Finally, the clinical importance of recommended guidelines for glycemic exposure, BP and lipid profiles was evaluated by the multivariate model adjusted for sex and use of ACE inhibitor or ARBs. The hazard ratio for the remission/regression of microalbuminuria increased with each increment in the number of factors at a salutary level (Fig. 1), when 3 factors, as compared with none, were at the salutary levels, the hazard ratio for remission/regression of microalbuminuria was 1.489 (95% CI: 0.625-3.550), but not at a significant level.

Discussion

Microalbuminuria in patients with type 2 diabetes mellitus has been considered the first step toward overt proteinuria and renal failure. However, our results indicate that microalbuminuria can improve to normal levels in some Japanese type 2 diabetes mellitus patients. Among 130 patients with microalbuminuria, only 20% of patients progressed to overt proteinuria, whereas 40% of patients improved to normoalbuminuria.

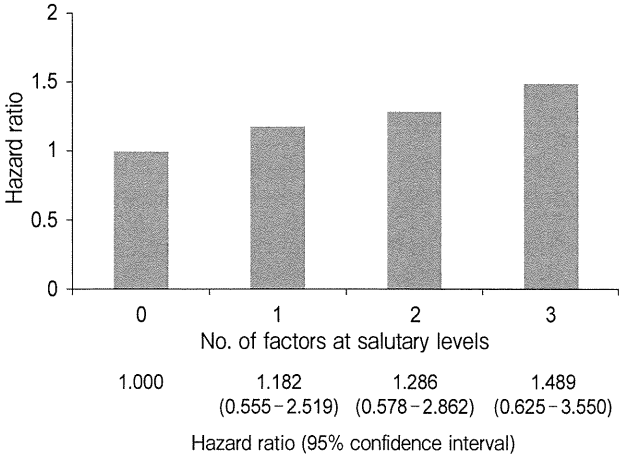

Fig. 1 Additive effects of factors at salutary levels on remission and/or regression of microalbuminuria. Salutary levels of the various factors were defined as < 6.5% for HbA1c, a combination of < 130 mmHg of systolic blood pressure and < 80 mmHg of diastolic blood pressure, and a combination of < 150 mg/dl of triglyceride, < 120 mg/dl of LDL cholesterol, and ≥ 40 mg/dl of HDL cholesterol. The reference category was considered to be the absence of a salutary level for any of the 3 factors. The estimates were adjusted for sex, and use of ACE inhibitor or ARBs. The number of patients having 0, 1, 2, and 3 factors at salutary levels were 23 (18.1%), 47 (37.0%), 40 (31.5%), and 17 (13.4%), respectively.

This study also provided evidence that rigorous control of glycemic exposure, HDL cholesterol and SBP, and female sex were independently associated with the remission and/or regression of microalbuminuria. Improvement of microalbuminuria has recently been reported in patients with type 1 and type 2 diabetes mellitus. Perkins *et al.* found frequent regression, with 58% (95% CI: 52-64) incidence at the 6-year follow-up in patients with type 1 diabetes mellitus [16]. Araki *et al.* also found frequent remission and/or regression, with ~50% incidence at the 6-year follow-up in patients with type 2 diabetes mellitus [17]. These observations strongly indicate that microalbuminuria frequently regresses, contrary to our expectations. In this study, we additionally identified the factors associated with the reduction of microalbuminuria in type 2 diabetes mellitus.

The level of blood glucose seems to be the strongest factor influencing the onset of microalbuminuria. This has been demonstrated in several observational studies [13, 14, 20-23] as well as in clinical trials [24-27]. In our study, the lower HbA1c (≤ 6.0%) was independently associated with remission/regression of microalbuminuria. This cutoff value is lower than the recommended therapeutic target (< 7.0%) for HbA1c [19, 28]. Additionally, the Steno type 2 randomized study [29] showed that the target HbA1c level to prevent progression of diabetic nephropathy was < 6.5%. In our study, the ORs for remission/regression of microalbuminuria in subjects with HbA1c < 7.0% was not statistically significant (OR: 3.68, 95% confidence interval: 0.71-17.33). Our participants' mean HbA1c at baseline (7.2 ± 1.0%) was lower than in other previous studies.

Essential hypertension [30-33], elevated SBP [23, 32], cigarette smoking [14, 34], elevated levels of serum cholesterol and triglycerides [22], and genetic susceptibility are risk factors for diabetic nephropathy [35]. Maintaining BP < 130/80 mmHg is recommended in diabetic patients for preservation of renal function and reduction of cardiovascular events [19, 28, 36]. In our study, lower SBP (≤ 130 mmHg) and lower DBP (≤ 80 mmHg) were not independently associated with remission/regression of microalbuminuria because our participants' mean SBP and DBP at baseline (130.6 ± 10.8 mmHg, 75.3 ± 8.5 mmHg) were comparably well controlled. In lipid profiles, HDL cholesterol was independently associated with

improvement of microalbuminuria. We thought that LDL cholesterol might not have been statistically significant in our study because LDL cholesterol was well controlled by the use of statins, especially in participants with progression of microalbuminuria. These results suggest that normalization of the lipid profile may be associated with remission/regression of type 2 diabetes mellitus with microalbuminuria.

Sex is one of the risk factors for microvascular complications, as well as blood glucose, BP, lipids and cigarette smoking. However, there have been few reports relating sex to the incidence/prevalence and the pathological condition of microvascular complications. There have been a few studies regarding sex as a risk factor for nephropathy, with different results. In a German study of type 1 diabetes mellitus patients, male sex was a risk factor for evident nephropathy [37]. In a study of UKPDS, male sex was a risk factor of an evident albumin urocrisis [38]. In the report of Takane *et al.*, the time when diabetes mellitus and nephropathy were diagnosed tended to be earlier in men than in women, and the dialysis induction age was significantly lower in men [39]. Daniels *et al.* showed that female sex associated with an increased frequency of microalbuminuria in children and adolescents with type 1 diabetes mellitus [40]. In this study, the rate of remission/regression of microalbuminuria was significantly higher in female patients. Future studies investigating sex-specific interventions to reduce the incidence and rate of progression of diabetic nephropathy are required.

Potential limitations still remain in our study. First, the definition of regression of microalbuminuria is not generally recognized. We defined regression of microalbuminuria as a decrease in ACR of $\geq 50\%$ from baseline, as in the Joslin Diabetes Center study and Shiga University study [16, 17]. This definition does not always reflect changes in renal function. It remains unclear whether a decrease in ACR of ≥ 50 percent reflects improvements in morphological abnormalities. Second, our study did not clarify whether remission and/or regression of microalbuminuria finally results in a reduction of the incidence of end-stage renal disease (ESRD) or cardiovascular mortality. Because the average observation period was short, continuation of the observation over the long term is necessary to confirm the results. Third, we examined biochemical measurements in spot blood samples, so blood glucose and triglycerides were not evaluated. Fourth, the small sample size made it difficult to prove the factors that are associated with remission/regression of microalbuminuria, or to investigate the importance of each clinical practice recommendation separately.

In conclusion, we revealed the conditions for improvement of microalbuminuria in patients with type 2 diabetes mellitus at a typical Japanese clinic. These results suggest that management of blood glucose and BP are beneficial and useful measures for the remission and/or regression of microalbuminuria. Lipid profiles, especially higher level of HDL cholesterol, might also be important for remission/regression of microalbuminuria. In addition, the possibility of sex-specific interventions to reduce the incidence and rate of progression of diabetic nephropathy was suggested.

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Short Communication

Lifestyle Modification Is Associated with Improving Estimated Glomerular Filtration Rate (eGFR) and Proteinuria in Japanese with Proteinuria

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The link between lifestyle modification and changes in both proteinuria and estimated glomerular filtration rates (eGFRs) was evaluated in Japanese subjects with proteinuria who were not taking medications. We used data from 51 men (35.8±10.0 years) and 74 women (38.0±11.0 years) with proteinuria at baseline and a 1-year follow up. eGFR was defined by a new equation developed specifically for Japanese subjects. Subjects were given advice for dietary and lifestyle improvement at the initial appointment. At the 1-year follow up, eGFR was increased in both sexes, but not at significant levels. (men: $p=0.7709$, women: $p=0.2180$). Proteinuria was also improved in many subjects. A decrease in proteinuria may be associated with improving eGFR in Japanese.

