Table 4. Comparison of various parameters in albuminuria stages of chronic kidney disease in type 2 diabetes patients (n = 85).

	A1	A2	A3	Total	Kruskal-Wallis
Number (male/female)	36 (19/17)	25 (15/10)	24 (15/9)	85 (49/36)	
Age (years)	63.8±11.3	61.0±12.5	63.3±12.3	62.9±11.3	0.006*
BMI (kg/m^2)	24.8±5.1	25.7±4.5	24.2±3.9	24.9±4.6	0.543
SBP (mmHg)	124.0±12.6	129.5±20.5	126.0±19.7	126.2±17.3	0.484
DBP (mmHg)	73.9±10.3	72.6±8.1	69.1±14.4	72.2±11.1	0.261
HbA1c (%)	7.31±0.64	7.24±0.90	7.38±1.17	7.31±0.87	0.850
Total protein (g/L)	70.4±4.3	70.7±4.8	66.1±6.5	69.3±5.4	0.003*
Albumin (g/L)	42.9±2.5	41.2±3.2	35.7±7.0	40.4±5.3	1.80×10^{-16}**
Cr (μmol/L)	66.4±13.3	78.3±26.3	144.2±70.3	91.9±52.3	4.86×10^{-10}**
UN (μmol/L)	5.5±1.5	7.1±2.7	10.0±3.8	7.3±3.3	5.92×10^{-8}**
Uric acid (μmol/L)	305.8±61.5	352.8±96.2	396.2±68.0	344.6±83.1	9.68×10^{-5}**
T-Cho (mmol/L)	5.09±0.94	4.86±0.84	5.06±1.14	4.99±0.97	0.689
TG (mmol/L)	1.65±0.92	1.70±1.10	2.16±1.74	1.81±1.26	0.780
HDL-C (mmol/L)	1.49±0.41	1.35±0.31	1.23±0.39	1.38±0.39	0.031*
LDL-C (mmol/L)	2.85±0.81	2.70±0.65	2.80±0.95	2.79±0.80	0.271
eGFR (mL/min)	74.5±16.3	67.9±19.2	42.4±19.0	63.5±22.4	6.66×10^{-9}**
ACR (mg/gCr)	12.7±6.0	114.3±72.6	1424±996	441.2±812	1.81×10^{-16}**
Fetuin-A (ng/gCr)	0.40±0.43	0.60±0.53	1.57±1.13	0.79±0.87	7.29×10^{-8}**
α1-microglobulin (μg/gCr)	4.24±4.03	6.30±5.12	17.83±18.08	8.68±11.74	8.84×10^{-9}**
Orosomucoid (ng/gCr)	17.5±9.1	17.9±8.7	91.4±87.2	38.5±57.0	3.34×10^{-8}**

BMI, body mass index; SBP, Systolic Blood Pressure; DPB, Diastolic Blood Pressure; Cr, serum creatinine; UN, serum urea nitrogen; T-Cho, Total cholesterol; TG, Triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; eGFR, estimated glomerular filtration ratio; ACR, albumin/creatinine ratio;
*$p<0.05$;
**$p<0.01$.
doi:10.1371/journal.pone.0077118.t004

peptides were extracted once in 1% formic acid and subsequently twice in 5% formic acid and in 50% acetonitrite. Peptides were separated by nanoUPLC (nanoACQUITY UPLC, Waters, Milford, MA) and analyzed with Q-Tof micro (Waters). nanoUPLC was equipped with 5.0 μm Symmetry C18, 180 μmID×2 cm precolumn and 1.7 μm BEH 130 C18, 100 μmID×10 cm column. Mobile phase A was water with 0.1% formic acid whilst mobile phase B was 0.1% formic acid in acetonitrile. Using MassLynx 4.1 (Waters) the MS/MS raw data were transformed into peak lists (.pklfiles) and they were searched thorough NCBInr and Swiss-Prot by using Mascot (Matrix Science, Boston, MA).

Blood Sampling and Assays

We measured overnight fasting serum levels of total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides (L Type Wako Triglyceride H, Wako Chemical, Osaka, Japan), uric acid, creatinine (Cr), and urea nitrogen (UN). We also measured plasma glucose and HbA1c. Urinary albumin was measured in random spot urine samples by standard immuno-nephelometric assay. The urinary albumin-creatinine ratio (ACR) was calculated. Estimated glomerular filtration rate (eGFR) was calculated by equation; eGFR (ml/min/1.73 m^2) = $194\times Cr^{-1.094}\times age^{-0.287}$ in male and eGFR (ml/min/1.73 m^2) = $194\times Cr^{-1.094}\times age^{-0.287}\times 0.739$ in female [14]. By using the definition and classification of chronic kidney disease [Kidney Disease: Improving Global Outcomes (KDIGO)] [2], all patients were classified into albuminuria and GFR category. In albuminuria stages, the patients were classified into three groups; A1 (<30 mg/gCr), A2 (30–299 mg/gCr) and A3 (≥300 mg/gCr). In GFR stages, they were classified into 4 groups; G1 (>90 ml/min/1.73 m^2), G2 (60–89 ml/min/1.73 m^2), G3 (30–59 ml/min/1.73 m^2), and G4 (15–29 ml/min/1.73 m^2). Urinary excretions of fetuin-A, α1-microglobulin, and orosomucoid were measured with ELISA kit for Human Fetuin-A (BioVender, Modrice, Czech Republic), LZ Test Eiken α1-M (Eiken Chemical Co., Tokyo, Japan), and N Antiserum to Human α1-acid Glycoprotein (Siemens Healthcare Diagnostics Inc., Marburg, Germany).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) values in tables. Urinary levels of fetuin-A, α1-microglobulin, and orosomucoid demonstrated non-normal distribution and medians with interquartile range were indicated in box plot in Figures. Spearman correlation coefficients were used to evaluate whether urinary levels of fetuin-A, α1-microglobulin, and orosomucoid correlated with various parameters. To determine the variables independently associated with urinary levels of fetuin-A, α1-microglobulin, and orosomucoid in the patients with type 2 diabetes, multiple regression analysis was performed by including estimated glomerular filtration rate (eGFR), albumin/creatinine ratio and HDL cholesterol (HDL-C) as independent variables. Urinary levels of fetuin-A, α1-microglobulin, orosomucoid and various clinical parameters in albuminuria and GFR stages were compared by Kruskal-Wallis test. Multivariate logistic regression analysis to access the urinary fetuin-A, α1-microglobulin, orosomucoid excretions as a risk for diabetic nephropathy with microalbuminuria or with GFR<60 mL/min. *P* values less than

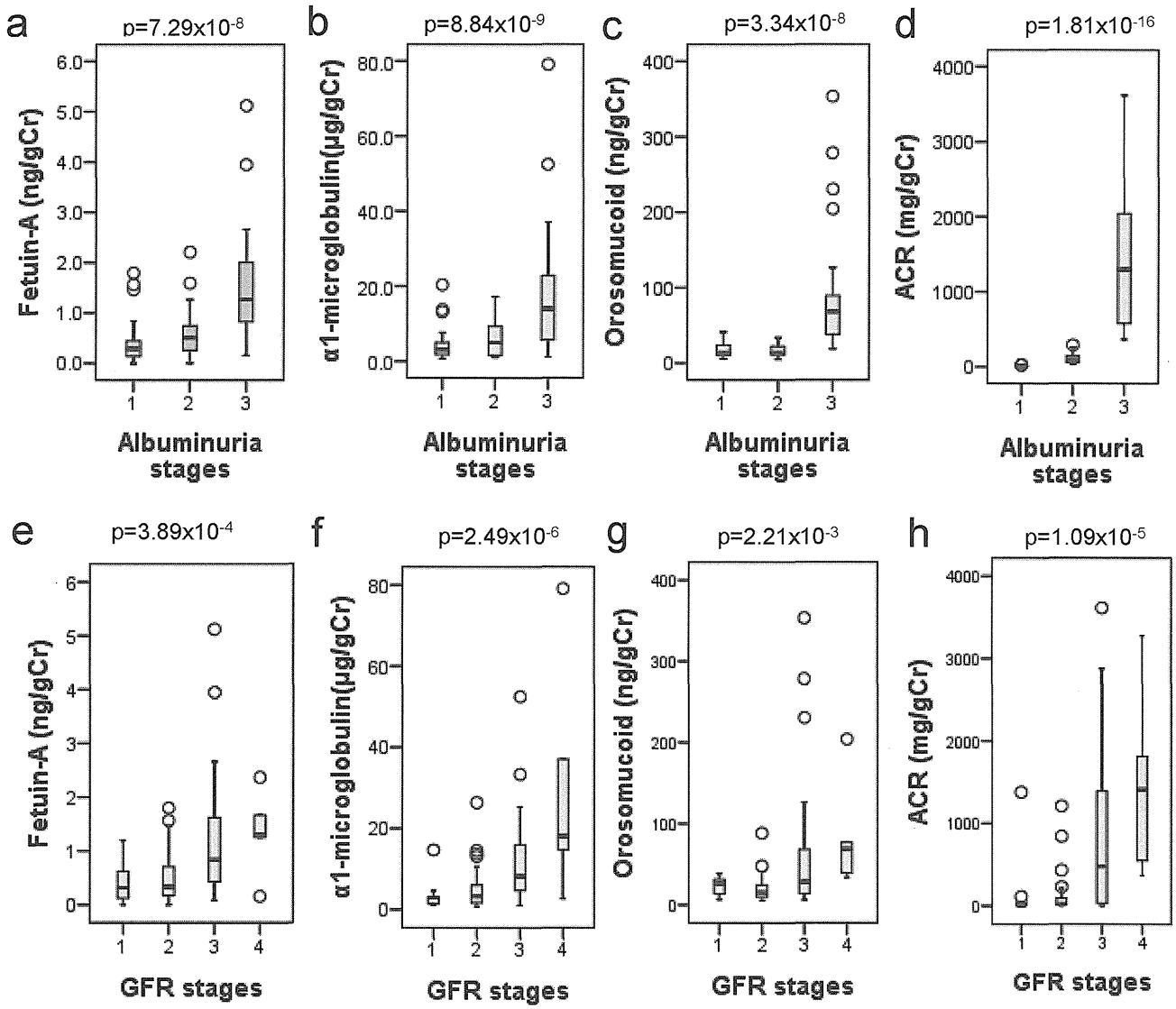

Figure 3. Urinary excretion of fetuin-A, α1-microglobulin, orosomucoid and albumin creatinine ratio (ACR) in various stages of diabetic nephropathy (n = 85). All of the urinary excretion of sialylated glycoprpteins such as fetuin-A, α1-microglobulin, and orosomucoid are compared by Kruskal-Wallis test.
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0.05 were considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics Base and IBM SPSS Regression (IBM, Armonk, NY).

