Table 1. Baseline Characteristics of Patients Enrolled in the Study

	Losartan group	Amlodipine group	*p* value
N	58	59	
Age (years)	55.7±13.6	57.5±11.9	NS*
Male/female	36/22	41/18	NS†
BMI (kg/m²)	23.9±3.7	22.9±3.2	NS*
Systolic BP (mmHg)	156.5±12.2	155.4±13.5	NS*
Diastolic BP (mmHg)	94.0±9.2	93.5±8.6	NS*
Serum creatinine (mg/dl)	2.04±0.48	1.97±0.52	NS*
Urinary protein (g/day)	2.85±2.65	2.50±2.07	NS*
Serum albumin (g/dl)	3.79±0.48	3.80±0.47	NS*
Diagnoses (No. of patients)			
Chronic glomerulonephritis	38 (11#)	41 (12#)	
Diabetic nephropathy	7	7	
Hypertensive nephrosclerosis	11	9	
Tubulointerstitial nephritis	1	0	
Polycystic kidney disease	1	0	
Renal amyloidosis	0	1	
Castleman's disease	0	1	

Mean±SD. * Unpaired *t*-test; † Fisher's exact test. # IgA nephropathy. BMI, body mass index; BP, blood pressure.

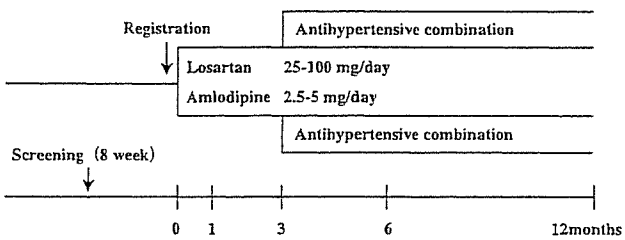

Fig. 1. *Study design for treatment of patients with proteinuric CKD and hypertension. Antihypertensive combination therapy was allowed after the first 3 months, if necessary. For this alternation, the target goal BP setting was <130/85 mmHg.*

tients.

Exclusion criteria were as follows:

1) DBP≥120 mmHg.
2) Renovascular hypertension or endocrine hypertension.
3) BP control treatment with antihypertensive agent(s).
4) Patients in whom antianxiety drugs could not be discontinued.
5) Pregnancy, possibility of pregnancy, or in a period of lactation.
6) Patients that the chief investigator judged not to be eligible.

BP was measured at patients' visit to the clinic with the patient in a sitting position.

A 24-h urine collection was performed from 8:00 AM of the day before to 8:00 AM of the day of the clinic visit, and was used to obtain the 24-h urine volume, urinary protein excretion, urinary creatinine level, and the amount of sodium excretion. The creatinine clearance (Ccr) was calculated as $Ccr = Ucr \times V/Scr \times 1.73/A$, where Ccr is the creatinine clearance (ml/min), Ucr is the urinary creatine (mg/dl), V is the urine volume (ml/min), Scr is the serum creatine (mg/dl), and A is the body surface area. The rate of renal impairment as a function of time was expressed with a reciprocal slope of Scr (1/Scr).

Protein intake was estimated by measurement of urea nitrogen plus protein concentration using the following formula: Protein intake (g/day)=[urinary urea nitrogen (g/day)+0.031(g)×BW(kg)]×6.25+urinary protein excretion (g/day) (*11*). Sodium chloride (NaCl) intake was measured by NaCl concentrations in the collected urine using the following formula: NaCl intake (g/day)=urinary sodium excretion (mEq/day)/17.