Key words: proteinuria, estimated glomerular filtration rate (eGFR), lifestyle modification

Chronic kidney disease (CKD) is a common disorder and has become a public health challenge [1]. We previously reported in a cross-sectional study that the estimated glomerular filtration rate (eGFR) [2] in men with abdominal obesity and in women with hypertension was significantly lower than that in subjects without these components of metabolic syndrome [3]. A longitudinal our analysis showed that decreasing systolic blood pressure was associated with improving eGFR in healthy Japanese women [4]. In addition, proteinuria has been closely linked to lower eGFR in Okayama prefecture, Japan [5].

However, whether decreases in proteinuria with lifestyle modification are beneficial for improving eGFR remains to be investigated in a longitudinal study.

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In this study, we evaluate the link between changes in eGFR and changes in proteinuria in Japanese at a 1-year follow up after lifestyle modification counseling was given.

Subjects and Methods

Subjects. We used data for 51 men (35.8±10.0 years) and 74 women (38.0±11.0 years) with proteinuria, who met the following criteria: (1) received a health check-up including special health guidance and a follow-up check-up 1-year later, (2) received anthropometric measurements as part of the annual health check-up, (3) received no medications for diabetes, hypertension, and/or dyslipidemia, and (4) provided written informed consent (Table 1).

At the first health check-up, all subjects were given instructions by well-trained medical staff on how to change their lifestyle as special health guidance. Nutritional instruction was provided with a well-

Table 1 Clinical profiles of enrolled subjects and changes in parameters with lifestyle modification after 1 year

	Men			Women		
	Baseline	Follow up	*p*	Baseline	Follow up	*p*
Number of subjects	51			74		
Age	35.8 ± 10.0			38.0 ± 11.0		
Height (cm)	169.6 ± 5.8			157.0 ± 5.7		
Body weight (kg)	72.7 ± 14.7	71.5 ± 13.2	0.1070	57.1 ± 10.1	56.7 ± 10.2	0.2960
Body mass index (kg/m^2)	25.3 ± 4.7	24.8 ± 4.2	0.0690	23.2 ± 4.0	23.0 ± 4.0	0.1746
Abdominal circumference (cm)	83.1 ± 12.7	82.3 ± 11.1	0.3030	73.1 ± 9.7	73.0 ± 9.9	0.6772
Hip circumference (cm)	95.2 ± 7.4	94.5 ± 6.4	0.1300	91.7 ± 6.2	91.5 ± 5.7	0.5469
Creatinine (mg/dl)	0.84 ± 0.13	0.82 ± 0.11	0.2034	0.63 ± 0.15	0.60 ± 0.10	**0.0247**
eGFR (ml/min/1.73m^2)	87.9 ± 17.9	88.4 ± 14.9	0.7709	89.6 ± 23.9	92.5 ± 20.2	0.2180

Mean ± SD

trained nutritionist, who planned a diet for each subject based on their data and provided simple instructions (*i.e.* not to eat too much and to consider balance when they eat). Exercise instruction was also provided by a well-trained physical therapist, who encouraged each subject to increase their daily amount of steps walked. At the the second health check-up, medical staff subjectively evaluated changes in their lifestyle and subjects with proteinuria were encouraged to receive medications.

Ethical approval for the study was obtained from the Ethical Committee of Okayama Health Foundation, Okayama, Japan.

Anthropometric and body composition measurements. Anthropometric and body compositions were evaluated based on the following parameters: height, body weight and abdominal circumference. Body mass index (BMI) was calculated by weight/[height]^2, in kg/m^2. Abdominal circumference was measured at the umbilical level and the hip was measured at the widest circumference over the trochanter in standing subjects after normal expiration [6].

Urine examination. Urine samples were collected before 10 a.m. as second urine of the day and were subjected to examination within 1h. The urine examination was performed using urine test strips (BAYER, Tokyo, Japan). The reagent strip was dipped directly into the urine sample. Just after dipping, the sample was graded as negative (−), trace positive (±), or +, 2+, 3+or 4+positive at levels of 30, 100, 300 or 1,000mg/dl, respectively, based on color chart found on the container's label [7].

Blood sampling and assays. We measured overnight fasting serum levels of creatinine (Cr) (enzymatic method). eGFR was calculated using the following equation: eGFR (ml/min/1.73m^2)=194×Cr$^{-1.094}$×Age$^{-0.287}$×0.739 (a constant derived specifically for women) [2]. Reduced eGFR was defined as an eGFR <60ml/min/1.73m^2.

Statistical analysis. Data are expressed as means±standard deviations (SDs). Statistical analysis was performed using a paired *t* test: $p<0.05$ was considered to be statistically significant.

Results

The clinical parameters at baseline and 1-year follow up are summarized in Table 1. Anthropometric and body composition parameters such as body weight, BMI, abdominal circumference and hip circumference were reduced with lifestyle modification after one year, but not at a significant level in both sexes. Cr was significantly decreased in women; eGFR was increased, but not at a significant level in either sex (Table 1). Only one man and 5 women were diagnosed below normal-range eGFR at baseline, and one men and one woman at the 1-year follow up.

We further evaluated the changes in proteinuria (Table 2). At baseline, 36 men and 57 women were diagnosed with trace proteinuria (±), 8 men and 11 women as+, 5 men and 4 women as 2+, and 2 men and 2 women as 3+. However, after lifestyle modifications at the 1-year follow up, 38 men and 56 women were diagnosed as proteinuria negative (−), 7 men and 10 women as trace (±), 2 men and 5 women as+, 3 men and 2 women as 2+, and 1 man and 1 woman as 3+. Proteinuria was increased in only 3 men

Table 2 Changes in proteinuria with lifestyle modification in subjects with proteinuria after 1 year

		Follow up				
Men		Proteinuria (−)	Proteinuria (±)	Proteinuria (+)	Proteinuria (2+)	Proteinuria (3+)
Baseline	Proteinuria (±)	31	3	1	0	1
	Proteinuria (+)	5	1	1	1	0
	Proteinuria (2+)	2	2	0	1	0
	Proteinuria (3+)	0	1	0	1	0
Women						
Baseline	Proteinuria (±)	45	8	3	0	1
	Proteinuria (+)	7	2	1	1	0
	Proteinuria (2+)	2	0	1	1	0
	Proteinuria (3+)	2	0	0	0	0

Table 3 Changes in eGFR in subjects whose proteinuria increased in the 1-year periodr

		Proteinuria		eGFR(ml/min/1.73m^2)	
Subjects	Sex	Baseline	Follow up	Baseline	Follow up
1	Men	+	2+	80.4	75.0
2	Men	±	3+	62.8	52.6
3	Men	±	+	133.2	94.7
4	Women	+	2+	80.8	87.2
5	Women	±	3+	82.1	81.5
6	Women	±	+	69.7	118.9
7	Women	±	+	152.7	133.1
8	Women	±	+	99.1	98.3

eGFR: estimated glomerular filtration rate

(5.9%) and 5 women (6.8%) after 1 year.

Finally, we evaluated the changes in eGFR in the 8 subjects mentioned above with increased proteinuria at the 1-year follow up (Table 3). Their eGFR was decreased at the 1-year follow up, but not at a significant level (Baseline: 95.1±31.8ml/min/1.73m^2, Follow up: 92.7±25.2ml/min/1.73m^2, p=0.7916).

Discussion

The main objective of this study was to explore the link between changes in eGFR and changes in proteinuria in Japanese with proteinuria at a 1-year follow up.

It is well known that an unhealthy lifestyle is closely associated with CKD. Lower physical activity was reported to be a risk for death in patients with CKD [8]. Shankar *et al.* showed that heavy drinking was also closely linked to CKD [9]. In addition, several studies have shown that restriction of protein intake is useful for preventing and improving CKD [10-12]. Swift *et al.* also reported that modest salt reduction reduces blood pressure and urine protein excretion in black hypertensives [13]. Therefore, it seems reasonable to suggest that simple improvements in lifestyle might result in the amelioration of CKD in some Japanese. However, these are few studies on the link between lifestyle modification and CKD using the new equation developed in Japan, so the hypothesis that CKD may be improving by lifestyle modification has not yet been confirmed in the Japanese population.