Results

Lectin Microarray Analyses Demonstrated the Increased Binding Activity to Siaα2-6-Gal/GalNAc

We performed lectin microarray analyses and compared the urine samples of the healthy subjects without type 2 diabetes (n = 12) and the patients with type 2 diabetes with various stages of normoalubuminuria (n = 7), microalbuminuria (n = 5) and macroalbuminuria (n = 5). The reactivity to the many lectins, such as fucose binder (PSA, LCA, AOL, and AAL), Lac/LacNAc binder [PHA(L), ECA, RCA120, PHA(E)], α- or β-Gal binder (BPL, ABA, PNA, ACA), chitobiose binder (DSA, LEL, STL, UDA, PWM, WGA), and α- or β-GalNAc binder (Jacalin, WFA, MPA, VVA, DBA, SBA, PTL-I, GSL-IA4), significantly declined at the stage of macroalbuminuria (**Figure 1**). Among them, lectins which bind to N-glycosylation, RCA120, PHA(E), DSA, demonstrated the increased binding activity at the stage of microalbuminuria. Notably, in contrast to majority of the lectins, the binding to Siaα2-6-Gal/GalNAc (SNA, SSA, TJA-1) progressively increased in the albuminuria stages of diabetic nephropathy (**Figure 1, red box**). Since we identified specific increase in the biding activity to Siaα2-6-Gal/GalNAc in urine samples in the patients with diabetic nephropathy, we next screened the sialylated glycoproteins in the urine samples of diabetic nephropathy.

Fetuin-A, α1-microglobulin and Orosomuioid were Identified by SSA-Agarose Column Chromatography and LC/MS-MS Analyses

SNA- and SSA-agarose were commercially available and we could isolate the glycoproteins by SSA-agarose in preliminary experiments. Thus, we performed SSA-Agarose column chromatography and the effluents were subjected to SDS-PAGE in

Table 5. Comparison of various parameters in glomerular filtration stages of chronic kidney disease in type 2 diabetes patients (n = 85).

	G1	G2	G3	G4	Total	Kruskal-Wallis
Number (male/female)	9 (6/3)	42 (22/20)	29 (19/10)	5 (2/3)	85 (49/36)	
Age (years)	51.9±13.9	62.9±10.3	66.5±9.2	59.6±15.3	62.9±11.3	0.647
BMI (kg/m^2)	27.6±8.1	25.1±4.6	24.1±3.1	22.6±2.2	24.9±4.6	0.155
SBP (mmHg)	129.7±13.8	127.6±17.3	124.0±19.1	120.2±11.1	126.2±17.3	0.640
DBP (mmHg)	76.5±13.8	74.6±8.0	69.5±12.4	59.4±9.2	72.2±11.1	0.006
HbA1c (%)	7.54±0.79	7.27±0.83	7.40±0.94	6.68±0.82	7.31±0.87	0.323
Total protein (g/L)	70.4±3.7	70.9±4.4	67.6±6.2	63.0±5.0	69.3±5.44	0.002*
Albumin (g/L)	41.4±4.6	42.3±2.9	38.3±6.6	33.4±5.0	40.4±5.3	1.10×10^{-4}**
Cr (μmol/L)	60.9±16.0	65.9±11.6	115.7±40.7	227.1±88.2	91.9±52.3	1.89×10^{-17}**
UN (μmol/L)	5.5±2.2	5.8±1.6	8.6±2.7	14.6±5.1	7.3±3.3	9.85×10^{-12}**
Uric acid (μmol/L)	329.1±39.5	312.8±74.4	388.7±82.6	391.4±99.8	344.6±83.1	6.59×10^{-4}**
T-Cho (mmol/L)	5.11±0.96	4.97±0.89	5.06±1.02	4.52±1.39	4.99±0.97	0.695
TG (mmol/L)	2.04±1.30	1.68±1.06	1.95±1.57	1.58±0.63	1.81±1.26	0.487
HDL-C (mmol/L)	1.25±0.26	1.44±0.37	1.32±0.41	1.45±0.51	1.38±0.39	0.427
LDL-C (mmol/L)	2.96±0.95	2.79±0.67	2.83±0.92	2.28±0.87	2.79±0.80	0.740
eGFR (mL/min)	96.2±15.6	74.8±8.1	44.4±9.2	20.6±8.3	63.5±22.4	1.16×10^{-30}**
ACR (mg/gCr)	179.7±451.6	108.3±227.7	824.5±0.80	1484±1168	441.2±812	1.09×10^{-5}**
Fetuin-A (ng/gCr)	0.39±0.39	0.49±0.45	1.25±1.18	1.34±0.80	0.79±0.87	3.89×10^{-4}**
α1-microglobulin (μg/gCr)	3.74±4.26	4.94±4.92	11.90±11.04	30.32±29.93	8.68±11.74	2.49×10^{-6}**
Orosomucoid (ng/gCr)	22.5±11.4	19.7±14.8	62.7±84.3	84.4±69.4	38.5±57.0	2.21×10^{-3}**

BMI, body mass index; SBP, Systolic Blood Pressure; DPB, Diastolic Blood Pressure; Cr, serum creatinine; UN, serum urea nitrogen; T-Cho, Total cholesterol; TG, Triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; eGFR, estimated glomerular filtration ratio; ACR, albumin/creatinine ratio;
*$p<0.05$;
**$p<0.01$.
doi:10.1371/journal.pone.0077118.t005

Figure 2. We confirmed that the bands visualized with Coomassie Brilliant Blue staining increased in the patients with CKD stage of A3G4 compared with the patients A3G3. The effluents were subjected to LC/MS-MS and raw data of the proteins hit by Mascot program searching through NCBInr and Swiss-Prot database were indicated in **Tables 2 and 3** in the patients with CKD stages of A3G3 and A3G4, respectively. The listed proteins demonstrated the serum major proteins such as albumin, immunoglobulins, complements, α1-antitrypsin, transferrin, and haptoglobin. However, we identified three sialylated glycoprrpteins such as α1-microglobulin (Protein AMBP), α1-acid glycoprotein (orosomucoid) and fetuin-A (α2-HS-glycorptein). Fetuin-A [15], α1-microglobulin [16], and orosomucoid [17] have been reported as sialylated glycoproteins and we further validated the significance of urinary excretion of sialylated glycoproteins as biomarkers for diabetic nephropathy.

Elevated Urinary Fetuin-A Excretion is a Risk for the Development of Diabetic Nephropathy

We investigated urinary excretion of sialylated glycoproteins in various stages of diabetic nephropathy (n = 85). In albuminuria stages, age, serum total protein, serum albumin, Cr, UN, uric acid, HDL-C, eGFR, ACR were significantly changed revealed by Kruskal-Wallis test (**Table 4**). All of the urinary excretion of sialylated glycoprrpteins such as fetuin-A, α1-microglobulin, and orosomucoid significantly increased during the progression of A1 to A3 stages (**Table 4 and Figure 3a–d**). During the progression of GFR stages, serum total protein, serum albumin, Cr, UN, uric acid, eGFR, and ACR were significantly altered by Kruskal-Wallis test (**Table 5**). Like albuminuria stages, the urinary excretion of fetuin-A, α1-microglobulin, and orosomucoid significantly increased in the GFR stages from G1 to G4 revealed by Kruskal-Wallis test (**Table 5 and Figure 3e–h**).

All of the urinary excretion of fetuin-A, α1-microglobulin, and orosomucoid positively correlated with Cr, UN and ACR and negatively correlated with serum albumin, HDL-C and eGFR with statistically significant differences (**Table 6 and Figure 4**). The linear regression analyses were followed by a multiple regression analysis using the urinary excretion of fetuin-A, α1-microglobulin, and orosomucoid as the dependent variables to further analyze the significant predictors (**Table 6**). eGFR, ACR and HDL-C were used as independent variables. eGFR and ACR independently and significantly predicted urinary excretion of fetuin-A and α1-microglobulin. For urinary excretion of orosomucoid, ACR and HDL-C were significantly determinants in multiple regression models in **Table 7**. Finally, multivariate logistic regression analysis was employed to assess three urinary sialylated glycoproteins as a risk for diabetic nephropathy with microalbuminuria or GFR<60 mL/min. We used the forward stepwise method and the variable whose addition causes the largest statistically significant change in −2 Log Likelihood is added to the model. The final models are indicated in **Tables 8** and only fetuin-A was demonstrated as a risk factor for both microalbuminuria and reduction of GFR in diabetic nephropathy with the odds ratio (95% confidence intervals) of 4.721 (1.881–11.844) and 3.739 (1.785–7.841), respectively.

Table 6. Simple correlation of urinary sialylated glycoprotein excretions with various clinical parameters in the patients with type 2 diabetes (n = 85).

	Fetuin-A (ng/gCr)	α1-microglobulin (μg/gCr)	Orosomucoid (ng/gCr)
Age (years)	R = 0.009, p = 0.937	R = 0.123, p = 0.261	R = −0.008, p = 0.944
BMI (kg/m^2)	R = −0.139, p = 0.205	R = −0.067, p = 0.541	R = −0.032, p = 0.770
SBP (mmHg)	R = 0.043, p = 0.693	R = −0.005, p = 0.964	R = 0.103, p = 0.348
DBP (mmHg)	R = −0.145, p = 0.186	R = −0.214, p = 0.049*	R = −0.027, p = 0.807
HbA1c (%)	R = 0.113, p = 0.307	R = 0.110, p = 0.318	R = 0.056, p = 0.612
Total protein (g/L)	R = −0.261, p = 0.017*	R = −0.275, p = 0.012*	R = −0.213, p = 0.053
Albumin (g/L)	R = −0.377, p = 4.36×10^{-4}**	R = −0.376, p = 4.67×10^{-4}**	R = −0.394, p = 2.28×10^{-4}**
Cr (μmol/L)	R = 0.368, p = 5.23×10^{-4}**	R = 0.388, p = 2.40×10^{-4}**	R = 0.399, p = 1.53×10^{-4}**
UN (μmol/L)	R = 0.405, p = 1.31×10^{-4}**	R = 0.439, p = 2.96×10^{-5}**	R = 0.363, p = 6.85×10^{-4}**
Uric acid (μmol/L)	R = 0.079, p = 0.474	R = 0.073, p = 0.509	R = 0.295, p = 0.006**
T-Cho (mmol/L)	R = −0.099, p = 0.372	R = −0.080, p = 0.471	R = 0.062, p = 0.576
TG (mmol/L)	R = 0.060, p = 0.582	R = 0.055, p = 0.615	R = 0.186, p = 0.088
HDL-C (mmol/L)	R = −0.313, p = 0.004**	R = −0.258, p = 0.017*	R = −0.244, p = 0.025*
LDL-C (mmol/L)	R = −0.007, p = 0.948	R = −0.043, p = 0.697	R = 0.067, p = 0.544
eGFR (mL/min)	R = −0.395, p = 1.80×10^{-4}**	R = −0.472, p = 5.23×10^{-6}**	R = −0.431, p = 3.90×10^{-5}**
ACR (mg/gCr)	R = 0.548, p = 5.76×10^{-8}**	R = 0.466, p = 7.02×10^{-6}**	R = 0.652, p = 1.40×10^{-11}**