All values were expressed as the mean±SD. The baseline characteristics of the enrolled patients were tested for comparability between the losartan group and the amlodipine group using unpaired *t*-test or Fisher's exact test. The differences in changes in SBP and DBP between the two groups were tested by repeated-measures analysis of variance with treatment effect, period effect, and the interaction between treatment and period effect. Changes in urinary protein excretion, Scr, and Ccr within each group were analyzed by paired *t*-test. Unpaired *t*-test was used to compare the percent changes of urinary protein excretion, Scr, and Ccr between the losartan group and the amlodipine group. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

In all patients enrolled during the term from December 1999

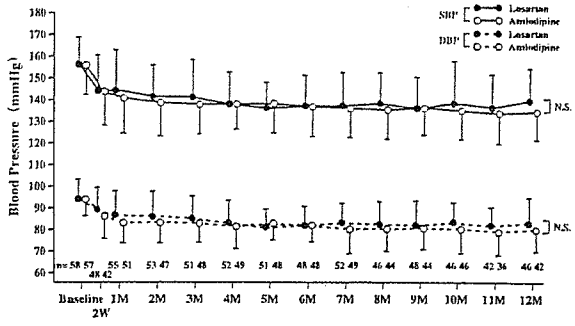

Fig. 2. *SBP and DBP changes (mmHg) throughout 12 months in groups treated with losartan and amlodipine. Circles and bars indicate the mean and SD. SBP and DBP were not significantly different between the losartan and amlodipine groups.*

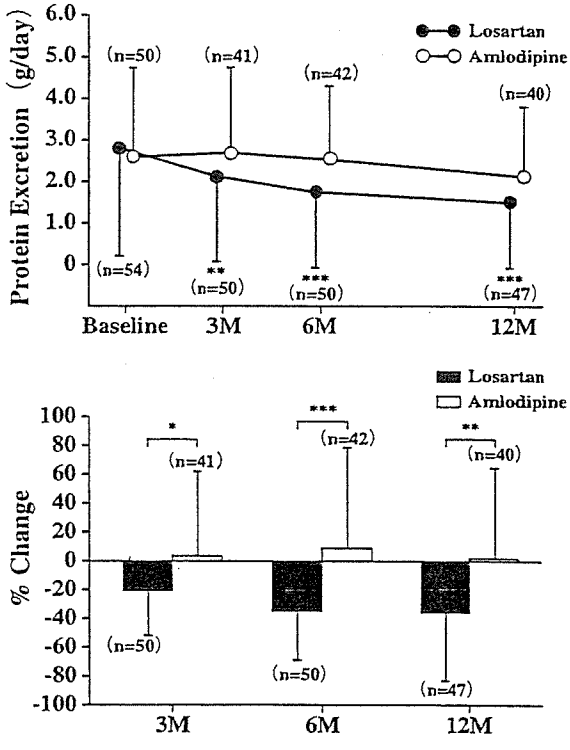

Fig. 3. *Changes in 24-h urinary protein excretion (upper panel) and respective percent changes (lower panel) from baseline. Circles and bars indicate the mean and SD.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

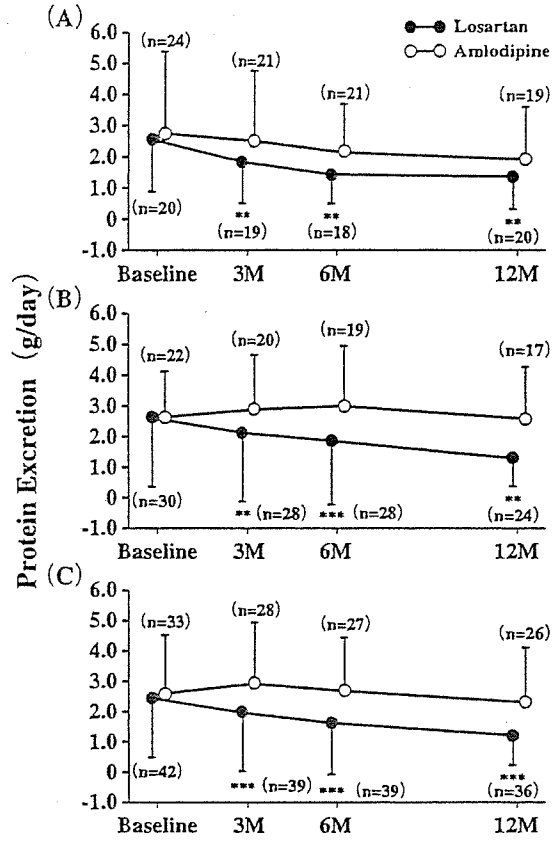

Fig. 4. *Changes in urinary protein excretion in patients stratified in response to BP control measured at month 3. (A) BP<140/90 mmHg. (B) BP≥140/90 mmHg. (C) BP≥130/85 mmHg. Note that patients in group C are included in either the group A or B because of respective BP ranges, as a consequence. Circles and bars indicate the mean and SD.* ** $p<0.01$, *** $p<0.001$.