We previously evaluated the relationship between eGFR and proteinuria in a cross-sectional analysis [5]. The prevalence of proteinuria was closely linked to reduced eGFR. eGFR in subjects with proteinuria at the + level or greater was significantly lower than that in subjects without proteinuria for both sexes. About 15.0% men and women in subjects with proteinuria at the + level or greater were diagnosed as having reduced eGFR. Iseki K *et al.* also identified a strong, graded relationship between end-stage renal disease and dipstick urinalysis positivity for proteinuria, with an adjusted odds ratio was 2.71 [14]. Therefore, proteinuria is a strong, independent predictor of end-stage renal disease. However, in this study, lifestyle modification alone (without medication) in subjects with proteinuria resulted in increased eGFR and improved proteinuria at the one-year follow-up compared to the initial visit. Although we could not evaluate pathophysiological concerns such as IgA nephropathy and nephrotic syndrome, only 8 subjects developed massive proteinuria. Taken together, lifestyle modification alone may be a useful method for improving eGFR in Japanese subjects with proteinuria.

Potential limitations remain in our study. First, the small sample size in our study makes it difficult to infer causality between eGFR and lifestyle modification. eGFR was not significantly improved, especially in men. At the first health check-up, all subjects were given instructions by well-trained medical staff on how to change their lifestyle. However, the changes in participants' lifestyles were not quantitatively evaluated, and the gender differences in lifestyle and improvements in eGFR were also not clearly illuminated. The difference in eGFR between the participants who actually altered their lifestyles and the participants who did not would have been helpful. In addition, eGFR whose proteinuria increased over the one-year period was not significantly decreased in this study. Second, we also could not reveal the mechanism of the linkage between eGFR and lifestyle modification. Third, most of the enrolled subjects were not diagnosed as below-normal-range eGFR at baseline. Therefore, the results in this study may not apply for all patients with CKD.

In conclusion, a decrease in proteinuria with simple lifestyle modification and no medication changes appeared to be associated with an increase in eGFR. Therefore, lifestyle modification alone may be a useful measure for the improvement of CKD.

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Dipeptidyl peptidase-4 inhibitor ameliorates early renal injury through its anti-inflammatory action in a rat model of type 1 diabetes

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ABSTRACT

Introduction: Dipeptidyl peptidase-4 (DPP-4) inhibitors are incretin-based drugs in patients with type 2 diabetes. In our previous study, we showed that glucagon-like peptide-1 (GLP-1) receptor agonist has reno-protective effects through anti-inflammatory action. The mechanism of action of DPP-4 inhibitor is different from that of GLP-1 receptor agonists. It is not obvious whether DPP-4 inhibitor prevents the exacerbation of diabetic nephropathy through anti-inflammatory effects besides lowering blood glucose or not. The purpose of this study is to clarify the reno-protective effects of DPP-4 inhibitor through anti-inflammatory actions in the early diabetic nephropathy.

Materials and methods: Five-week-old male Sprague–Dawley (SD) rats were divided into three groups; non-diabetes, diabetes and diabetes treated with DPP-4 inhibitor (PKF275-055; 3 mg/kg/day). PKF275-055 was administered orally for 8 weeks.

Results: PKF275-055 increased the serum active GLP-1 concentration and the production of urinary cyclic AMP. PKF275-055 decreased urinary albumin excretion and ameliorated histological change of diabetic nephropathy. Macrophage infiltration was inhibited, and inflammatory molecules were down-regulated by PKF275-055 in the glomeruli. In addition, nuclear factor-κB (NF-κB) activity was suppressed in the kidney.

Conclusions: These results indicate that DPP-4 inhibitor, PKF275-055, have reno-protective effects through anti-inflammatory action in the early stage of diabetic nephropathy. The endogenous biological active GLP-1 might be beneficial on diabetic nephropathy besides lowering blood glucose.

1. Introduction

Diabetic nephropathy has become the most common cause of end-stage renal disease worldwide [1]. Many mechanisms have been proposed to explain the pathogenesis of diabetic nephropathy. Recently, accumulated data have emphasized that an inflammation plays a crucial role in the pathogenesis of diabetic nephropathy [2,3]. Actually, we have shown that inflammatory molecules and mediators are important in the early stage of diabetic nephropathy by using intercellular adhesion molecule-1 (ICAM-1) -deficient mice and macrophage scavenger receptor-A-deficient mice, and by an administration of methotrexate [4–6].

Currently, incretin-based drugs are being used to achieve better glycemic control in patients with type 2 diabetes. GLP-1 receptor agonists that enhance resistance to the degradation by DPP-4 enzyme, strongly and steadily stimulate GLP-1 receptor in the pharmacological level. On the other hand, DPP-4 inhibitors that block the activity of DPP-4, reinforce the endogenous biological actions of incretin.

The incretin receptors have been found in multiple organs including kidney [7]. Previous reports and our study [8] revealed that GLP-1 receptor was expressed in glomeruli and renal tubule. To our knowledge, gastric inhibitory polypeptide (GIP) receptor was not expressed in the kidney [9]. We studied the effects on diabetic nephropathy animal model by using GLP-1 receptor agonist called exendin-4. Exendin-4 reduced the urinary albumin

Abbreviations: DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; SD, Sprague–Dawley; NF-κB, nuclear factor-κB; GIP, gastric inhibitory polypeptide; ICAM-1, intercellular adhesion molecule-1; PAM, periodic acid-methenamine silver; cAMP, cyclic AMP; CRP, c-reactive protein; IL, interleukin; CD, cluster of differentiation; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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excretion, and attenuated the histological parameters of glomerular injuries characterized by mesangial extracellular matrix expansion and glomerular hypertrophy. It also attenuated inflammatory molecules and mediators, such as ICAM-1, macrophage infiltration, cytokines and NF-κB activity, in the kidney. Furthermore, the effects were shown, observed directly through the GLP-1 receptor in culture cells [8]. The other groups also presented that GLP-1 receptor agonist was beneficial on diabetic nephropathy [10–12].

Recently, the other investigator reported that DPP-4 inhibitor ameliorated diabetic nephropathy by inhibition of apoptosis and sclerosis [13]. Therefore, the endogenous biological effects of GLP-1 would ameliorate renal injuries. However, it is not obvious whether DPP-4 inhibitor prevents the exacerbation of diabetic nephropathy through anti-inflammatory effects in the early stage model of diabetic nephropathy. The purpose of this study is to clarify the reno-protective effects of DPP-4 inhibitor through anti-inflammatory actions in the early diabetic nephropathy.

2. Methods

2.1. Animals

Male SD rats (Charles River, Yokohama, Japan) were purchased from Charles River (Yokohama, Japan). SD rats aged 4 weeks were divided into the following groups: negative control group, non-diabetes (NDM); positive control group, diabetes (DM); and test group, diabetes treated with DPP-4 inhibitor (DM + D) (n = 7 per group). At the age of 5 weeks, rats chosen for the DM and DM + D groups were injected intravenously with streptozotocin (Sigma–Aldrich, St. Louis, MO, USA) at 65 mg/kg body weight in citrate buffer (pH 4.5). Only rats with blood glucose concentrations >300 mg/dl at 7 days after streptozotocin injection were used in the diabetes groups. The NDM group received injections of citrate buffer alone. The DM + D group was given DPP-4 inhibitor (PKF275-055 [14]; Novartis, Basel, Switzerland) orally at 3 mg/kg body weight daily for 8 weeks, starting at 1 week after streptozotocin injection. All rats had free access to standard chow and tap water. All procedures were approved by the Committee for Ethics and Animal Experimentation of Nihon Bioresearch Inc. All rats were killed at 9th week after the induction of diabetes, and the kidneys were harvested.

2.2. Metabolic variables

Systolic blood pressure was measured by tail-cuff plethysmography (Softron, Tokyo, Japan). Food intake was calculated as the average over 3 days. Urine samples were collected over a 24 h period in individual metabolism cages. Urinary albumin excretion was measured by nephelometry using anti-rat albumin antibody (ICN Pharmaceuticals, Aurora, OH, USA). Creatinine clearance (ml min^{-1} kg^{-1}) was calculated as described previously [15]. Serum active GLP-1 levels were measured by using ELISA kit (AKMGP-011, Shibayagi, Gunma, Japan) at the pre-prandial (after about 12 h of fasting) and post-prandial (2 h) time. 24 h urinary cyclic AMP (cAMP) excretion was measured by using cAMP Complete Enzyme Immunometric Assay kit (Enzo Life Sciences, Ann Arbor, USA) according to the manufacturer's instructions. Serum c-reactive protein (CRP) levels were measured by rat CRP ELISA kit (Life Diagnostics, PA, USA) according to the manufacturer's instructions. Serum interleukin (IL)-6 levels were measured by rat IL-6 ELISA kit (Uscn Life Science, China) according to the manufacturer's instructions.

