<0.01
(1+)	21	22	25	
(2+)	5	15	21	
(3+)	0	3	5	
Renal arteriolar hyaline change, %				
(−)	80	68	56	<0.05
(1+)	14	21	23	
(2+)	1	10	16	
(3+)	5	1	5	
Renal interstitial fibrosis, %				
(−)	76	41	10	<0.01
(+/−)	18	46	54	
(1+)	6	13	27	
(2+)	0	0	9	

Abbreviation: %GGS, percentage of global glomerulosclerosis.
The significance of the relationship between various renal histology grades and each tertile of %GGS was evaluated by linear regression analysis. The values are expressed as frequency (%).
[a]*P* values are versus lowest tertile of %GGS.

cardio–renal damage generally, and of renal structural damage and LVH in particular.[28,29] Genetic or environmental factor may affect the degree of cardio–renal injury in healthy individuals.

Age is the most important factor in the relationship between nephrosclerosis and LVH. Age-related renal change is associated with the declining renal blood flow as shown by the xenon washout technique.[30] Its blunted vasodilatory response to intrarenal acetylcholine administration suggests that age-related decline in renal blood flow has a vascular cause. Conversely, left ventricular dysfunction is sensed by baroreceptors, which activate the sympathetic nervous system. Impaired cardiovascular hemodynamic variables, such as increased end-diastolic pressure, increased peripheral vessel resistance and decreased stroke volume, potentially impair the peripheral blood flow in the kidneys.[1] Although aging is the strongest risk factor for LVH, we speculate that left ventricular remodeling and arterial stiffness accelerate the age-related decline in renal blood flow and that this interaction produces a vicious cycle. Age-dependent GFR decreases are reportedly more pronounced in individuals with concentric LVH than in those with hypertension and normal left ventricular mass.[4,31] In addition, we identified by univariate analysis that pulse pressure increases along with tertiles of %GGS and found a weak correlation between left ventricular mass index and pulse pressure (Supplementary Figure 1). On the other hand, the association between decreased numbers of intact glomeruli and LVH may indicate that sodium retention contributes to these pathophysiological phenomena.[32]

This study has several limitations. First, there is a selection bias toward subjects with a family history of end-stage renal disease. The causes of renal diseases include hypertension and diabetes mellitus. Moreover, the subjects were self-selected by their agreement to serve as living kidney donors. Second, the biopsy specimens included only the superficial renal cortex. In the present study, the frequency of GGS was relatively higher than that reported by an autopsy study.[5] However, according to Ito[33], in the early stages of hypertension, renal injury occurs predominantly in the juxtamedullary nephrons, the majority of other nephrons remaining relatively intact. On the other hand, in an autopsy study, Kasiske[34] reported that GGS occurs uniformly across all zones of the cortex. We could not confirm that the %GGS in our biopsy specimens is representative of the whole kidney. Finally, because

Table 3 Multivariate logistic regression analysis of LVH

		Model 1 (unadjusted)		*Model 2 (age- and sex-adjusted)*		*Model 3 (multivariate-adjusted)*	
Tertiles of %GGS	*Number*	*OR (95% CI)*	*P-value*	*OR (95% CI)*	*P-value*	*OR (95% CI)*	*P-value*
Low (0–3.45%)	80	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Moderate (3.46–11.76%)	78	5.3 (0.83–103)	0.08	4.3 (0.61–85)	0.15	1.8 (0.22–39)	0.60
High (≥ 11.77%)	80	15.8 (3.0–291)	<0.01	13.9 (2.64–257)	<0.01	7.0 (1.03–132)	<0.05

Abbreviations: CI, confidence interval; GFR, glomerular filtration rate; LVH, left ventricular hypertrophy; OR, odds ratio; %GGS; percentage of global glomerulosclerosis.
Subjects in the highest tertile of %GGS had a worse risk profile for LVH than did subjects in the lowest tertile, even in the age- and sex-adjusted (Model 2) and multivariate-adjusted models (Model 3). Model 1, unadjusted analysis; Model 2, adjusted for age and sex; Model 3, adjusted for age, sex, systolic blood pressure, history of diabetes mellitus, total serum cholesterol and GFR determined by a radioisotopic technique. The values are ORs and their 95% CI's versus the lowest tertile.

this study is cross-sectional in nature, we cannot reach cause-and-effect conclusions. Further prospective studies are needed to clarify the long-term prognostic significance of the relationship between LVH and GGS.

CONCLUSION

We have demonstrated a correlation between the GGS and LVH in subjects with normal renal function. This relationship is independent of age, sex, blood pressure, renal function and other conventionally recognized atherogenic factors. A cardio–renal interaction may exist before there is an obvious decline in renal function.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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