to March 2002, 117 patients (58 for losartan and 59 for amlodipine) were eligible, as their baseline characteristics are shown in Table 1. A large number of patients were diagnosed with chronic glomerulonephritis, including IgA nephropathy. Patients with diabetic nephropathy and hypertensive nephrosclerosis were also included. The characteristics of the two treatment groups were similar. Forty-seven patients in the losartan group and 40 patients in the amlodipine group completed the 12-month study for measurement of urinary protein endpoint. The dietary compliance assessment of 24-h urinary urea nitrogen plus proteins and sodium showed that, there was no significant difference in total protein and NaCl intake between the two drug treatment groups at baseline and no change from baseline to month 3, as reported previously (*9*). At month 12, again, there was no change from baseline and therefore no difference between the losartan group and the amlodipine group in protein intake or NaCl intake (protein [g/day]: losartan, 50.7±19.7; amlodipine, 53.5±17.0; NaCl [g/day]: losartan, 8.0±3.8; amlodipine, 9.6±3.5).

The BP-lowering effect, in both systole (SBP) and diastole (DBP), was similar with losartan and amlodipine. Figure 2 shows changes in SBP and DBP measured at week 2 and at every month. In the losartan group, SBP was reduced from 156.5±12.2 mmHg at baseline to 139.5±14.8 mmHg at month 12 (−11.3±9.2%), and DBP from 94.0±9.2 mmHg at baseline to 83.0±11.7 mmHg at month 12 (−12.2±10.8%), and in the amlodipine group, the reduction in SBP

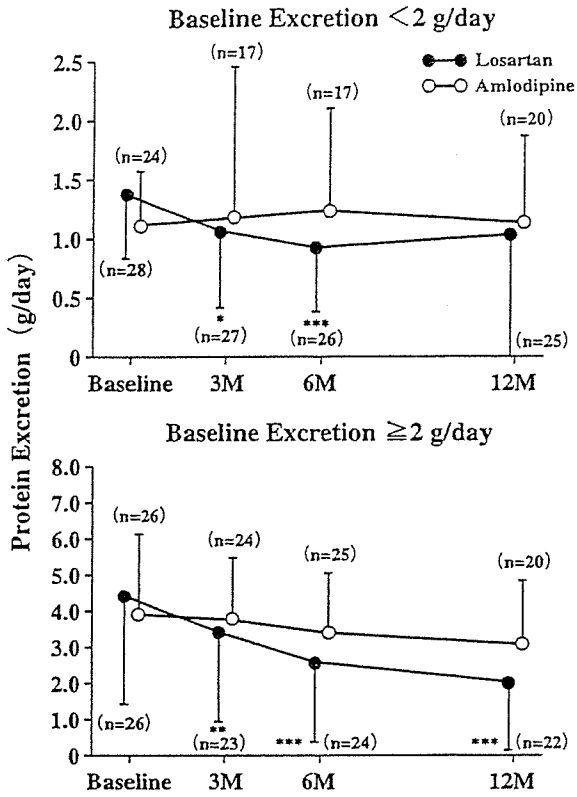

Fig. 5. *Changes in urinary protein excretion from baseline in patients stratified into two groups showing proteinuria of <2 g/day (upper panel) and ≥2 g/day (lower panel) as measured at baseline. Circles and bars indicate the mean and SD.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