2.3. Light microscopy

Periodic acid-methenamine silver (PAM)-stained slice were analyzed as described previously [8]. To evaluate the glomerular size and mesangial matrix area, we examined randomly selected ten glomeruli per animal (n = 7 per group). Quantitative analysis for all staining was performed in a blinded manner.

2.4. Immunoperoxidase staining

Immunoperoxidase staining was performed as described previously [16]. Primary antibody was monoclonal antibody against rat monocytes/macrophages (ED1, 1:50; Serotec, Oxford, UK), which was applied for 12 h at 4 °C. Secondary antibody was biotin-labelled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature. Intraglomerular ED1-positive cells were counted in ten glomeruli per animal (n = 7 per group). Quantitative analysis for all staining was performed in a blinded manner.

2.5. RNA extraction, quantitative real-time PCR

Total RNA was extracted from glomeruli isolated by the mechanical sieving technique as previously reported [17], and by using a kit (RNeasy plus Mini; Qiagen, Valencia, CA, USA). Real-time PCR was performed as described previously [8]. The amount of PCR products was normalized with β-actin. The specific oligonucleotide primer sequences are shown in Supplementary Table S1.

2.6. Cytokines and chemokines in the kidney

High-throughput multiplex immunoassays in the renal cortex were performed with the Procarta cytokine assay kit (Panomics Inc., CA, USA) according to the manufacturer's instructions, and analyzed by using Bio-plex (Bio-Rad, Tokyo, Japan).

2.7. Nuclear factor-κB activity

Nuclear proteins were extracted from the kidney tissues with a nuclear extract kit (Active motif, Carlsbad, CA) according to the manufacturer's instructions. NF-κB p65-dependent DNA-binding activity was determined by TransAM NF-κB p65 (Active motif) according to the manufacturer's instructions.

2.8. Statistical analysis

All values are expressed as the means ± SEM. Differences between groups were examined for statistical significance by using one-way ANOVA followed by Scheffe's test. For comparisons between two groups, an un-paired t test was used to assess statistical significance. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Metabolic characteristics of experimental animal models

At 8th week after induction of diabetes, HbA1c, food intake, creatinine clearance and kidney weight were elevated to the same level in both the diabetic groups compared with the NDM group (Table 1). Body weight was decreased to the same level in both the diabetic groups. However, there was no significant difference between the DM and DM + D groups. Systolic blood pressure remained at the same level in the three groups. It is noteworthy that PKF275-055 treatment significantly reduced urinary albumin excretion compared with the DM group.

In this experimental animal model, serum active GLP-1concentration was significantly increased in the DM + D group compared with the NDM group at both the pre-prandial and post-prandial

Table 1
Physiological and metabolic characteristics of non-diabetes, diabetes and diabetes treated with DPP-4 inhibitor, PKF275-055, at the 8th week.

	NDM	DM	DM+D
HbA1c (%)	4.4 ± 0.1**	9.9 ± 0.5	9.7 ± 0.2
Body weight (g)	449 ± 13.3**	254 ± 16.2	260 ± 16.7
Food intake (g/day)	24.9 ± 0.9**	49.0 ± 1.5	51.7 ± 3.5
Systolic blood pressure (mmHg)	135 ± 2	133 ± 4	130 ± 2
Urinary albumin excretion (μg/day)	135 ± 24.4**	699 ± 48.7	420 ± 93.3#
Creatinine clearance (ml/min/kgBW)	7.6 ± 0.2*	12.6 ± 1.5	12.5 ± 1.3
Kidney weight (g/kgBW)	3.7 ± 0.1**	7.2 ± 0.6	7.7 ± 0.6

NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055; BW, body weight. Values are presented as means ± SEM. $n = 7$ per group.
* $P < 0.05$.
** $P < 0.001$ vs DM, DM + D.
# $P < 0.05$.
$P < 0.001$ vs DM.

time. There was significant difference at only the post-prandial time between the DM and DM + D groups (Fig. 1A).

To examine the association with the GLP-1 receptor signal pathway, we measured urinary cAMP excretion known as the second messenger of GLP-1 receptor. Urinary cAMP excretion was significantly elevated in the DM + D group compared with the other groups (Fig. 1B).

3.2. DPP-4 inhibitor ameliorated glomerular morphology in the early stage of diabetic nephropathy

Glomerular hypertrophy was observed in both the diabetic groups as compared with the NDM group. There was no significant difference in glomerular size between the DM and DM + D groups (Fig. 2D). Mesangial matrix expansion was also observed in both the diabetic groups as compared with the NDM group. However, PKF275-055 treatment significantly prevented mesangial matrix expansion compared with the DM group. The prevention of mesangial matrix expansion was not at the same level to the NDM group (Fig. 2E).

3.3. The reno-protective effects of DPP-4 inhibitor through anti-inflammatory action

To evaluate the anti-inflammatory effect of DPP-4 inhibitor in the glomeruli, we examined inflammatory-related factor. The

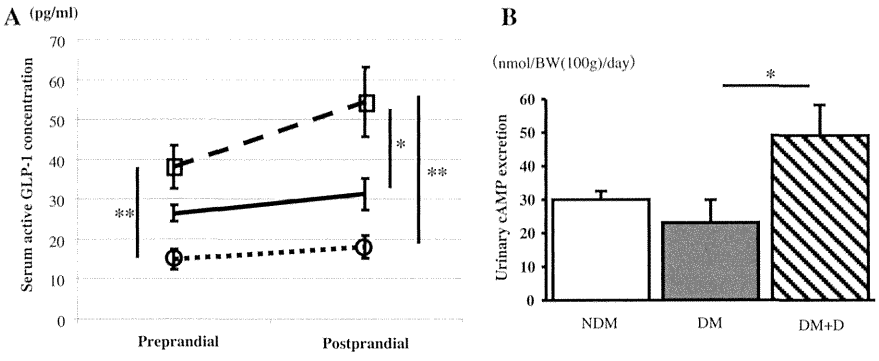

Fig. 1. Serum active GLP-1 concentration and urinary cAMP excretion in this model. (A) The concentration of the serum active GLP-1 at the preprandial and postprandial condition. Data are means ± SEM $n = 7$ per group. Dotted line (○), NDM; solid line, DM; Dotted line (□), DM + D. *$P < 0.05$; **$P < 0.001$. (B) Urinary cAMP excretion for 24 h. Data are means ± SEM $n = 7$ per group. *$P < 0.05$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

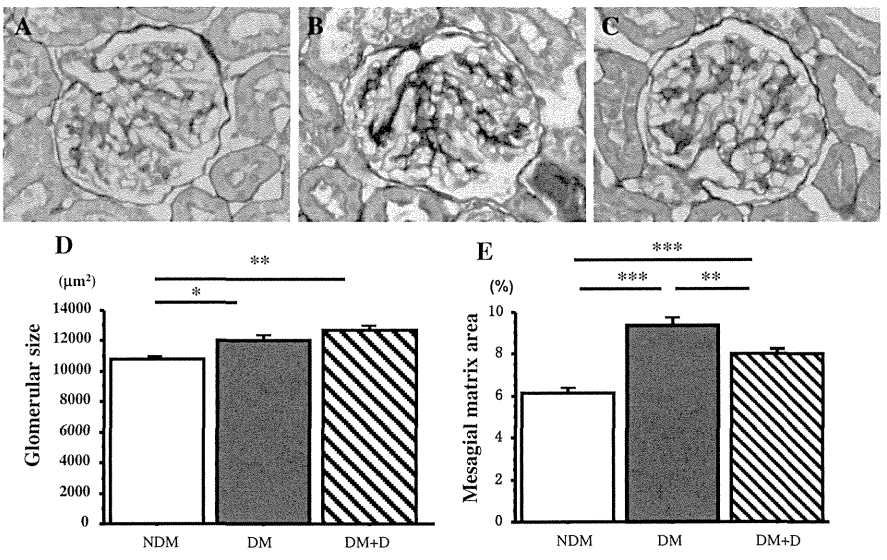

Fig. 2. The effects of DPP-4 inhibitor against glomerular morphology. Periodic acid-methenamine silver (PAM)-stained slice in glomeruli. (A) non-diabetic group, (B) diabetes control, (C) diabetes treated with PKF275-055. Randomly selected ten glomeruli per animal ($n = 7$ per group) were measured the glomerular size (D) and mesangial matrix area (E). Quantitative analysis for all staining was performed in a blinded manner. Data are means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

average number of macrophages (ED-1 positive cells) per glomerulus was markedly increased in the DM group compared with the NDM group, whereas macrophage infiltration was significantly inhibited by PKF275-055 treatment (Fig. 3A–D). Likewise, the mRNA level of cluster of differentiation (CD) 68 (a cell surface marker of macrophages) was significantly up-regulated in the DM group compared with the NDM group, and then significantly down-regulated in the DM + D group (Fig. 3E).