BMI, body mass index; SBP, Systolic Blood Pressure; DPB, Diastolic Blood Pressure; Cr, serum creatinine; UN, serum urea nitrogen; T-Cho, Total cholesterol; TG, Triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; eGFR, estimated glomerular filtration ratio; ACR, albumin/creatinine ratio;
*p<0.05;
**p<0.01.
doi:10.1371/journal.pone.0077118.t006

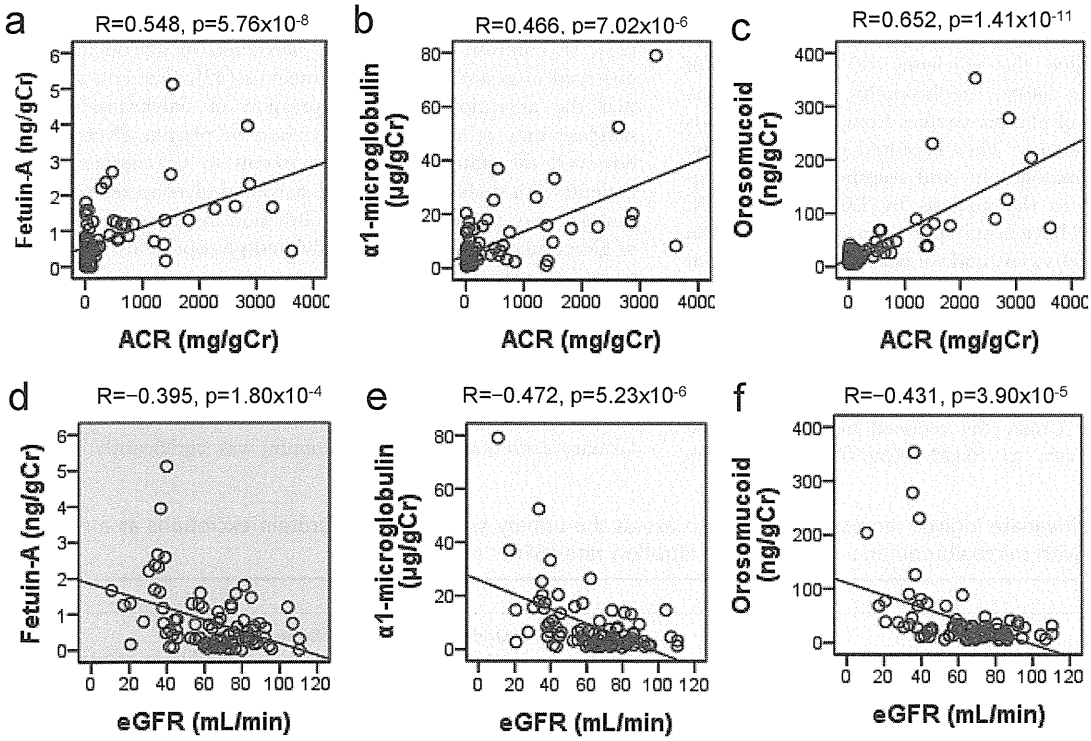

Figure 4. Simple correlation of urinary excretion of fetuin-A, α1-microglobulin, orosomucoid with estimated glomerular filtration ratio (eGFR) and urinary albumin creatinine ratio (ACR) in the patients with diabetic nephropathy (n = 85). Spearman correlation coefficients are used to evaluate whether urinary levels of fetuin-A, α1-microglobulin, and orosomucoid correlate with eGFR and ACR.
doi:10.1371/journal.pone.0077118.g004

Table 7. Multiple linear regression analysis using urinary sialylated glycoprotein excretions as dependent variables in the patients with type 2 diabetes (n = 85).

Dependent variable	Independent variable	Unstandardized coefficient		Standardized coefficient	t value	P value	Adjusted R^2
		B	Standard Error	Beta			
Fetuin-A	eGFR (mL/min)	−0.076	0.042	−0.196	−1.813	0.074	0.335
(ng/gCr)	ACR (mg/gCr)	0.004	0.001	0.395	3.645	4.71×10^{-4}**	
	HDL-C (mmol/L)	−4.048	2.035	−0.182	−1.989	0.050	
α1-microglobulin	eGFR (mL/min)	−0.138	0.053	−0.263	−2.617	0.011*	0.423
(μg/gCr)	ACR (mg/gCr)	0.007	0.001	0.461	4.560	4.71×10^{-5}	
	HDL-C (mmol/L)	−1.443	2.548	−0.048	−0.566	0.573	
Orosomucoid	eGFR (mL/min)	−0.136	0.212	−0.053	−0.642	0.523	0.605
(ng/gCr)	ACR (mg/gCr)	0.049	0.006	0.703	8.405	1.19×10^{-12}**	
	HDL-C (mmol/L)	−26.65	10.240	−0.183	−2.603	0.011	

Estimated glomerular filtration rate (eGFR), albumin/creatinine ratio and HDL cholesterol (HDL-C) are used as independent variables in stepwise multiple linear regression analysis in model 1. In model 2, all parameters are included in the analysis.
*p<0.05;
**p<0.01.
doi:10.1371/journal.pone.0077118.t007

Discussion

Glycans have important roles in living organisms with their structural diversity; however, glycan profiling studies have not been extensively performed because it is technically challenging. Recently, the genome-wide association study identified hepatocyte nuclear factor 1-α (HNF1A) as a key regulator of fucosylation and the DG9-glycan index, which is the ratio of fucosylated to nonfucosylated triantennary glycans, display altered fucosylation of N-linked glycans on plasma proteins. Thus, the glycan biomarkers could improve the efficiency of a diagnosis of HNF1A-MODY [18]. In diabetic nephropathy, Ahn J.M. *et al.* performed glycan profile of plasma samples from normal subjects and the patients with diabetes. They captured glycoproteins by multi-lectin affinity chromatography and trypsin-digested glycoproteins were subjected to the analysis by LC-MS/MS [19]. However, no other studies have been reported to survey the glycan profile of the urine samples so far, and we believe that the current investigation is the first study to perform glycan profiling of urines samples from the patients with diabetic nephropathy. As a result, we have found that global reduction of the bindings to lectins, such as fucose, Lac/LacNA, α- or β-Gal, chitobiose, and α- or β-GalNAc binders in urine samples of diabetic nephropathy at macroalbuminuria stage. Unlike the reduced bindings to these lectins, the biding activity to Siaα2-6-Gal/GalNAc binders progressively increased at micro- and macroalbuminruia stages. In the patients with type 1 diabetes, the reduction of sialidase activities was observed in mononuclear leucocytes and they speculated that diabetes-associated changes in sialylation of functional cell surface glycolconjugates may have important clinical consequences in diabetes [20]. The analysis of sialylation of insulin-like growth factor-binding protein (IGFBP)-3 from the poorly controlled patients with type 2 diabetes, increased binding of IGFBP-3 to SNA suggesting increased sialylation of IGFBP3 [21]. In contrast, reduced α2-6 sialylation of glycodelin-A was observed in gestational diabetes mellitus [22]. One can speculate that the alterations in the expression of sialyltransferases or sialidase may influence the sialylation of plasma glycoproteins; however, the status of sialylation seems to be complex in the patients with diabetes. Increased sialylated glycoproteins in urine samples may also be due to the alteration in the permselectivities of glomerular capillary, since sialylated glycoproteins are characterized by negative charge.

α1-microglobulin, also known as protein HC (for Heterogeneous Charge), was initially suggested as a marker for the detection of proximal tubular dysfunction by cadmium poisoning [23]. α1-microglobulin is a small protein with up to 31 kDa and it is filtered through glomeruli and reabsorbed by the proximal tubules [24]. Urinary excretion of α1-microglobulin was significantly higher in

Table 8. Stepwise multivariate logistic regression analysis to assess the urinary sialylated glycoprotein excretions as a risk for diabetic nephropathy with microalbuminuria or glomerular filtration rate (GFR)<60 ml/min.

Risk factor for microalbuminuria	B	Standard error	p	Odds ratio (95% confident intervals)	Predictive accuracy
Fetuin-A (ng/gCr) (1SD increments)	1.784	0.539	9.424×10^{-4}**	4.721 (1.881–11.844)	74.1%
Risk factor for GFR<60 mL/min	B	Standard error	p	Odds ratio (95% confident intervals)	Predictive accuracy
Fetuin-A (ng/gCr) (1SD increments)	1.516	0.434	4.755×10^{-4}**	3.739 (1.785–7.841)	72.9%

**p<0.01.
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the early stages of the disease process, while albumin excretion was still in the normal range [25]. Thus, it may serves as early marker for tubular damages in diabetic nephropathy and may precede albumin excretion to the urine [25–27]. Low molecular weight markers of tubular dysfunction such as α1-microglobulin, therefore, appear as markers of renal dysfunction that may complement markers of glomerular dysfunction such as albumin [7]. Orosomucoid, α-1 acid glycoprotein with 41 kDa, is an acute-phase protein and the serum concentrations correlated with low-grade inflammation in the patients with diabetes [28,29]. Urine excretion of orosomucoid was increased in the patients with diabetes and normoalbuminuria and it correlated with markers of inflammation such as CRP [29–31] and markers for endothelial dysfunction [32]. Type 2 diabetic patients with elevated urinary orosomucoid excretion exhibited normal glomerular and tubular function, suggesting the possibility of local renal production of orosomucoid due to chronic low-grade inflammation rather than hyperfiltration [33].