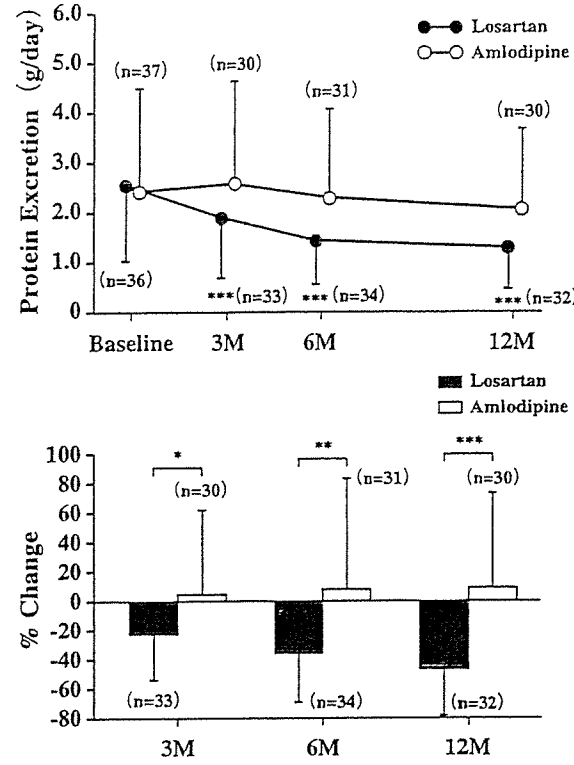

Fig. 6. *Changes in urinary protein excretion (upper panel) and respective percent changes (lower panel) in patients with chronic glomerulonephritis. Circles and bars indicate the mean and SD.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

was from 155.7±13.6 mmHg at baseline to 134.3±13.1 mmHg at month 12 (−12.7±10.0%), and that of DBP was from 94.1±7.9 mmHg at baseline to 79.7±10.1 mmHg at month 12 (−15.1±12.5%), respectively.

However, urinary protein excretion was significantly reduced only in the losartan group. The upper panel of Fig. 3 shows the change in urinary protein excretion and the lower panel shows the percent change from the respective baselines. The apparent changes in percent were −20.7%, −35.2%, and −35.8% at months 3, 6, and 12, respectively. We then analyzed the relationship between BP control and reduction of proteinuria in patients treated with losartan.

The responsiveness to the drug was assessed by BP measured at month 3. In this analysis, patients whose BP was controlled to <140/90 mmHg as well as those whose BP was not controlled at month 3 showed a statistically significant reduction in urinary protein excretion from baseline at each of months 3, 6, and 12. Although the JNC-VI guidelines recommend a BP goal of <130/85 mmHg for hypertensive patients with CKD (*12*), patients in whom this goal was not achieved still showed a statistically significant reduction in urinary protein excretion by losartan (Fig. 4). In the losartan group with a BP of <130/85 mmHg, there was an apparent reduction in urinary protein excretion, but without statistical significance.

Although at baseline there was no statistically significant difference between treatment groups in the ratio of males to females (Table 1), the number of female patients in the amlodipine group decreased during the study. However, in the losartan group, changes in proteinuria were almost comparable between males and females: −21.0% ($n=31$) and −20.2% ($n=19$) at month 3, −35.5% ($n=31$) and −34.6% ($n=19$) at month 6, and −35.2% ($n=29$) and −36.9% ($n=18$) at month 12 in males and females, respectively. Likewise, although no effect was observed with amlodipine, changes in the amount of proteinuria in males and females were +7.1% (n=31) and −8.0% ($n=10$) at month 3, +13.6% ($n=30$) and −4.6% ($n=12$) at month 6, and −1.5% ($n=30$) and +10.6% ($n=10$) at month 12, respectively.