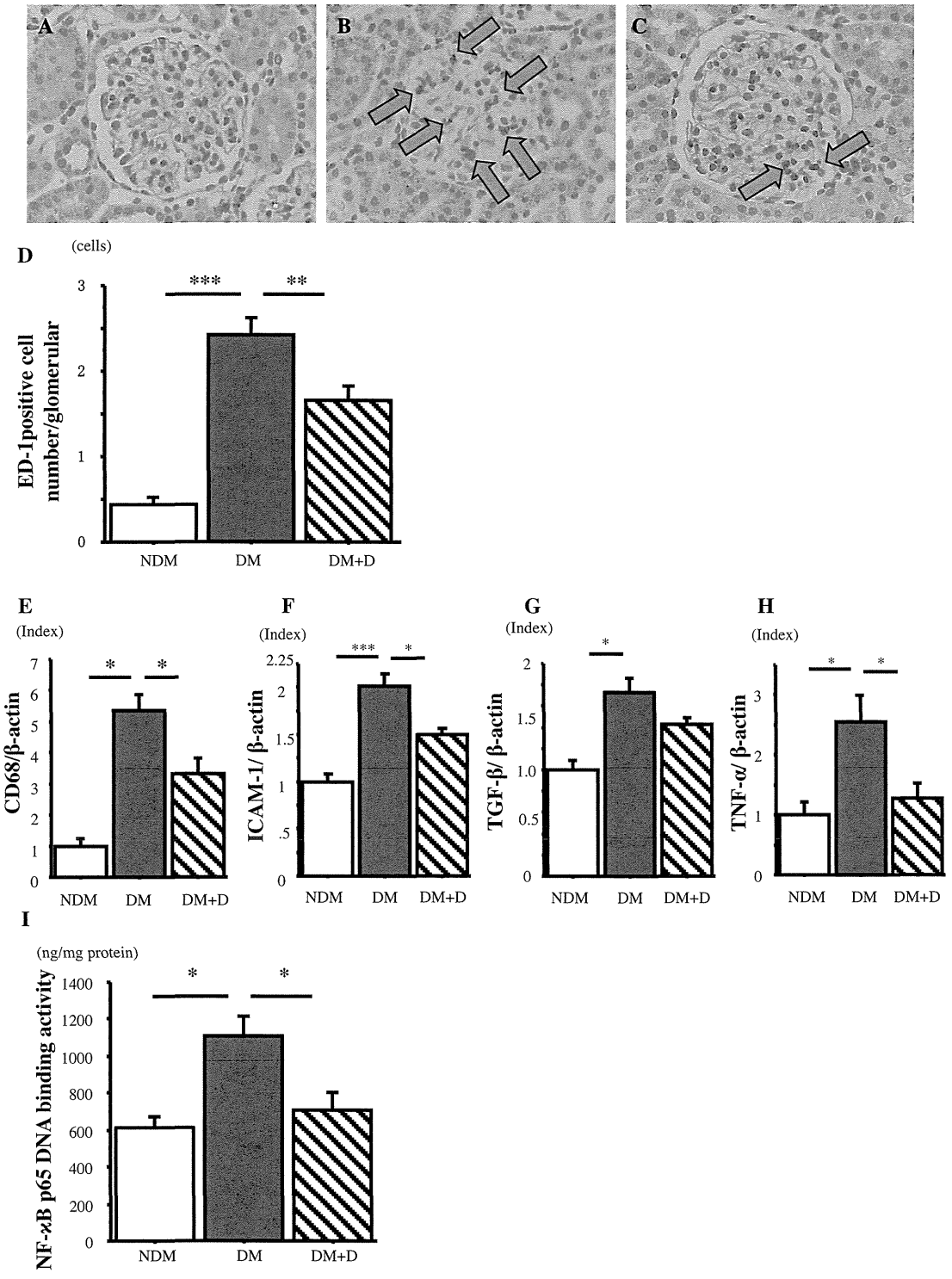

Fig. 3. The anti-inflammatory effects of DPP-4 inhibitor. (A–C) Immunoperoxidase staining for macrophage infiltration into glomeruli. (A) non-diabetic group, (B) diabetes control, (C) diabetes treated with PKF275-055. Arrow head is presented as macrophage. (D) Randomly selected ten glomeruli per animal ($n = 7$ per group) were measured the number of macrophages per glomerulus. Quantitative analysis for all staining was performed in a blinded manner. (E–H) The mRNA levels in the glomeruli ($n = 6$ per group). Values (means ± SEM) are presented as the ratio of NDM. (I) The NF-κB p65 DNA binding activity in the kidney. $n = 5$ per group. Absorbance was normalized to milligram protein. Data are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. NDM, non-diabetic group; DM, diabetes control; DM + D, diabetes treated with PKF275-055.

In addition, the mRNA levels of ICAM-1, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α were significantly up-regulated in the DM group as compared with the NDM group. PKF275-055 treatment significantly down-regulated the mRNA levels of ICAM-1 and TNF-α compared with the DM group (Fig. 3F–H).

To evaluate the effect of DPP-4 inhibitor against the NF-κB activity, we examined NF-κB p65-dependent DNA-binding activity in the kidney. The NF-κB p65-dependent DNA-binding activity was significantly increased in the DM group compared with the NDM group. PKF275-055 treatment significantly decreased the NF-κB p65-dependent DNA-binding activity (Fig. 3I).

In order to clarify the influence of cytokines and chemokines which are cleaved by DPP-4 enzyme, we examined the production of eotaxin, Il-2, IL-1β, IP-10, MCP-1, MCP-3 and RANTES in the cortex. These cytokines and chemokines were not affected by PKF275-055 treatment (Supplementary Fig. S1 A–G).

To evaluate systemic inflammation, we measured the levels of serum CRP and IL-6. The level of serum CRP was significantly elevated in the DM group compared with the NDM group, but not attenuated by PKF275-055 treatment. The level of serum IL-6 was not affected (Supplementary Table S2).

4. Discussion

Our results indicated that DPP-4 inhibitor, PKF275-055, reduced urinary albumin excretion, and histological change of diabetic nephropathy, and prevented the macrophage infiltration and inflammatory molecules in the glomeruli. In addition, it inhibited the NF-κB activity in the kidney. DPP-4 inhibitor treatment didn't have an impact on glucose control, blood pressure and body weight which affect nephropathy. These results demonstrated that DPP-4 inhibitor ameliorated diabetic nephropathy through anti-inflammatory effects.

We should consider the following contents against the anti-inflammatory action of DPP-4 inhibitor. (1) The increase of active GLP-1 and GIP, (2) the inhibition of DPP-4 itself, (3) the influence of the other substrate of DPP-4 except for an incretin.

In terms of the influence of active GLP-1 and GIP, several previous reports have already shown that GLP-1 has reno-protective effects through anti-inflammation and anti-oxidative stress [8,10,11]. On the other hand, GIP receptor is not expressed in the kidney to our knowledge [9]. Therefore, it would be unlikely about the effects through GIP.

Recently, the inhibition of DPP-4 itself suppressed the secretion of cytokine from macrophage and NF-κB activity induced by lipopolysaccharide [18]. Therefore, the inhibition of DPP-4 itself might ameliorate diabetic nephropathy through anti-inflammatory effect by DPP-4 inhibitor. It is not directly observed in this study. Thereafter, it is necessary to examine it by using tissue-specific DPP-4 deficient animal model.

We examined the influence of cytokines and chemokines which are cleaved by DPP-4 enzyme. There are eotaxin, IL-2, IL-1β, IP-10, MCP-1, MCP-3 and RANTES as the substrate of DPP-4 related to inflammation. As for our result, at least DPP-4 inhibitor did not significantly aggravate inflammation in the kidney.