Fetuin-A has been principally studied as an inhibitor for ectopic calcium deposition in the renal field and it is also an important promoter for insulin resistance. Fetuin-A, a liver secretory glycoprotein with 64 kDa, has been shown that it acts as carrier of free fatty acids (FFAs) and they are the intrinsic ligands for Toll-like receptor 4 (TLR4), which induces adipose tissue inflammation and insulin resistance [34]. Fetuin-A binds to the residues of Leu100-Gly123 and Thr493-Thr516 of TLR4 through the terminal galactoside moiety [34]. Thus, FFA-Fetuin-A induced TLR4 activation is very important in the lipid-induced inflammation and insulin resistance and type 2 diabetes. In addition, fetuin-A and adiponectin mediate the crosstalk between adipose tissues, liver and kidney. Fetuin-A suppresses mRNA expression of adiponectin in cultured human adipocytes and treatment of wild-type mice with fetuin-A lowered serum adiponectin levels [35]. Higher fetuin-A and lower adiponectin levels may contribute the development of insulin resistance, diabetes and subsequent obesity-related CKD and diabetic nephropathy [36]. Serum concentration of fetuin-A in type 2 diabetes patients has been reported and they positively correlated with macrovascular late complications in high-risk type 2 diabetes patients, while no association with metabolic status or microvascular complications [37]. Recent study indicated that serum fetuin-A is lower in microalbuminuria patients compared with normo- and mascroalbuminuric patients [38]. In other studies, lower serum levels of fetuin-A are associated with peripheral arterial disease in patients with type 2 diabetes [39] and serum fetuin-A levels are negatively associated with atherosclerotic calcified plaques [40]. Thus, the significance of serum fetuin-A levels is controversial whether it is a good marker for diabetic micro- and macrovascular complications. Unfortunately, we failed to detect changes in binding activities to various lectins in the sera from the patients with various stages of diabetic nephropathy (data not shown), we did not get a chance to measure the serum levels of fetuin-A. However, we demonstrated that the urinary excretion of fetuin-A is a candidate for the biomarker to predict the progression of diabetic nephropathy. Although two previous published studies identified fetuin-A in urines samples of the patients with diabetic nephropathy, the quantifications were limited to inaccurate estimations by fluorescence 2-D differential in-gel electrophoresis [41] and capillary electrophoresis coupled to mass spectrometry [42]. In contrast to previous studies, we firstly used stable ELISA kit to quantify the urinary excretion of fetuin-A. Urinary concentration of fetuin-A may be depending on the production from the liver, alterations in permeability through glomerular basement membrane by capillary damages and changes in tubular reabsorption. Higher excretion of fetuin-A into urine has been reported to reflect the insulin resistance and inflammatory responses in obesity and type 2 diabetes [43] and it may reflect the increase in the serum levels of fetuin-A and alterations in the changes in the permeability of glomerular capillaries. Fetuin-A is reported to pass through the slit diaphragm and re-introduced to proximal tubular cells by megalin-mediated endocytosis [44]. Zhou et al. also reported that urinary exosomal fetuin-A was increased in the rats treated with cisplatin injection and in the ICU patients with acute kidney injury [45]. Thus, an alternative explanation for increased urinary fetuin-A excretion in diabetic nephropathy could be due to the tubular injury. In our study, multivariate regression analysis indicated that higher urinary fetuin-A excretion demonstrated a higher risk for the development of microalbuminuria and reduction of renal function and future cohort study is required to further confirm this notion.

Conclusions

In summary, the glycan profiling studies using urine samples from the patients with diabetic nephropathy is useful to identify the new biomarkers to predict the progression of diabetic nephropathy. We demonstrated that global reduction of the bindings to lectins, such as fucose, Lac/LacNA, α- or β-Gal, chitobiose, and α- or β-GalNAc binders in urine samples of diabetic nephropathy at macroalbuminuria stage, and in contrast increased biding activity to Siaα2-6-Gal/GalNAc binders. Further, we identified three sialylated glycoprrpteins such as α1-microglobulin (Protein AMBP), α1-acid glycoprotein (orosomucoid) and fetuin-A (α2-HS-glycorptein) by SSA-Agarose column choromatography and LC/MS-MS analysis. Finally, we have newly shown that higher urinary excretion of fetuin-A is a risk factor for both microalbuminuria and reduction of GFR in diabetic nephropathy.

Author Contributions

Wrote the paper: JW KI HM. Designed and performed most of the experiments: KI JW. Recruited the patients: JE AN ST KM DO TT AK AT II KH. Performed lectin microarray analysis: MY TO. Conceived the study: JW KI HM.

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Combination therapy with an angiotensin-converting-enzyme inhibitor and an angiotensin II receptor antagonist ameliorates microinflammation and oxidative stress in patients with diabetic nephropathy

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ABSTRACT

Aims/Introduction: Recent studies have pointed to the effectiveness of combination therapy with an angiotensin-converting-enzyme inhibitor (ACEI) and an angiotensin receptor blocker (ARB) for diabetic nephropathy. However, some controversy over this combination treatment remains and the mechanisms underlying its renoprotective effects have not been fully clarified. Therefore, we compared the renoprotective effects of imidapril (ACEI) and losartan (ARB) combination therapy with losartan monotherapy in patients with diabetic nephropathy. We also compared the anti-inflammatory and anti-oxidative stress effects of these two treatments.

Materials and Methods: A total of 32 Japanese patients with type 2 diabetes and nephropathy were enrolled. Patients were randomized to either 100 mg/day losartan (n = 16) or 50 mg/day losartan plus 5 mg/day imidapril (n = 16). We evaluated clinical parameters, serum concentrations of high-sensitivity C-reactive protein (hs-CRP), soluble intercellular adhesion molecule-1 (sICAM-1), interleukin-18 (IL-18) and monocyte chemotactic protein-1 (MCP-1), and the urinary concentrations of IL-18, MCP-1 and 8-hydroxy-2′-deoxyguanosine (8-OHdG) at 24 and 48 weeks after starting treatment.

Results: Blood pressure was not significantly different between the two groups. The serum levels of hs-CRP, sICAM-1 and IL-18, as well as urinary excretion of albumin, IL-18 and 8-OHdG decreased significantly in the combination therapy group at 48 weeks. The percent decreases in serum IL-18 concentrations and urinary IL-18 and 8-OHdG were significantly greater in the combination therapy group than in the monotherapy group.

Conclusions: Combination therapy with an ACEI and an ARB could be beneficial for treating diabetic nephropathy through its anti-inflammatory and anti-oxidative stress effects. (J Diabetes Invest doi: 10.1111/jdi.12004, 2013)

KEY WORDS: Combination, Diabetic nephropathy, Renin–angiotensin system

INTRODUCTION

It is widely accepted that chronic inflammation is profoundly involved in the development of atherosclerosis[1]. Adhesion molecules, pro-inflammatory cytokines and chemokines, including soluble intercellular adhesion molecule-1 (sICAM-1), interleukin-18 (IL-18) and monocyte chemotactic protein-1 (MCP-1), are involved in the pathogenesis of diabetic nephropathy as well as atherosclerosis[2,3]. C-reactive protein (CRP) is a good marker for cardiovascular risk[4], and is a precipitating factor for diabetic nephropathy[5]. Angiotensin II, which is produced by the renin–angiotensin system (RAS), is known to promote inflammation[6]. Inhibition of the RAS and associated inflammation might be renoprotective in chronic renal diseases, including diabetic nephropathy[6,7].

Oxidative stress is a critical pathogenic component of atherosclerosis and diabetic nephropathy[8]. After the onset of renal disorders, the levels of pro-inflammatory cytokines and oxidative stress begin to increase, inducing cardiovascular diseases through vascular endothelial dysfunction[9]. Furthermore, an increase in

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oxidative stress has been reported in hyperglycemic rats[10]. While activation of the RAS increases oxidative stress, angiotensin-converting enzyme inhibitors (ACEI)[11] and angiotensin II type 1 receptor blockers (ARB)[12] inhibit oxidative stress.

Combination therapy with an ACEI and an ARB has been considered in several renal diseases to protect the kidney by potently inhibiting RAS activity[13]. In the context of diabetic nephropathy[6,13,14], combination therapy was found to reduce albuminuria. However, most of the studies testing ACEI/ARB combination therapy in diabetic nephropathy were short-term observational studies, and clinical studies attempting to elucidate the mechanisms underlying these renoprotective effects are not sufficient[15].

In patients with diabetic nephropathy, ACEI/ARB combination therapy is expected to have more potent anti-inflammatory and anti-oxidative stress effects than monotherapy at the systemic and local levels in the kidney. Combination therapy might also inhibit the development or progression of atherosclerosis and diabetic nephropathy more potently than monotherapy. However, to our knowledge, no clinical studies have evaluated the anti-inflammatory and anti-oxidative stress effects of combination therapy. Therefore, we carried out a randomized controlled study of ACEI/ARB combination therapy vs ARB monotherapy in patients with type 2 diabetes and early nephropathy to compare the anti-inflammatory and anti-oxidative stress effects of these therapies.

MATERIALS AND METHODS

Study Design

Patients meeting the following inclusion criteria were eligible for the present study: age 30–74 years, diagnosed with type 2 diabetes, disease duration ≥7 years, diagnosis of diabetic neuropathy and retinopathy, creatinine clearance (C_{Cr}; determined using the Cockcroft–Gault formula) >60 mL/min and urinary albumin/creatinine ratio (ACR) >30 mg/gCr. The diagnosis of type 2 diabetes was made according to the World Health Organization criteria.

Patients meeting any of the following criteria were excluded from the present the study: patients with chronic inflammatory disease or malignancies, pregnant women or women who wished to become pregnant, patients with renovascular hypertension, patients with hemoglobin A1C (A1C; National Glycohemoglobin Standardization Program) >9.4%; patients with blood pressure (BP) >180/110 mmHg and patients who had participated in another clinical trial within 3 months before enrolling in the present study. At the discretion of the investigator, patients who had previously used an ACEI or ARB that could not be washed out were also excluded. Concomitant use of steroids, potassium-sparing diuretics, digoxin, or antiarrhythmic drugs except for β-blockers and calcium channel blockers was not allowed. Concomitant use of new statins or thiazolidinedione derivatives was not allowed during the washout or observation periods. Blood glucose levels were to be controlled by adjusting the dose of medications already in use.

Written informed consent was obtained from all participants. The present study was approved by the Ethics Committee of Okayama Saiseikai General Hospital.

The design of the present study is summarized in Figure 1a. During the washout period, previous ACEIs or ARBs were discontinued and switched to amlodipine (5 mg/day). The observation period was started 8 weeks after starting treatment with amlodipine. In the present study, we treated patients with losartan (ARB) and imidapril (ACEI) or losartan alone during the observation period. Amlodipine was stopped when starting combination or monotherapy.

For losartan monotherapy, the losartan dose was 100 mg/day. In combination therapy, the doses of losartan and imidapril were 50 and 5 mg/day, respectively. The first patient enrolled in the study was allocated to monotherapy, with subsequent patients allocated to one of the two therapies.

During the observation period, patients were to be discontinued or withdrawn from the study if BP could not be maintained at <180/110 mmHg, C_{Cr} decreased to 60 mL/min, serum creatinine increased from baseline by ≥30%, or if an adverse drug reaction possibly related to therapy occurred.