In order to examine whether the magnitude of proteinuria affected the result of treatments with losartan and amlodipine, we stratified patients into two subgroups: those with proteinuria <1 g/day and those with proteinuria ≥1 g/day at baseline. In these subgroups, the change in urinary protein excretion from baseline was not significantly different between the losartan group and the amlodipine group. We next stratified patients with proteinuria levels of <2 g/day and

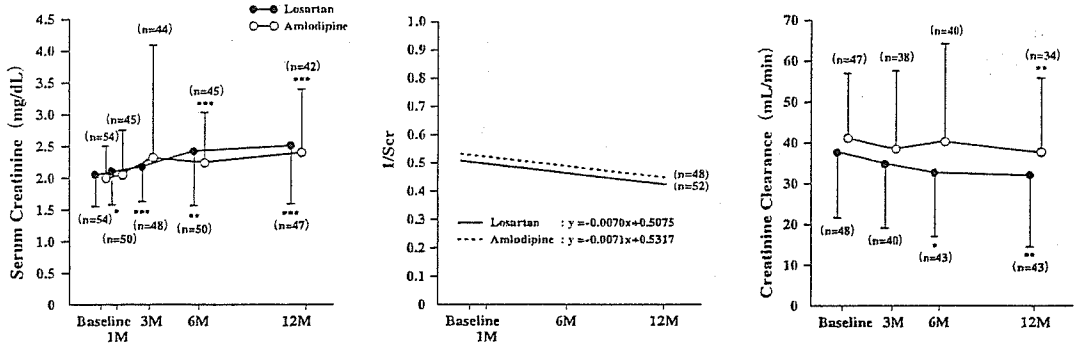

Fig. 7. *Changes in Scr (left panel), 1/Scr (middle panel), and creatinine clearance (right panel) in patients treated with losartan for Scr and 1/Scr. Circles and bars indicate the mean and SD.* * $p<0.05$, ** $p<0.01$, *** $p<0.001$. *There was no difference for the slope of 1/Scr between the losartan and amlodipine group.*

≥2 g/day at baseline. As shown in Fig. 5, the reduction in urinary protein excretion was evident in losartan groups of both <2 g/day and ≥2 g/day. Again, amlodipine did not significantly reduce urinary protein excretion in both groups of <2 g/day and ≥2 g/day.

With respect to the diagnosis of patients, 38 patients in the losartan group and 41 in the amlodipine group had chronic glomerulonephritis, and 7 in the losartan group and 7 in the amlodipine group had diabetic nephropathy. Analysis of the patients with diabetic nephropathy revealed an apparent decrease from baseline in urinary protein excretion in the two treatment groups, with no statistically significant difference between the groups (data not shown). Analysis of the subgroup with chronic glomerulonephritis exhibited a statistically significant reduction in proteinuria in the losartan group at months 3, 6, and 12. Because amlodipine did not reduce proteinuria in patients with chronic glomerulonephritis, there was a prominent difference in the percent reduction in urinary protein excretion from baseline between the two treatment groups (Fig. 6).

Changes in Ccr and Scr and the slope of 1/Scr did not differ between the two treatment groups. Scr slightly increased from the baseline to month 3 in both groups. Ccr showed a tendency of decline (Fig. 7).

Adverse events considered to be possibly related to the study were reported for increases in aspartate aminotransferase (AST; GOT) (2 cases), alanine aminotransferase (ALT; GPT) (1 case) and γ-GTP (4 cases). These changes were mild and the incidence was almost the same between the losartan group and the amlodipine group. An increase in serum uric acid (2 cases) was reported in the amlodipine group, but was not observed in the losartan group. Hyperkalemia ranging from 5.1 to 6.9 mEq/l was reported in the losartan group (3 cases) and in the amlodipine group (2 cases). Two cases of dizziness and 1 case of transient ischemic attack were reported in the losartan and amlodipine groups. No fatal adverse events were observed in either group during the 12-month study.

Discussion

The present study demonstrated that, in patients with proteinuric CKD and hypertension, losartan effectively reduced proteinuria while amlodipine did not. It is noteworthy that the potency of BP-lowering of losartan and amlodipine was same throughout the entire 12-month study period. Allocation of patients resulted in an almost comparable male to female ratio between the treatment groups at baseline. However, more number of female patients decreased in the amlodipine group than in the losartan group as the study progressed. Consequently, at month 12, in the losartan group, the male/female ratio was 29/18, while in the amlodipine group it was 30/10. Although the losartan group included a greater number of female patients than the amlodipine group at months 3, 6, and 12, the percent reduction in urinary protein excretion in males was comparable to that in females in the losartan group. Therefore, it was unlikely that a sex hormone such as estrogen played a role in the vascular protection in this study. The fact that a large majority of female patients in the losartan group at baseline were aged (22 females: 54–59 year-old, 4; in their 60's, 9; in her 70's, 1) may warrant this discussion, because female patients of mid-50's or older were probably undergoing menopause.