A better glycemic control prevents the onset and progression of diabetic nephropathy. In the previous report, DPP-4 inhibitor actually ameliorated glucose control in patients with type 2 diabetes [19] and animal model [20], and resulted in reducing urinary albumin excretion. Decreases of blood pressure and body weight are also useful for nephropathy. Incretin-based drugs have the inhibitory action of sodium reabsorption in renal proximal tubule under the conditions of salt-sensitive models and salt load [21,22]. However, it is considered that such action was not observed in the condition of this study. There was also no influence in body weight. It does not contradict with the clinical data [23].

Liu and the members [13] administrated DPP-4 inhibitor, LAF237 (vildagliptin), to same nephropathy model and suppressed urinary albumin, and reduced renal injury. In their examination, several parameters were evaluated at 12th and 24th week after the treatment. The stage was the period of drop of creatinine clearance. On the other hand, our examination at 8th week was at the more early stage, which indicated glomerular hyperfiltration. To our knowledge, our examination is the first report that DPP-4 inhibitor has protective effect at the early stage. Their results indicated that DPP-4 inhibitor suppressed urinary 8-Hydroxydeoxyguanosine excretion known as the marker of oxidative stress. Our result was not the same, which DPP-4 inhibitor could not significantly reduce the oxidative stress elevated by the hyperglycemia (data not shown). The longer period of DPP-4 inhibitor treatment might be required to decrease the oxidative stress.

In our previous report on GLP-1 receptor agonist [8], there were improvements on glomerular hyperfiltration in the same stage of nephropathy model, but the same result did not appear in this study. This reason is considered to be due to the difference between the pharmacological effect expected by GLP-1 receptor agonist and the biological effect expected by DPP-4 inhibitor. According to the previous report examining about the inhibitory action of sodium reabsorption in the kidney between GLP-1 receptor agonist and DPP-4 inhibitor, GLP-1 receptor agonist elevated more urinary cAMP concentration than DPP-4 inhibitor, and the effect of it depended on the cAMP levels[24]. However, the details are still unclear whether GLP-1 signal contributes to glomerular hemodynamics or not. Therefore, a further study is necessary about it.

The serum CRP level was not improved by DPP-4 inhibitor in this study. The result caused a question about whether PKF275-055 treatment in this study affects the systemic inflammation. We proposed the difference of the DPP-4 expression in each organ. The previous report [25] described that DPP-4 activity was strongly expressed in the kidney compared with the other tissues. Furthermore, the prevention of DPP-4 itself by DPP-4 inhibitor attenuated the local inflammation [18]. Therefore, the anti-inflammatory effect on kidney through DPP-4 inhibitor might be more affected compared with whole body. In addition, the long-term treatment might be necessary to decrease the systemic inflammation similarly to oxidative stress. In our previous study on GLP-1 receptor agonist [8], oxidative stress was attenuated through the same treatment period, and in the same animal model. Taken together, the anti-inflammatory effect expected by PKF275-055 treatment might be weaker than GLP-1 receptor agonist. However, further examination is necessary to investigate this regard.

In conclusion, we showed that DPP-4 inhibitor, PKF275-055, have reno-protective effects through anti-inflammatory action in the early stage of diabetic nephropathy. The endogenous biological active GLP-1 might be beneficial on diabetic nephropathy besides lowering blood glucose.

Conflict of interest statement

This work was partially supported by research fund from Novartis Pharma K.K. KS receives speaker honoraria from Astellas, MSD, Eli Lilly Japan, Novartis Pharma, NovoNordisk, Ono, Sanofi and Tanabe Mitsubishi, and receives grant support from Tanabe Mitsubishi. HM is a consultant for AbbVie, Astellas, and Teijin, receives speaker honoraria from Astellas, MSD, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, MSD, Novo Nodisk, Takeda and Kyowahakko-Kirin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.049

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SPECIAL ARTICLE

A new classification of Diabetic Nephropathy 2014: a report from Joint Committee on Diabetic Nephropathy

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Abstract The Joint Committee on Diabetic Nephropathy has revised its Classification of Diabetic Nephropathy (Classification of Diabetic Nephropathy 2014) in line with the widespread use of key concepts such as the estimated glomerular filtration rate (eGFR) and chronic kidney disease. In revising the Classification, the Committee carefully evaluated, as relevant to current revision, the report of a study conducted by the Research Group of Diabetic Nephropathy, Ministry of Health, Labour and Welfare of Japan. Major revisions to the Classification are summarized as follows: (1) eGFR is substituted for GFR in the Classification; (2) the subdivisions A and B in stage 3 (overt nephropathy) have been reintegrated; (3) stage 4 (kidney failure) has been redefined as a GFR less than 30 mL/min/1.73 m^2, regardless of the extent of albuminuria; and (4) stress has been placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Keywords Diabetic nephropathy · Chronic kidney disease (CKD) · Albuminuria · Proteinuria · Glomerular filtration rate (GFR)

Japan Diabetes Society, Japanese Society of Nephrology, Japanese Society for Dialysis Therapy, and Japan Society of Metabolism and Clinical Nutrition established the Joint Committee on Diabetic Nephropathy, which published the revised Classification of Diabetic Nephropathy 2014 in Japanese [1–4]. This is the English version of that revision.

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Introduction

Diabetic nephropathy became the leading cause of chronic dialysis in 1998. Since then, the incidence of this condition has increased with only a recent plateau. However, diabetic nephropathy continues to account for a large proportion of all cases of chronic kidney disease (CKD) and remains by far the most common underlying cause of chronic dialysis among all kidney diseases [5], consequently leading to the escalation of healthcare costs, thus representing a compelling medico-social issue of interest.

The Classification of Diabetic Nephropathy (hereafter "Classification") developed earlier by the Research Group

of Diabetic Nephropathy at the Ministry of Health, Labour and Welfare (MHLW) [6] and later revised by the Joint Committee on Diabetic Nephropathy (hereafter "Committee") [7] is widely used in Japan. However, as the concept of CKD was proposed, followed by the classification of CKD stages [8], it became clear that there exists a subpopulation of patients with discrepant classifications of diabetic nephropathy and CKD. This is thought to be due to the fact that diabetic nephropathy is primarily classified according to the extent of albuminuria in addition to the glomerular filtration rate (GFR) (i.e., creatinine clearance [CCr]), whereas CKD is primarily classified based on the estimated GFR [estimated GFR (eGFR)]. Meanwhile, eGFR has become increasingly used to assess GFR, and a new classification of CKD was developed in 2012 [9]. Against this background, the Committee therefore discussed issues of interest in depth and sought to develop a revision of the Classification.

Development of the 2014 Classification (Revised Classification) (see Table 1)

Prior to revising the Classification, as part of a MHLW-subsidized project on kidney disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan", a "historical cohort study" was conducted by the Research Group of Diabetic Nephropathy, MHLW, involving a total of 4,355 subjects with type 2 diabetes from 10 participating healthcare facilities with the aim of evaluating renal events (i.e., a decrease in eGFR to half the baseline level and/or the need for dialysis), cardiovascular events and all-cause mortality [10, 11]. Summarized below are the major findings of this study (for detailed information, please access the MHLW website http://www.mhlw.go.jp/ or refer to the literature cited above).

1. Renal and cardiovascular events and all-cause mortality were significantly increased in the subjects with micro- or macroalbuminuria compared to that observed in the subjects with normoalbuminuria.
2. In those with renal impairment (defined as a GFR less than 60 mL/min/1.73 m^2):
   a. The risk of renal events increased in association with the onset of microalbuminuria and further increased with the onset of macroalbuminuria in the subjects;
   b. The risk of cardiovascular events was increased in those with micro-/macroalbuminuria; and
   c. All-cause mortality was increased in the subjects with macroalbuminuria as well as those with normoalbuminuria and microalbuminuria who exhibited a GFR of less than 30 mL/min/1.73 m^2.

While that study was not a true prospective study and involved only a limited number of facilities and patients from a population known to be less prone to cardiovascular

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events than those in Western countries, the findings provide important insight into the prognosis of diabetic nephropathy in Japanese patients. Therefore, in seeking to revise the Classification, the Committee gave due consideration to the above findings. At the same time, the following considerations were also taken into account.

1. The bulk of evidence for the classification of diabetic nephropathy comes from randomized controlled studies enrolling patients with diabetic nephropathy as defined based on the extent of albuminuria, and very little evidence is available for diabetic nephropathy as defined based on GFR.
2. The current "Medical Service Fee Schedule for Guidance on Preventing Diabetes-Associated Dialysis" was developed with the Classification in mind.
3. The "Guidelines for Clinical Efficacy Evaluation of Pharmacological Agents for Diabetic Nephropathy (Draft)" currently in use were developed with the Classification in mind.