Data Collection

After the baseline visit, patients were instructed to visit the hospital at 8, 16, 24 and 48 weeks. At each visit, physical findings, BP, hematological parameters, urinalysis parameters and complications were assessed (Figure 1b). Venous blood and urine samples were obtained in the early morning after an overnight fast. BP was measured by the same person in the morning. After a 5-min rest in a sitting position, BP was measured in triplicate to calculate the mean for assessment.

Analysis of Biomarkers

The primary objective of the present study was to evaluate the anti-inflammatory and anti-oxidative stress effects of imidapril/losartan combination therapy by measuring the serum concentrations of high-sensitivity CRP (hs-CRP), sICAM-1, IL-18, MCP-1 and aldosterone, as well as the urinary concentrations of IL-18, MCP-1, aldosterone and 8-hydroxy-2′-deoxyguanosine (8-OHdG). Blood and urine samples were centrifuged immediately after collection, and the supernatants were stored at −80 and −30°C, respectively, until analysis. The concentrations of the pro-inflammatory biomarkers were analyzed using samples obtained at baseline and at weeks 24 and 48. All samples were measured together once all specimens were collected.

An immunoturbidimetric assay was used for urinary albumin concentrations (Nitto Boseki Co. Ltd, Tokyo, Japan) and an immunonephelometric assay kit was used for hs-CRP concentrations (Dade Behring, Marburg, Germany). Enzyme-linked immunosorbent assay kits were used to measure IL-18 (MBL, Nagoya, Japan), sICAM-1 concentration and MCP-1 concentrations (R&D Systems, Inc, Minneapolis, MN, USA), and 8-OHdG concentration (Nikken SEIL Co. Ltd, Tokyo, Japan). A radioimmunoassay kit was used to measure aldosterone

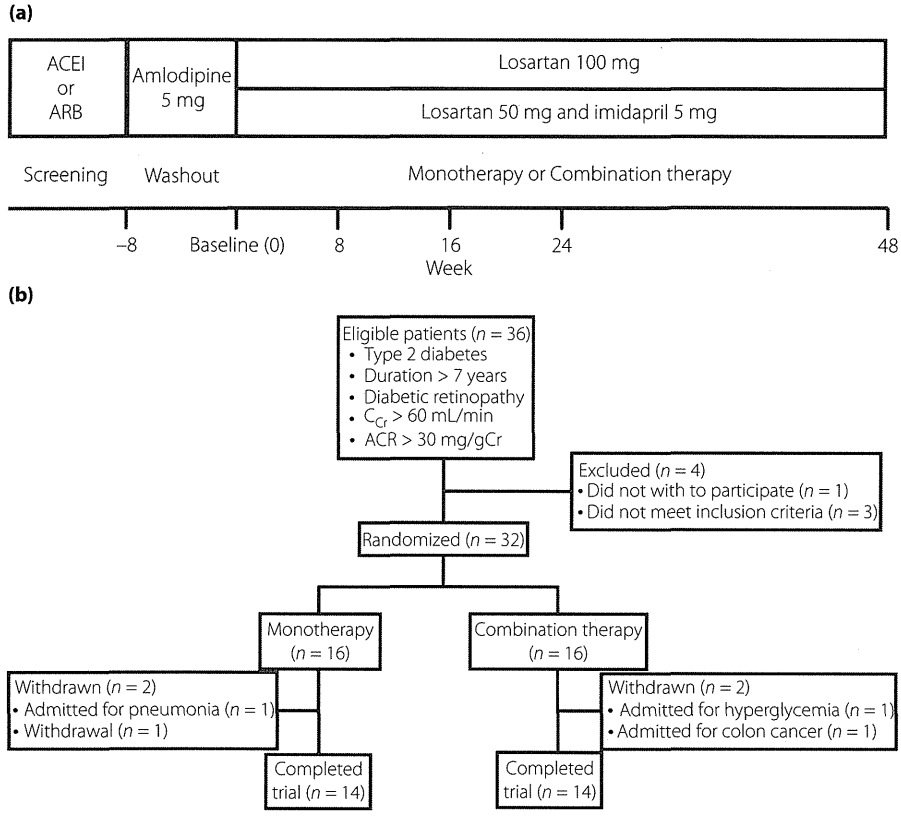

Figure 1 | (a) Study design. During the washout period, angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) were discontinued and switched to amlodipine (5 mg/day). At the end of the washout period, patients were allocated to either monotherapy (100 mg/day losartan) or combination therapy (50 mg/day losartan plus 5 mg/day imidapril) and followed for 48 weeks. (b) Patient disposition. Four patients were excluded from this study during the washout period, including one patient who refused to participate in the study, two patients with an ACR < 30 mg/gCr and one patient with C_{Cr} < 60 mL/min. Of the 32 patients who started the observation period, 28 were treated for 48 weeks and were included in the analysis.

concentrations (TFB Co. Ltd, Tokyo, Japan). A1C was measured using a high-pressure liquid chromatography method and is expressed in National Glycohemoglobin Standardization Program values. The urinary markers were divided by the urinary creatinine concentration to calculate the creatinine ratio for assessment.

Statistical Analysis

Comparisons of baseline factors between the two therapeutic groups were analyzed by Student's unpaired *t*-test for continuous parameters or chi-squared-test for categorical parameters (sex and previous therapy). Baseline concentrations of pro-inflammatory markers, serum and urinary aldosterone concentrations, and urinary 8-OHdG concentrations were compared using Student's unpaired *t*-test. The percent change (% change) from baseline for pro-inflammatory biomarkers, serum and urinary aldosterone concentrations, urinary 8-OHdG concentration, A1C, BP,and C_{Cr} at 24 and 48 weeks was calculated and analyzed by analysis of variance (ANOVA). The differences in the percent change of these parameters at each time between the two therapeutic groups were analyzed by ANOVA. All analyses were two-sided with a significance level of 5%. Data are expressed as the number for categorical variables, mean change from baseline for percent changes and mean ± standard deviation for continuous variables.

RESULTS

A total of 36 patients who met the inclusion criteria were screened (Figure 1b). Before screening, all of the patients had received an ACEI and/or ARB. Four patients were excluded from the study during the washout period, including one patient who refused to participate in the study, two patients with an ACR < 30 mg/gCr and one patient with C_{Cr} < 60 mL/min. Therefore, 32 patients started the study. In two patients allocated to combination therapy, the doses of losartan and imidapril were 25 and 2.5 mg/day, respectively, for the first 4 weeks because their systolic BP (SBP) was <120 mmHg. The doses in these patients were subsequently increased to 50 and 5 mg/day, respectively. During the observation period, two patients allocated to combination therapy were

excluded from the study; one patient was hospitalized for hyperglycemia and one patient underwent surgery for colon carcinoma. In the monotherapy group, two patients were excluded during the observation period; one patient was hospitalized for pneumonia and one patient stopped visiting the hospital. Consequently, 14 patients in each therapeutic group, a total of 28 patients, completed 48 weeks of observation and were included in the analysis (Table 1). There were no significant differences in baseline parameters between the two therapeutic groups, including age, sex, duration of diabetes, body mass index, A1C, Ccr, SBP, diastolic BP (DBP), ACR and previous therapy.

None of the patients developed new cerebrovascular disease, cardiovascular disease, arteriosclerosis obliterans, progression of nephropathy, excessive BP decrease or hyperkalemia during the study. Urinalysis showed no evidence of ketonuria, hematuria or urinary tract infection.

Time-Course of A1C, BP and C_{Cr}

There were no significant differences in percent change in A1C at 8, 16, 24 or 48 weeks between the monotherapy (7.8 ± 1.6, 8.0 ± 1.2, 8.0 ± 1.3 and 7.9 ± 1.2%, respectively) and combination therapy groups (7.8 ± 0.6, 8.1 ± 1.0, 8.1 ± 1.0 and 7.8 ± 0.8%, respectively). Similarly, there were no significant differences in SBP (monotherapy 137 ± 7, 136 ± 8, 135 ± 7 and 134 ± 8 mmHg; combination therapy 134 ± 12, 133 ± 10, 137 ± 10 and 134 ± 9 mmHg), DBP (monotherapy 80 ± 6, 79 ± 7, 81 ± 7 and 79 ± 5 mmHg; combination therapy 79 ± 8, 80 ± 8, 80 ± 8 and 78 ± 8 mmHg) or C_{Cr} (monotherapy 84.6 ± 18.6, 85.2 ± 20.4, 82.8 ± 18.6 and 84.6 ± 21.6 mL/min; combination therapy 85.2 ± 31.8, 87.6 ± 33.6, 86.4 ± 34.2 and 86.4 ± 30.6 mL/min) at 8, 16, 24 and 48 weeks of treatment.

Table 1 | Baseline background of patients in each group

	Mono	Combination
No. patients	14	14
Age (years)	61.4 ± 8.8	61.7 ± 5.3
Sex (male/female)	9/5	10/4
Duration of diabetes (years)	11.7 ± 4.7	15.6 ± 6.7
Body mass index (kg/m^2)	25.4 ± 2.9	24.5 ± 5.1
A1C (%)	7.8 ± 1.5	7.8 ± 0.8
Creatinine clearance (mL/min)	85.4 ± 16.1	90.1 ± 36.1
Systolic blood pressure (mmHg)	136 ± 6	134 ± 12
Diastolic blood pressure (mmHg)	79 ± 6	79 ± 7
Albumin creatinine ratio (mg/gCr)	224 ± 197	270 ± 202
Medication (*n*)		
Insulin	5	4
Oral hyperglycemic agents	5	9
Statins	5	4

A1C, hemoglobin A1C; Mono, monotherapy.
Data are *n* or mean ± standard deviation.

Time-Course of Serum Concentrations of Pro-Inflammatory Biomarkers and Aldosterone

At baseline, we found no significant differences in the serum concentrations of hs-CRP, sICAM-1, IL-18, MCP-1 or aldosterone between the two groups (Table 2).