In the present study, we first stratified patients into 3 subgroups with regard to BP reduction measured at month 3. The first 3 months was a meaningful period because no other drugs was added on either losartan or amlodipine during this period. Losartan reduced both BP and proteinuria. However, it was also true that not all patients responded to losartan to reach the goal BP of <130/85 mmHg that was recommended by the JNC-VI (*12*). In fact, the goal BP was achieved in only 8 patients in the losartan group and 13 patients in the amlodipine group. It was expected that patients who reached the goal BP of <130/85 mmHg would show a prominent decrease in urinary protein excretion. However, there was no significant change in urinary protein excretion from baseline in either the losartan group or the amlodipine group, al-

though in the losartan group urinary protein tended to decrease. The reason for this finding is unclear; however, since the number of patients in each group was very small, this might be the reason why we failed to demonstrate statistical significance, especially in the losartan group. Nonetheless, even in patients who did not accomplish the BP goal, reduction of proteinuria was evident. Likewise, patients who achieved a BP of $<140/90$ mmHg represented the anti-proteinuric effect of losartan. A striking evidence was that patients who did not accomplish the level of BP $<140/90$ mmHg also showed the reduction in proteinuria, the degree of which did not largely differ from those in the group of BP $<140/90$ mmHg.

It must not be a conclusion that, in patients with CKD and hypertension, it is sufficient to pursue a reduction in proteinuria without a corresponding reduction in BP. It should be emphasized that BP control is still an important strategy in treating patients with CKD and hypertension, as the JNC-VI recommends. Our results can only be taken to indicate that losartan may still be effective to reduce proteinuria, even if BP can not reach the BP goal of the JNC-VI guidelines (*12*). In this aspect, losartan should be used in clinical practice under the condition of exerting anti-hypertensive effect. The goal BP of $<130/80$ mmHg for patients with CKD which was currently recommended by JNC-VII guideline (*13*) should also be taken into account. Thus, the use of losartan will bring better outcomes for patients with CKD and hypertension with concomitant BP control.

Although we failed to find a difference in anti-proteinuric effect between losartan and amlodipine when patients were stratified with the baseline proteinuria of <1 g/day and ≥ 1 g/day, further stratification with levels of <2 g/day and ≥ 2 g/day clearly demonstrated the anti-proteinuric effect of losartan at all assay points in the group of ≥ 2 g/day. These results suggest that losartan was effective to reduce severe proteinuria of probably glomerular origin. The effect was still observable in the group of <2 g/day at months 3 and 6, but was not statistically significant at month 12, probably due to a wide range of standard deviation from the mean value. Very recently, Tojo *et al.* (*14*) reported that, in streptozotocin-induced diabetic rats, intervention of actions of angiotensin II by either an ACE inhibitor or an angiotensin II antagonist restored albumin reabsorption in the proximal tubules without changing blood glucose *via* restoration of the expression of megalin, a glycoprotein responsible for reabsorption of proteins in the proximal tubules, resulting in the reduction in urinary protein excretion. The authors suggested that expression of megalin is suppressed in the proximal tubules when the kidney is impaired for tubular dysfunction. This evidence may explain, at least in part, our results on the effect of losartan on proteinuria, a part of which may be of tubular origin.