Therefore, after giving due consideration to all of these issues during the course of several sessions, the Committee decided to leave the Classification essentially unchanged for now (Table 1), while showing how it may be aligned with the widespread CKD classification based on GFR (eGFR) ("see Appendix"). The former is not, however, presented as a heat map, due to the limitations of the study referred to above, which involved a small number of patients with diabetic nephropathy and included no dialysis patients, providing the basis for this revision. Again, as all kidney diseases affecting patients with diabetes are covered in the Classification, the Committee called for attention with notes included which were required, in order to highlight the importance of the differential diagnosis between diabetic nephropathy and non-diabetic kidney disease in all stages. The differential diagnosis calls for collaboration with nephrologists; such collaboration is not limited to cases requiring a renal biopsy. Furthermore, given that the disease may not always progress in some patients, numerous notes were included in the table in order to call attention to these cases. Additionally, in view of the potential need to use multiple anti-diabetic drugs over time, "Key Precautions in View of Drug Use" are included below the table. The major revisions to the Classification are summarized below:

1. eGFR is now substituted for GFR in the Classification.

Table 1 Classification of Diabetic Nephropathy 2014

Stage	Urinary albumin (mg/g Cr) or urinary protein (g/g Cr)	GFR (eGFR) (mL/min/1.73 m^2)
Stage 1 (pre-nephropathy)	Normoalbuminuria (< 30)	≥30[a]
Stage 2 (incipient nephropathy)	Microalbuminuria (30–299)[b]	≥30
Stage 3 (overt nephropathy)	Macroalbuminuria (≥ 300) or Persistent proteinuria (≥ 0.5)	≥30[c]
Stage 4 (kidney failure)	Any albuminuria/proteinuria status[d]	<30
Stage 5 (dialysis therapy)	Any status on continued dialysis therapy	

Diabetic nephropathy does not always progress from one stage to the next. The revised classification takes into account findings on the prognosis of type 2 diabetic patients from a "historical cohort study" conducted as part of the MHLW-subsidized Project on Kidney Disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan" [10, 11]

[a] While a GFR of less than 60 mL/min/1.73 m^2 is consistent with the diagnosis of CKD, underlying causes other than diabetic nephropathy may be involved in patients with a GFR below 60 mL/min/1.73 m^2 thus calling for the differential diagnosis between diabetic nephropathy and any other potential non-diabetic kidney diseases

[b] Patients with microalbuminuria are to be diagnosed as incipient nephropathy after the differential diagnosis based on the criteria for an early diagnosis of diabetic nephropathy

[c] Precautions are required in patients with macroalbuminuria, in whom renal events (e.g., a decrease in eGFR to half its baseline value, the need for dialysis) have been shown to increase as the GFR decreases below 60 mL/min/1.73 m^2

[d] All patients with a GFR of less than 30 mL/min/1.73 m^2 are classified as exhibiting kidney failure, regardless of their urinary albumin/protein values. However, in those with normoalbuminuria and microalbuminuria, the differential diagnosis is required between diabetic nephropathy and any other potential non-diabetic kidney diseases

Key Precautions in View of Drug Use: This table is intended, first and foremost, as a classification of diabetic nephropathy and not as a guide to drug use. All drugs, including anti-diabetic drugs, particularly renally metabolized agents, are to be used in accordance with their prescribing information, with due consideration to relevant factors such as GFR in each patient

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2. The stages used in the Classification have been simplified to include normoalbuminuria, microalbuminuria, macroalbuminuria and kidney failure.
3. The division between A and B (early versus late macroalbuminuria) in stage 3 has been abandoned and A and B have been reintegrated, due to the paucity of evidence for proteinuria of 1 g/day as the threshold for dividing the stage.
4. Kidney failure has been redefined in all cases as a GFR less than 30 mL/min/1.73 m^2, which represents the threshold value for kidney failure obtained by quantifying the existing definition of kidney failure in the Classification based on the Classification of the Japanese Society of Nephrology (JSN) [12] with all other pre-kidney failure conditions redefined as a GFR of 30 mL/min/1.73 m^2 or greater.
5. Qualifying or illustrating phases in parentheses, such as "e.g., incipient nephropathy", have been retained throughout the Classification, as they have become common currency in the field, although their removal from the Classification was suggested during the process of revision.
6. Stress is now placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Of note, the American Diabetes Association (ADA) proposed in its Clinical Practice Recommendations 2013 that all cases of albuminuria of 30 μg/mg Cr (=mg/g Cr) be defined as "increased urinary albumin excretion", thus abandoning the division between micro- and macroalbuminuria [13]. Again, while this concept was retained in the Clinical Practice Recommendations 2014, the ADA further proposed that microalbuminuria and macroalbuminuria be redefined as persistent albuminuria of 30–299 mg/24 h and ≥300 mg/24 h, respectively [14]. While this change may result in the terms micro- and macroalbuminuria ceasing to be common currency in the clinical setting in the US, to avoid confusion, the Committee has chosen not to follow suit and rather err on the side of caution, thereby retaining these terms in the Classification, given that they are less likely to no longer be used in scientific publications and are expected to remain common currency in Japan.

Last but not least, with a number of multicenter prospective studies currently underway, including the Japan Diabetes Complication and Prevention prospective (JDCP) study, JSN registries, Japan Diabetes Clinical Data Management (JDDM) studies and Japan Diabetes Optimal Integrated Treatment for 3 Major Risk Factors of Cardiovascular Diseases (J-DOIT3) randomized study, the Committee also plans to further revise the Classification in a timely fashion as required, as relevant evidence becomes available from these and other studies.

Conclusions

In order to resolve the discrepancy between the existing Classification of Diabetic Nephropathy and the current Classification of CKD stages, the Joint Committee on Diabetic Nephropathy revised its Classification of Diabetic Nephropathy. The new classification has already been uploaded onto the website of each member society represented on the Joint Committee as of January 10, 2014. Again, in view of further revisions in the years to come, the Joint Committee has termed the revised classification as the "Classification of Diabetic Nephropathy 2014."

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Conflict of interest Masakazu Haneda has received speaker honoraria from pharmaceutical companies Boehringer Ingelheim GmbH, Mitsubishi Tanabe Pharma Corporation, Novo Nordisk Pharma Ltd., Daiichi-Sankyo Co., Ltd., Taisho Pharmaceutical Co., Ltd., Sanofi K.K., Merck Sharp & Dohme, Astellas Pharma Inc., Kyowa Hakko Kirin Co., Ltd., Kowa Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Novartis Pharma K.K., scholarship grants from Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., Merck Sharp & Dohme, Boehringer Ingelheim GmbH, and Eli Lilly and Company; Daisuke Koya has received speaker honoraria from pharmaceutical companies Mitsubishi Tanabe Pharma Corporation, Boehringer Ingelheim GmbH, and Eli Lilly and Company, research grants from Mitsubishi Tanabe Pharma Corporation, Boehringer Ingelheim GmbH, Japan Tabacco Inc., Eli Lilly and Company, and Ono Pharmaceutical Co., Ltd.; Tetsuya Babazono has received speaker honoraria from pharmaceutical company Merck Sharp & Dohme; Tatsumi Moriya has received travel expenses from pharmaceutical companies Astellas Pharma Inc., Takeda Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., and Daiichi-Sankyo Co., Ltd.; Hirofumi Makino has received speaker honoraria from pharmaceutical companies Teijin Pharma Limited, Chugai Pharmaceutical Co., Ltd., AbbVie GK, Astellas Pharma Inc., Boehringer Ingelheim GmbH, Daiichi-Sankyo Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Merck Sharp & Dohme, Novartis Pharma K.K., Pfizer Japan Inc., Takeda Pharmaceutical Co., and Mitsubishi Tanabe Pharma Corporation, research grants from Project for accelerating Practice and Research on Community Medicine in Okayama Prefecture, scholarship grants from Astellas Pharma Inc., Daiichi-Sankyo Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Merck Sharp & Dohme, Takeda Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., Novo Nordisk Pharma Ltd., and Mitsubishi Tanabe Pharma Corporation; Kenjiro Kimura has received research grants from pharmaceutical companies Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Teijin Pharma Limited, Boehringer Ingelheim GmbH, Baxter International Inc., and Sekisui Medical Co., Ltd.; Takashi Wada has received speaker honoraria from pharmaceutical company Daiichi-Sankyo Co., Ltd., scholarship grants from Chugai pharmaceutical Co., Ltd.; Susumu Ogawa has received speaker honoraria from pharmaceutical companies Daiichi-Sankyo Co., Ltd., Eli Lilly and Company, and Novo Nordisk

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Human rights statement and Informed consent This article does not contain any studies with human or animal subjects performed by the any of the authors.