In all patients, the serum hs-CRP concentration decreased significantly from baseline by 23.5% at 48 weeks. In the combination therapy group, the serum hs-CRP concentration decreased significantly from baseline by 28.0 and 31.0% at 24 and 48 weeks, respectively. The serum hs-CRP concentration decreased by 16.6% at 48 weeks in the monotherapy group, although not significantly (Table 2). The serum sICAM-1 concentration decreased significantly by 10.6% at 48 weeks in

Table 2 | Change of the serum levels of inflammatory molecules and aldosterone

Serum molecules	Week 0	Week 24	Week 48
hsCRP (mg/L)			
All patients	1.33 ± 0.77	1.04 ± 0.79	0.71 ± 0.60
% change		−13.7	−23.5*
Mono	1.39 ± 0.93	1.24 ± 1.01	1.04 ± 0.75
% change		−0.4	−16.6
Combination	1.26 ± 0.56	0.83 ± 0.39	0.78 ± 0.36
% change		−28.0*	−31.0‡
sICAM-1 (pg/mL)			
All patients	275 ± 76	256 ± 74	250 ± 54
% change		−5.9*	−6.4
Mono	282 ± 96	265 ± 100	262 ± 65
% change		−5.4	−2.1
Combination	269 ± 52	247 ± 35	238 ± 38
% change		−6.4	−10.6‡
IL-18 (pg/mL)			
All patients	195 ± 62	209 ± 89	194 ± 75
% change		+7.2	0.0
Mono	180 ± 49	214 ± 107	207 ± 73
% change		+16.4	+11.6
Combination	211 ± 72	204 ± 69	182 ± 53
% change		−2.0	−11.4*§
MCP-1 (pg/mL)			
All patients	308 ± 95	283 ± 78	282 ± 63
% change		−6.3*	−5.6*
Mono	331 ± 114	295 ± 84	291 ± 71
% change		−7.8	−8.0
Combination	285 ± 68	270 ± 74	273 ± 56
% change		−4.8	−3.2
Aldosterone (pmol/L)			
All patients	2470 ± 1180	2230 ± 930	1760 ± 950
% change		−5.4	−27.0‡
Mono	2680 ± 1220	2510 ± 840	1860 ± 1210
% change		+1.4	−31.2‡
Combination	2260 ± 1150	1960 ± 960	1650 ± 610
% change		−12.1*	−22.7‡

Data are means ± SD or frequencies (%).*$P < 0.05$, †$P < 0.01$, ‡$P < 0.005$ versus baseline. §$P < 0.05$ for combination versus monotherapy (Mono).

the combination therapy group, but did not change significantly in the monotherapy group (Table 2). The serum IL-18 concentration decreased significantly by 11.4% at 48 weeks in the combination therapy group, but tended to increase in the monotherapy group, resulting in a significant difference in the percent change between the two groups at 48 weeks (Table 2). In all patients, the serum MCP-1 concentration decreased significantly from baseline by 6.3 and 5.6% at 24 and 48 weeks, respectively. The serum MCP-1 concentration decreased in both therapeutic groups, although the percent change was not significant. The serum aldosterone concentration decreased significantly from baseline at 48 weeks in all patients and in both groups (Table 2).

Time-Course of ACR and Urinary Concentrations of IL-18, MCP-1, Aldosterone and 8-OHdG

At baseline, there were no significant differences between the two groups in ACR or urinary concentrations of IL-18, MCP-1, aldosterone or 8-OHdG (Table 3).

In all patients, ACR decreased significantly by 8.3 and 11.0% at 24 and 48 weeks, respectively. ACR decreased significantly by 6.2% at 48 weeks in the monotherapy group and by 12.8 and 15.4% at 24 and 48 weeks, respectively, in the combination therapy group. The percent change (decrease) in ACR was significantly greater in the combination therapy group than in the monotherapy group at both times (Table 3). In all patients, the urinary IL-18 concentration tended to decrease over time, although not significantly. In the combination therapy group, the urinary IL-18 concentration decreased significantly by 55.3% at 48 weeks, and the percent change was significantly different from that in the monotherapy group (Table 3). In all patients, the urinary MCP-1 concentration decreased by 15.4% at 24 weeks, but increased significantly by 22.4% at 48 weeks. In both groups, the urinary MCP-1 concentration was increased at 48 weeks. The urinary aldosterone concentration was significantly decreased at all times in all patients and in both treatment groups; however, there were no significant differences in the percent changes between the two groups (Table 3). In all patients, the urinary 8-OHdG concentration decreased significantly from baseline by 28.6 and 26.6% at 24 and 48 weeks, respectively. The urinary 8-OHdG concentration decreased significantly by 23.8% at 24 weeks in the monotherapy group and by 33.5 and 37.8% at 24 and 48 weeks, respectively, in the combination therapy group. The percent change in 8-OHdG concentration at 48 weeks was significantly greater in the combination therapy group than in the monotherapy group (Table 3).

Table 3 | Change of the urinary levels of albumin/creatinine ratio, inflammatory molecules, aldosterone and 8-hydroxy-2′-deoxyguanosine

Urinary molecules	Week 0	Week 24	Week 48
ACR (mg/gCr)			
All patients	252 ± 197	200 ± 202	175 ± 159
% change		−8.3‡	−11.0‡
Mono	234 ± 197	209 ± 221	182 ± 128
% change		−3.5	−6.2*
Combination	270 ± 202	192 ± 189	168 ± 191
% change		−12.8‡§	−15.4‡§
IL-18 (pg/mL/Cr)			
All patients	57 ± 62	33 ± 34	13 ± 14
% change		−15.7	−24.3
Mono	39 ± 35	25 ± 17	19 ± 17
% change		−7.3	+6.8
Combination	75 ± 77	40 ± 45	8 ± 8
% change		−24.1	−55.3‡§
MCP-1 (pg/mL/Cr)			
All patients	318 ± 192	205 ± 101	340 ± 244
% change		−15.4	+22.4*
Mono	341 ± 220	216 ± 109	351 ± 216
% change		−15.4	+32.8
Combination	294 ± 164	195 ± 95	329 ± 277
% change		−15.4	+12.0
Aldosterone (pmol/L/Cr)			
All patients	100 ± 70	48 ± 45	68 ± 54
% change		−50.9‡	−29.1‡
Mono	125 ± 79	68 ± 52	89 ± 59
% change		−35.7*	−22.6*
Combination	77 ± 68	29 ± 28	47 ± 41
% change		−65.1‡	−35.2
8-OHdG (pg/mL/Cr)			
All patients	12.8 ± 5.0	8.2 ± 4.2	8.6 ± 3.4
% change		−28.6‡	−26.6‡
Mono	13.7 ± 3.8	9.7 ± 4.3	10.7 ± 3.1
% change		−23.8*	−15.4‡
Combination	12.0 ± 5.9	6.8 ± 3.6	6.5 ± 2.3
% change		−33.5†	−37.8‡§

Data are means ± SD or frequencies (%). *$P < 0.05$, †$P < 0.01$, ‡$P < 0.005$ versus baseline. §$P < 0.05$ for combination versus monotherapy (Mono).

DISCUSSION

The present study showed that treatment with losartan and imidapril was more effective than treatment with amlodipine in decreasing ACR as well as inflammatory and oxidative stress markers in serum and urine in Japanese patients with type 2 diabetes and nephropathy. These effects were maintained for 48 week. Combination therapy with losartan and imidapril was more useful in decreasing ACR, and pro-inflammatory and oxidative stress markers than losartan administered at a double dose, without differences in BP or glycemic control. These results suggest that the losartan/imidapril combination might be useful to prevent the progression of atherosclerosis and nephropathy in patients with type 2 diabetes by exerting anti-inflammatory and anti-oxidative effects through inhibition of the RAS.

The RAS and downstream inflammatory activities are involved in the progression of atherosclerosis[16]. In the present study, treatment with losartan and imidapril did not significantly change A1C or BP, but hs-CRP levels at 24 and

48 weeks, and sICAM-1 levels at 48 weeks were significantly lower than those at baseline; these changes were not observed in the monotherapy group. Amlodipine was reported to have anti-inflammatory and anti-oxidative stress effects, and to inhibit the production of hs-CRP and sICAM-1[17,18]. RAS inhibitors also have anti-inflammatory effects, which are favorable cardiovascular effects, independent of their antihypertensive effects[19,20]. The present study showed that losartan and imidapril could inhibit the progression of atherosclerosis by their anti-inflammatory effects through inhibition of the RAS more potently than amlodipine.

The serum IL-18 concentration tended to be increased in the monotherapy group, but decreased significantly in the combination therapy group, resulting in a significant difference between the two groups at 48 weeks. Serum IL-18 is an important prognostic predictor of diabetic nephropathy and atherosclerosis[21]. It has been reported that ARBs and ACEIs decrease IL-18 by inhibiting the RAS[22]. In the present study, amlodipine was only administered during the washout period. Therefore, the efficacy of losartan and imidapril represented the change from treatment with amlodipine. Based on our searches of the literature, amlodipine has no known effect on serum IL-18 concentrations. These results indicate that imidapril/losartan combination therapy could have better anti-inflammatory efficacy in patients with type 2 diabetes with nephropathy, as compared with amlodipine or losartan monotherapy.

Similar to microinflammation, oxidative stress is profoundly involved in the development of atherosclerosis and diabetic complications[23]. 8-OHdG is a biomarker of systemic DNA damage in diabetic nephropathy[24]. In the present study, the urinary 8-OHdG concentration decreased significantly after treatment with losartan and imidapril, as compared with baseline levels. The decrease in urinary 8-OHdG concentrations was greater in the combination therapy group than in the monotherapy group. It has been reported that amlodipine[17,18], ACEI[11] and ARB[12,19] suppress oxidative stress. However, it is unclear which of these agents or which combination of these drugs elicits the greatest reduction in oxidative stress in patients with diabetic nephropathy. The present results suggest that imidapril/losartan combination therapy might be more effective in preventing the development or progression of diabetic nephropathy because of its more potent inhibitory effects on oxidative stress than losartan monotherapy. These effects were maintained for up to 48 weeks.

The urinary aldosterone concentration was also decreased more markedly by combination therapy than monotherapy in the present study, which suggests that combination therapy more potently inhibits the RAS in the kidney. The urinary IL-18 concentration was also significantly decreased by combination therapy as compared with monotherapy. We previously reported that serum and urine IL-18 concentrations are predictors of diabetic nephropathy[21]. IL-18 secretion in monocytes and macrophages is stimulated by inflammation and oxidative stress associated with hyperglycemia[25]. These results suggest that the combination of losartan and imidapril might be useful to prevent the development of early nephropathy in patients with type 2 diabetes by decreasing ACR, and reducing inflammation and oxidative stress in the kidney through more potent inhibition of the RAS.

The main limitation of the present study was that the observation period was not long enough to evaluate renal or cardiovascular outcomes. The Olmesartan Reducing Incidence of Endstage Renal Disease in Diabetic Nephropathy Trial (ORIENT)[26], and Ongoing Telmisartan Alone and in Combination with Ramipril Global End Point Trial (ONTARGET)[27] studies both showed that combination therapy with an ACEI and an ARB did not have significant benefits on renal outcomes. However, in the ORIENT study[26], combination therapy with olmesartan and an ACEI reduced proteinuria, as observed in the present study, but did not further improve renal or cardiovascular outcomes. The reason for these discrepancies remains unclear. Further studies are required to confirm the beneficial effect of combination therapy with an ARB and an ACEI on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy.