While the RENAAL study (*8*) was conducted in patients with type 2 diabetes, a large majority of the patients enrolled in the present study had chronic glomerulonephritis including cases of immunoglobulin A (IgA) nephropathy. In these patients, losartan effectively reduced urinary protein excretion. Chronic glomerulonephritis involves many factors in its etiology, and the complicated proteinuria is not solely a result of hyperfiltration of glomeruli. Rather, remodeling of the glomerulus must be considered. Since amlodipine did not affect the protein excretion in such patients, the present result is of particular interest in considering the direct actions of angiotensin II on the structure and functions of glomeruli. Patients with diabetic nephropathy in the losartan group and the amlodipine group were 7 and 5 on the day of start and only 5 and 4 patients completed the study, respectively. Because of this limited number of diabetic patients, there was no statistically significant change in urinary protein excretion in either drug treatment group, although the magnitude of the mean reduction of urinary protein ranged from -30% to -50%. We therefore cannot conclude from these results that these drugs have no anti-proteinuric effect in patients with diabetic nephropathy.

With respect to the pharmacotherapy of patients with CKD, the therapeutic benefit of interfering with the actions of angiotensin II has been extensively documented with ACE inhibitors over the last decade. The breakthrough evidence that direct blockade of angiotensin II receptors protects the kidney in patients with type 2 diabetic nephropathy was provided by the RENAAL study (*8*) with losartan, and the IDNT study with irbesartan (*15*).

Recent publications provided evidences that the angiotensin II receptor antagonist candesartan was effective in Japanese patients with type-2 diabetic nephropathy, with a dose as low as 4 mg/day to prevent aggravation of proteinuria (*16*), or reduce urinary protein excretion by combination therapy with amlodipine (*17*), supporting previous evidences on losartan and irbesartan for diabetic nephropathy. The results of our present study provide the additional information useful in clinical practice, that losartan is effective not only for patients with type 2 diabetic nephropathy, but also those with a variety of types of CKD. Nakao *et al.* (*18*) recently studied the effect of combination therapy and monotherapy with losartan and the ACE inhibitor trandolapril in patients with non-diabetic renal disease. They demonstrated that losartan as well as trandolapril effectively lowered urinary protein excretion, although the combination of these two drugs exerted a more favorable effect on proteinuria. Taken together, the antiproteinuric effect of losartan may play a major role in its renoprotective effect.

The therapeutic benefit of losartan for kidney diseases in comparison to other antihypertensive drugs is still not fully explained. As is indicated in the JNC-VI (*12*) and JNC-VII (*13*) guidelines and several clinical reports, aggressive blood pressure control is mostly important. On the other hand, many clinical trials have demonstrated that blood pressure control is not the only factor pertinent for renoprotection; rather, ACE inhibitors and angiotensin II receptor antagonists provide additional benefit in patients with kidney dis-

eases.

The RAS is now well understood to be involved in the pathogenesis of renal impairment independent of its vasoconstrictive actions, inducing disturbance of glomerular and tubular functions. The direct actions of angiotensin II in the kidney include an increase in tubular sodium reabsorption and an influence on glomerular filtration rate (GFR), but morphopathological changes such as accumulation of extracellular matrix and mesangial cell proliferation and hypertrophy (*19*, *20*) are of more importance for pathogenesis of renal impairment. These concepts clearly constitute the theory of usefulness of blocking the actions of angiotensin II in kidney diseases. Although the UK Prospective Diabetes Study (UKPDS) (*21*) concluded that the effects of ACE inhibitor captopril and the β-blocker atenolol were similar in reducing the risk of macrovascular and microvascular complications related to type 2 diabetes, the African-American Study of Kidney Disease and Hypertension (AASK) Study (*22*), which compared the effects of the ACE inhibitor ramipril, the calcium channel blocker amlodipine, and the β-blocker metoprolol on the progression of hypertensive renal disease in African-Americans, showed that ramipril induced a slower decline in GFR and a lower risk of clinical end points compared to amlodipine.