Appendix

Relationship between the 2014 categories for diabetic nephropathy stages and the CKD severity categories

	Albuminuria category	A1	A2	A3
	Quantitative urinary albumin estimation Urinary albumin/Cr ratio (mg/g Cr) (quantitative urinary protein estimation) (urinary protein/Cr ratio (g/g Cr)	Normoalbuminuria < 30	Microalbuminuria 30-299	Macroalbuminuria ≥300 (or increased proteinuria) (≥0.50)
GFR category (mL/min/1.73 m^2)	≥90 60-89 45-59 30-44	Stage 1 (pre-nephropathy)	Stage 2 (incipient nephropathy)	Stage 3 (overt nephropathy)
	15-29 < 15	Stage 4 (kidney failure)		
	(dialysis therapy)	Stage 5 (dialysis therapy)		

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ORIGINAL ARTICLE

Impact of kidney function and urinary protein excretion on pulmonary function in Japanese patients with chronic kidney disease

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Abstract
Background Although the cardiorenal relationship in chronic kidney disease has been investigated, information about the lung–kidney relationship is limited. Here, we investigated the impact of kidney function and urinary protein excretion on pulmonary dysfunction.
Methods The data from pulmonary function tests and kidney function (estimated glomerular filtration rate [eGFR] and urinary protein) between 1 April 2005 and 30 June 2010 were selected from our laboratory database. Data were classified into 4 categories according to eGFR and proteinuria. Category 1, eGFR $\geq$60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 2, eGFR <60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 3, eGFR $\geq$60 ml/min/1.73 m^2 and urinary protein $\geq$0.3 g/gCr; and category 4, eGFR <60 ml/min/1.73 m^2 and urinary protein $\geq$0.3 g/gCr. Pulmonary function data were evaluated according to these 4 categories.
Results A total of 133 participants without major respiratory disease, abnormal computed tomography and smoking history were enrolled. Hemoglobin (Hb)-adjusted percentage carbon monoxide diffusing capacity (%DL_{CO}) in category 4 (46.2 ± 7.5) and category 2 (63.6 ± 17.8) were significantly lower than in category 1 (75.8 ± 18.9) ($P < 0.05$). In addition, Hb-adjusted %DL_{CO} was weakly correlated with eGFR in participants with urinary protein <0.3 g/gCr ($R = 0.30$, $P = 0.001$). Hb-adjusted %DL_{CO} was strongly correlated with eGFR in participants with urinary protein $\geq$0.3 g/gCr ($R = 0.81$, $P < 0.001$). Other pulmonary function test markers (percentage (%) vital capacity, % forced expiratory volume in one second (FEV1), FEV1/forced vital capacity, % total lung capacity, and % residual volume) were not significantly different between categories.
Conclusion This study suggests that decreased eGFR is associated with decreased %DL_{CO} in proteinuric patients.

Keywords Kidney function · Pulmonary function tests · Chronic kidney disease · Urinary protein · DL_{CO} (carbon monoxide diffusing capacity) · Diabetic nephropathy

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Background

The kidneys maintain whole-body homeostasis through regulation of body fluid, blood pressure, electrolytes and acid–base balance. Thus, the kidneys work in cooperation with the heart, liver, lungs and other organs. Various mechanisms participate in the organ-to-organ networks,

including humoral factors, and neuronal network systems. Although several reports indicate a relationship between the kidneys and the heart [1, 2], limited information is available regarding crosstalk between the kidneys and lungs in chronic kidney disease (CKD).

In contrast to CKD, acute kidney injury (AKI) often leads to acute lung injury. These associations between the kidneys and lungs [3–6] result in deterioration of the general condition of patients in critical care. Recent animal studies indicate that AKI increases inflammatory cytokine levels in the injured kidney and serum, and these cytokines may induce lung injury [7–9]. However, inflammatory cytokine production is also detected in the injured kidneys in CKD as well as in AKI [10]. Therefore, it is reasonable to speculate that CKD may affect pulmonary function.

The present study was performed to examine the associations between parameters of CKD (estimated glomeular filtration rate [eGFR] and urinary protein) and the results of pulmonary function tests (PFTs).

Method

Subjects

The data from PFTs and kidney function parameters (eGFR and urinary protein) were selected from our laboratory database between 1 April 2005 and 30 June 2010. PFTs were evaluated by international criteria [11]. Vital capacity (VC), forced vital capacity (FVC), functional residual capacity (FRC), and carbon monoxide diffusing capacity (DL_{CO}) were measured using the PFT system CHESTAC-9800 (Chest M.I., Inc., Tokyo, Japan). Participants were coached regarding standard forced expiratory manoeuvres. Three technically acceptable blows were recorded and the best values were used as their data.

We selected participants with normal spirometry (both %VC >80 % and forced expiratory volume in one second (FEV1)/FVC >70 %) to evaluate the association between renal function and pulmonary function. Participants with abnormal spirometry, major respiratory disease (asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, interstitial pneumonia, neuromuscular disease, scleroderma, pulmonary resection, and pneumonedema) and abnormal computed tomography (CT) were excluded. Moreover, participants with a history of smoking were excluded to remove the influence of smoking on kidney [12] and lung fucntion [13]. PFT markers (%VC, %FEV1, % residual volume [RV], % total lung capacity [TLC], and $\%DL_{CO}$) were adjusted for age, height, and sex using prediction formula.

This study was approved by the ethics committee of Kanazawa University Hospital (Approval No. 907) and was conducted in accordance with the Declaration of Helsinki.

Method of DLco

We measured DLco in accordance with the recommendations [14]. In short, once the mouthpiece is in place, four to five tidal volumes are recorded to determine a regular end-expiratory baseline. The DLco manoeuvre then begins with exhalation to RV. At RV the subject's mouthpiece is connected to a source of test gas and the subject inhales rapidly to TLC. The volume of test gas inhaled is V_I. DL_{CO} should be measured near TLC.

Participants' lung and kidney data function

eGFR (ml/min/1.73 m^2) was calculated using the prediction formula $194 \times \text{creatinine (Cr)}^{-1.094} \times \text{age (year)}^{-0.287}$ (multiplied by 0.739 for females) developed by the Japanese Society of Nephrology. Urinary protein (g/gCr) was evaluated by spot urine protein–creatinine ratio.

Pulmonary function was evaluated using PFT markers of restrictive ventilatory impairment (%VC, %RV, and %TLC), PFT markers of obstructive ventilatory impairment (%FEV1 and FEV1/FVC), and PFT markers of pulmonary diffusing capacity ($\%DL_{CO}$). To avoid the effects of physical features (age, sex and height), we used the predicted value of all PFT markers. Because predicted value is corrected by physical features, PFT markers are not influenced by physical features [13]. Furthermore, to avoid the effects of renal anaemia on CKD, $\%DL_{CO}$ was corrected by haemoglobin (Hb-adjusted $\%DL_{CO}$). The compensation formula of DL_{CO} for males is $DL_{CO} \times (10.22 + \text{haemoglobin})/(1.7 \times \text{haemoglobin})$ [14, 15]. For females, it is $DL_{CO} \times (9.38 + \text{haemoglobin})/(1.7 \times \text{haemoglobin})$.

Participants were classified into 4 categories according to eGFR and urinary protein—category 1, eGFR ≥60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr, category 2, eGFR <60 ml/min/1.73 m^2 and urinary protein <0.3 g/gCr; category 3, eGFR ≥60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr; and category 4, eGFR <60 ml/min/1.73 m^2 and urinary protein ≥0.3 g/gCr. Participants with CKD had an eGFR <60 ml/min/1.73 m^2 or/and U-PCR >0.3 g/gCr for at least 3 months. Pulmonary function was compared using these 4 categories.

Statistics

One-way analysis of variance (ANOVA) and Dunnett's post hoc test were used to compare PFT markers in category 1 to those in other categories. The chi-squared test was used for categorical parameters. Spearman's rank correlation test and multiple linear regression analysis were used to examine relationships between PFTs (Hb-adjusted $\%DL_{CO}$) and renal function (eGFR and urinary protein).