In the present study, the sample size of 28 patients was small, meaning larger studies are required to confirm these results. Although the serum and urinary concentrations of several biomarkers decreased after monotherapy or combination therapy, some changes were not statistically significant, perhaps because of the small sample size. Although amlodipine was used during the washout period to prevent possible changes in BP, we measured ambulatory BP at a single time, so we cannot exclude the possible effects of BP on the decrease in ACR. A larger clinical study using 24-h BP monitoring might be required to verify the results of the present study.

In conclusion, a combination of an ACEI and an ARB could be beneficial for treating diabetic nephropathy through their anti-inflammatory and anti-oxidative stress effects.

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REVIEW ARTICLE

WCN 2013 Satellite Symposium "Kidney and Lipids"

Pathology of glomerular lipidosis

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Abstract Glomerular lipid deposition is sometimes associated with a particular kind of lipid metabolism disturbance. Ultrastructural analyses using electron microscopy often indicate a disease-specific aspect of intraglomerular lipid distribution.

Keywords Glomerular lipidosis · Foam cell · Lipoprotein glomerulopathy · LCAT deficiency · Fabry disease

Introduction

Lipid deposition is frequently observed in kidney biopsy specimens. Here, we focus attention on glomerular lipidosis in which there are several glomerular disorders associated with specialized lipid metabolism disturbance. Electron microscopic examination provides a number of interesting findings on the morphological character of lipid depositions. According to the ultrastructural localization, intraglomerular lipid distribution may be roughly classified into the following four categories—lipid accumulation in the infiltrated macrophages, in the glomerular capillary lumens without foam cells, in the mesangial and/or subendothelial area, and in the glomerular epithelial cells.

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Lipid accumulation in the infiltrated macrophages

This type of glomerular lipidosis is usually recognized as intracapillary foam cells (Fig. 1), and is typically observed in the early stage of focal segmental glomerulosclerosis [1] or diabetic glomerulosclerosis [2], and in familial type III hyperlipoproteinemia [3]. Similar findings are also found in various glomerular diseases with no particular specificity.

Lipid accumulation within the glomerular capillary lumens without foam cells

This form of glomerular lipidosis is typically seen in lipoprotein glomerulopathy [4]. In this disease, enlargement of glomerular capillary lumens is the most outstanding observation (Fig. 2a). Although each capillary lumen only looks like a vacuum space without any solid substances on light microscopy, electron microscopy reveals that the largely expanded lumens are filled with numerous cobblestone-like materials with no cellular components, like intracapillary lipid thrombi (Fig. 2b).

Lipid accumulation in the mesangial and/or subendothelial area

This type was representatively demonstrated in a patient in our institute with lecithin-cholesterol acyltransferase (LCAT) deficiency [5]. Electron microscopy revealed numerous lipid particles in the mesangial and subendothelial

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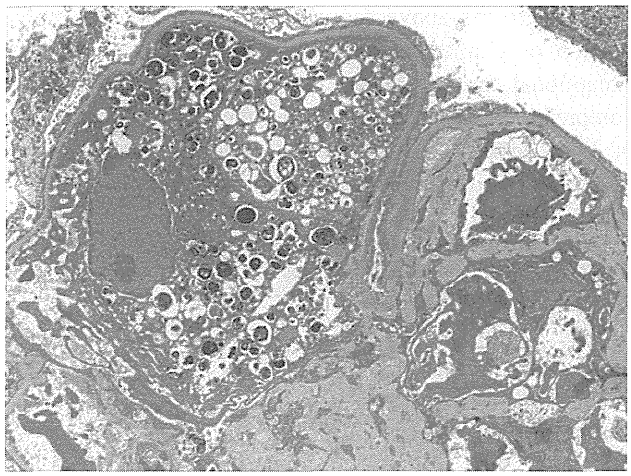

Fig. 1 Intracapillary foam cells in a patient with an early stage of focal segmental glomerulosclerosis. Electron microscopy, ×5,000

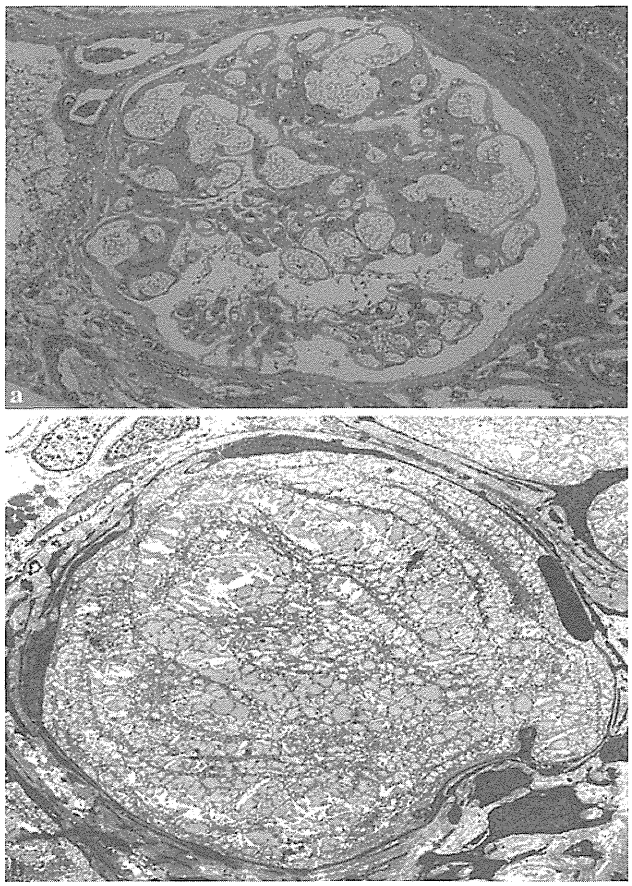

Fig. 2 **a** Marked enlargement of glomerular capillary lumens in a patient with lipoprotein glomerulopathy. Elastica-Masson stain, ×400. **b** Electron microscopy reveals numerous cobblestone-like materials fills the glomerular capillary lumen, ×4000

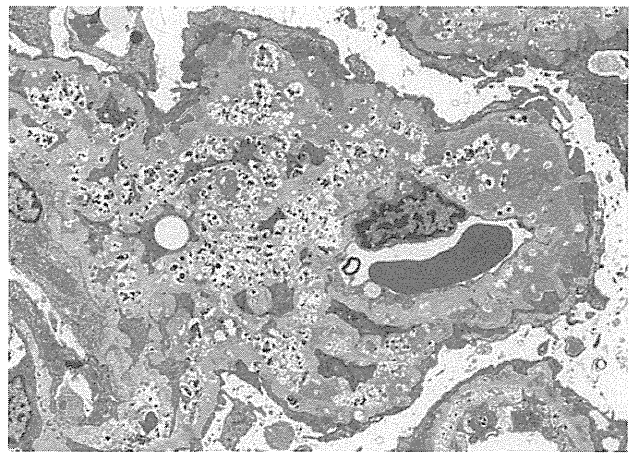

Fig. 3 Numerous lipid particles accumulated in mesangial and subendothelial space in a patient with LCAT deficiency. A number of subepithelial electron-dense deposits and spikes are also observed outside the glomerular basement membrane. Electron microscopy, ×4,000

space. A number of subepithelial electron-dense deposits and spikes were also observed outside the glomerular basement membrane, indicating a complication of stage II membranous nephropathy (Fig. 3).

Interestingly, this case showed only a clinical manifestation of LCAT deficiency without any genetic abnormalities specific to LCAT deficiency. Corticosteroid treatment improved not only the kidney-related manifestation including proteinuria but also all of the clinical phenomena associated with LCAT deficiency. It is speculated that some kind of immune-mediated non-genetic mechanisms played a key role on the impaired activity of LCAT although the precise mechanism has not yet been clarified. Takahashi et al. [6] recently reported a similar patient with acquired LCAT deficiency in which membranous nephropathy was complicated in the same way as in our case. They demonstrated the presence of an inhibitory anti-LCAT antibody in the patient's serum, and immunohistochemistry detected LCAT along parts of the glomerular capillary walls, suggesting that LCAT was the antigen responsible for the membranous nephropathy.

Lipid accumulation in the glomerular epithelial cells (Fig 4)

Fabry disease reveals a characteristic vacuolar degeneration of the glomerular epithelial cells deriving from an excessive deposition of neutral globotriaocylceramide (GL3).

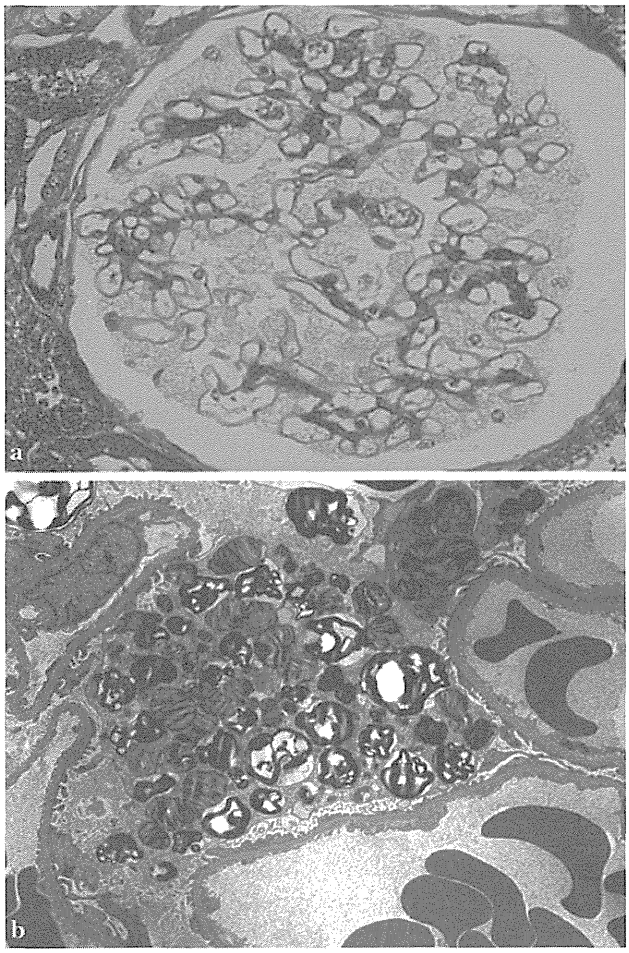

Fig. 4 **a** Bubbling appearance of the glomerular epithelial cells in a patient with Fabry disease. Azan-Mallory stain, ×400. **b** So-called zebra-bodies are recognized on electron microscopy, ×4,000

Although the above-described histopathological classification of glomerular lipidosis may contain only a few exceptional cases or borderline problems, an unequivocal comprehension of the intraglomerular distribution pattern of lipid deposition undoubtedly contributes to adequate understanding of lipid-associated kidney injuries.

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Conflict of interest None.

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ORIGINAL ARTICLE

The Japanese version of the modified ACR Preliminary Diagnostic Criteria for Fibromyalgia and the Fibromyalgia Symptom Scale: reliability and validity

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Abstract
Purpose The aim of this study is to investigate the reliability and validity of the Japanese version of the modified American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for Fibromyalgia (mACR 2010-J) and the Fibromyalgia Symptom Scale (mFS-J).
Methods According to the ACR 1990 classification criteria, patients with chronic pain were divided into the fibromyalgia group and nonfibromyalgia group (rheumatoid arthritis and osteoarthritis). Patients in both groups were assessed using mACR 2010-J and mFS-J.
Results 294 of 462 (64 %) patients in the fibromyalgia group met mACR 2010-J, whereas 4 % (9/231) of the nonfibromyalgia group did, with sensitivity of 64 %, specificity of 96 %, positive predictive value of 97 %, negative predictive value of 56 %, and positive likelihood ratio of 16.3. Mean total scores on mFS-J significantly differentiated the fibromyalgia from the nonfibromyalgia group. According to the value of the Youden index, the best cutoff score for the mFS-J was 9/10.
Conclusion Our findings indicate that mACR 2010-J as a positive test and mFS-J as a quantification scale might be suitable for assessing fibromyalgia among Japanese chronic pain populations.

Keywords Diagnostic criteria · Fibromyalgia · Symptom scale · Modified ACR Preliminary Diagnostic Criteria for Fibromyalgia

Introduction

Fibromyalgia (FM) is characterized by widespread musculoskeletal chronic pain, fatigue, poor sleep, frequent psychological difficulties, and multiple tender points on physical examination [1, 2]. In 1990, the American College

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of Rheumatology (ACR) presented FM criteria (ACR 1990) that required tenderness on pressure (tender points) in at least 11 of 18 specified sites and the presence of widespread pain for diagnosis [1]. Widespread pain was defined as axial pain, both left- and right-sided and with upper and lower segment pain. However, ACR 1990 had the serious problem of little variation in symptoms. To improve this shortcoming, new clinical criteria, which integrate variations in symptoms with severity scale (2010 ACR Preliminary Diagnostic Criteria for FM, ACR 2010) [3], have been presented. The diagnostic criteria for FM are satisfied if the following three conditions are met: (1) Widespread Pain Index (WPI) $\geq$7 and Symptom Severity Score (SS) $\geq$5, or WPI of 3–6 and SS $\geq$9; (2) symptoms have been present at a similar level for at least 3 months; and (3) the patient does not have a disorder that would otherwise explain the pain. The publication of ACR 2010 eliminated the tender point examination, thus making it possible to study FM in survey and clinical research.

Accordingly, we have validated the Japanese version of ACR 2010 [4]. In addition, we have originally validated the Japanese version of the Fibromyalgia Symptom Scale with the sum of WPI and the original SS, i.e., fatigue, waking unrefreshed, cognitive symptoms, and somatic symptoms in general consisting of 41 symptoms of the FS-J [4]. Both ACR 2010-J and FS-J have high reliability and validity, and are useful for assessing fibromyalgia among Japanese chronic pain populations.

Recently, Wolfe et al. [5] proposed a modification of the ACR 2010 (mACR 2010), deleting 38 out of 41 somatic symptoms in general from the original SS. Consequently, complete self-administration has become possible. Furthermore, they created the Fibromyalgia Symptom Scale with the sum of WPI and the new SS (FS). They reported that the criteria properly identified diagnostic groups, and that FS score $\geq$13 best separated criteria+ and criteria− patients.

The aim of this study is to investigate the reliability and validity of the Japanese version of the mACR 2010 (mACR 2010-J) and the Japanese version of the FS (mFS-J). Furthermore, our questions are whether mACR 2010-J would be more useful than ACR 2010-J for assessing fibromyalgia among Japanese chronic pain populations, and whether mFS-J is more suitable than FS-J as a positive test.

Subjects and methods

An experienced rheumatologist and an experienced psychiatrist had translated the mACR 2010 into Japanese with the author's permission and produced forward- and back-translations to create the mACR 2010-J.

We recruited FM patients who met the previous criteria of the ACR 1990 and were without psychiatric disorders according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [6] in a clinic specialized for FM, the Kasumigaseki Urban Clinic, in Tokyo, Japan, between August 1, 2010 and July 31, 2011. During the study period, other patients with diseases associated with chronic pain such as rheumatoid arthritis (RA) and osteoarthritis (OA) who had not been diagnosed previously with FM were recruited as control patients. To adjust the imbalance of number of patients, control patients were additionally recruited from May 30 to July 2, 2012. The diagnoses of RA and OA were made according to the 2010 rheumatoid arthritis classification criteria [7] and the American College of Rheumatology criteria for classification and reporting of osteoarthritis of the hand, hip, and knee [8–10]. The experienced rheumatologist and the experienced psychiatrist familiar with FM assessed these patients. This study was approved by the Institutional Review Board of Kasumigaseki Urban Clinic.

After obtaining informed consent from study participants, the rheumatologist rated patients with the mACR 2010-J. In order to assess interrater reliability, another rater independently rated a subset of the same subjects ($N = 19$) while blind to the diagnoses and scores of the other rater. The raters in this study were already fully trained in use of the scale and quite experienced in use of it. We therefore decided that only a small subsample was needed to reevaluate consistency across raters.

Statistics

Data were analyzed using SPSS 17.0-J software. Differences among groups in demographic and clinical characteristics were calculated with the unpaired t test. If data were not sampled from Gaussian distributions, a nonparametric test (Mann–Whitney U test) was used. To compare categorical data, we used Fisher's exact test.

In the present study, the control group was not healthy volunteer but consisted of chronic pain patients with RA and OA. It has been reported that the age-specific incidence of RA peaked in the 60–64 and 70–74 year age groups for females and males, respectively, in Taiwan [11]. Similarly, it has been reported that the peak prevalence of knee OA in women and men was $\geq$80 years in Japan [12]. In contrast, we have reported that the frequent age of onset of FM in women was 35–55 years based on our FM database including 3,500 Japanese patients with FM [13]. Among Asians, thus, patients with FM are much younger than those with RA and OA. Therefore, matching age of control patients with age of FM patients seems to be rather arbitrary. Accordingly, to control for the effect of age on the rate of patients meeting the mACR 2010-J, patients were divided into three age categories, i.e., 20–39, 40–59, and $\geq$60 years.

There were only eight FM patients and one non-FM patients less than 20 years of age, and there were only two FM patients and three non-FM patients 80 years or older. Then, the Mantel–Haenszel method was used to test the difference in the percentage of patients meeting the mACR 2010-J between the two groups. Also, to control for the effect of age on the score on the mFM-J, one-way analysis of covariance was used. The internal consistency for the mFM-J was calculated with Cronbach's α. Interrater reliability was measured with the intraclass correlation coefficient (ICC) for pairs of independent raters. Cutoff scores for the mFS-J were determined using receiver-operator characteristic (ROC) analyses to determine the Youden index when comparing the FM group with all non-FM subjects. Positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio (sensitivity/1 − specificity) were also calculated. All statistical tests were two-tailed. Statistical significance was set at $p < 0.05$.

Results

A total of 462 patients meeting the ACR 1990 (the FM group) and a total of 231 non-FM patients (RA patients, 196; OA patients, 35; the non-FM group) were enrolled. Demographic and clinical characteristics of the groups are presented in Table 1, showing that 294 of 462 (64 %) patients in the FM group met the mACR 2010-J, whereas 4 % (9/231) of the non-FM group did, including 4 % (8/196) of RA patients and 3 % (1/35) of OA patients. The percentage of patients meeting the mACR 2010-J criteria in the FM group was significantly higher than that of the non-FM group after adjusting for age (estimated odds ratio, 35.7, $p < 0.0001$; Table 1). The sensitivity, specificity, PPV, NPV, and positive likelihood ratio for comparison of the FM group with all non-FM subjects were 64, 96, 97, 56, and 16.3 %, respectively. The ICC between the two independent raters was very high for the mACR 2010-J, at 0.877.

The mean score (standard deviation, SD) of mFS-J in the FM group was 16.7 (6.5), while that in the non-FM group was 3.7 (4.1). The mean score of mFS-J in the FM group was significantly higher than that of the non-FM group after adjusting for age ($F = 605.1$, $p < 0.0001$; Table 1). Internal consistency was not high, with a Cronbach's α coefficient for the mFS-J (WPI + the modified SS) of 0.603. ROC analyses were performed for the mFS-J, comparing the FM group with the non-FM group. Table 2

Table 1 Demographic and clinical characteristics of the fibromyalgia group and nonfibromyalgia group

Group	Fibromyalgia ($N = 462$)	Nonfibromyalgia ($N = 231$)		p
		RA ($N = 196$)	OA ($N = 35$)	
Mean age (SD), years	50.6 (14.8)	61.3 (13.9)		<0.0001
		60.6 (14.4)	65.5 (10.2)	
Sex (female), N (%)	389 (84)	188 (81)		0.39
		160 (82)	28 (80)	
Patients meeting the mACR 2010-J,[a] N (%)	294 (64)	9 (4)		<0.0001[c]
		8 (4)	1 (3)	
Mean score (SD) of mFS-J[b]	16.7 (6.5)	3.7 (4.1)		<0.0001[d]
		3.7 (4.2)	3.9 (3.3)	

[a] The Japanese version of the modified 2010 ACR Preliminary Diagnostic Criteria for Fibromyalgia

[b] The Japanese version of the Fibromyalgia Symptom Scale (WPI + modified SS)

[c] To control for the effect of age on the rate of patients meeting the mACR 2010-J, patients were divided into three age categories. Then, the Mantel–Haenszel method was used to test the difference in the percentage of patients meeting the mACR 2010-J between the two groups

[d] To control for the effect of age on the score of the mFM-J, one-way analysis of covariance was used

SD standard deviation

Table 2 Sensitivity and specificity of the Japanese version of the Fibromyalgia Symptom Scale (mFS-J), based on receiver-operating characteristics (ROC) analysis: fibromyalgia group versus nonfibromyalgia (RA and OA) group

Cutoff score	Sensitivity (%)	Specificity (%)	Positive likelihood ratio	Youden index
8.5	87.7	89.2	8.1	0.769
9.5	84.8	92.2	10.9	0.770
10.5	82.0	92.2	10.5	0.742