The mechanism and mode of action of losartan and amlodipine to explain the exertion of different effect of renoprotection are not thoroughly explained and are controversial. Documents are available to explain the renoprotective efficacy of calcium channel blockers, including amlodipine. However, whether calcium channel blockers exert unique antiproteinuric effects is still controversial. In the AASK Study (*22*), proteinuria was not decreased with amlodipine. The Japan Multicenter Investigation of Antihypertensive Treatment for Nephropathy in Diabetes (J-MIND) study (*23*) reported that nifedipine retard and enalapril had a similar effect on nephropathy in hypertensive type 2 diabetic Japanese patients, but albumin excretion rate was not reduced with either drug despite the effective BP lowering. Kumagai *et al.* (*24*) reported the comparative evaluation of amlodipine with ACE inhibitors enalapril or captopril for renoprotective effect in hypertensive patients with renal dysfunction. They concluded that the effect of 1-year treatment with amlodipine on renal function was likely the same as that of ACE inhibitors. They also showed that urinary protein excretion tended to be reduced by either ACE inhibitor or amlodipine, but without statistical significance. These evidences suggest that, while a strong argument has been made for proteinuria as a risk factor for progression of renal disease (*25*), there is still a discrepancy between renoprotection as a final goal and urinary protein excretion as an important clinical sign for renal dysfunction.

There is thus a strong body of evidence suggesting that the pathways by which angiotensin II aggravates renal functions are mediated by angiotensin II type 1 (AT_1) receptors. Calcium channel blockers act to dilate the microvasculature, improving regional circulation by regulating the voltage-dependent calcium channels. The blockade of angiotensin II receptors results in a reduction in renal perfusion pressure in addition to dilation of the efferent arterioles to a greater extent than the afferent arterioles because of their different manner of constriction in response to angiotensin II, and thus angiotensin II antagonists reduce the glomerular filtration pressure to same extent. On the other hand, the action of angiotensin II is not solely to constrict macrovascular and microvascular trees, but a variety of cellular actions are evident. A number of reports have described roles of angiotensin II through AT_1 receptors to produce extracellular matrix as well as to stimulate proliferation and/or hypertrophy of many types of cells; *via* the direct stimulation of mitogen-activated protein kinase (MAPK), transforming growth factor (TGF-β), nuclear factor (NF-κB), induction of proto-oncogenes, and so on (*19*, *20*, *26*). Thus, although there is still no confirmatory theory, wider biological functions of angiotensin II may explain the diversity of renoprotective activity of the two drugs without depending on their BP lowering efficacy. The precise mechanism of the action of these drugs should be further investigated.

In the present study, there was no change in Ccr either in the losartan or amlodipine groups. Andersen *et al.* (*27*) conducted a 2-month, randomized, double-blind cross-over clinical trial to evaluate the effect of losartan and the ACE inhibitor enalapril in patients with type 1 diabetic nephropathy, and reported that angiotensin II blockade reduced urinary protein excretion without changing GFR. In the RENAAL study (*8*), the risk of a doubling of the serum creatinine concentration in the losartan treatment group and the placebo group was almost the same until 12 months from initiation of the study, although the reduction in urinary protein excretion was observed in the losartan treatment group within 6 months. The IDNT study (*15*) with irbesartan also reported no difference in the change in serum creatinine in comparison to placebo and amlodipine within 12 months. Thus, it is likely that effects on proteinuria and on Ccr differ in response to blockade of angiotensin II receptors, although the reason is not explained. The present study was completed at 12 months. It might be expected that longer-term treatment of the patients with CKD and hypertension with losartan would have more beneficial effects on renal functions such as improvement of GFR in patients beyond the effect to reduce proteinuria.

In conclusion, a term of total 12 months treatments of Japanese patients with proteinuric CKD and hypertension with losartan reduced proteinuria more effectively than amlodipine, although BP lowering effect was not different between the two drug-treated groups. Since the effect was beyond the blood pressure control, losartan is effective in patients with CKD manifesting proteinuria and hypertension.

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Serum Interleukin-18 Levels Are Associated With Nephropathy and Atherosclerosis in Japanese Patients With Type 2 Diabetes

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OBJECTIVE — Interleukin (IL)-18 is a proinflammatory cytokine secreted from mononuclear cells. Serum concentration of IL-18 is a strong predictor of death in patients with cardiovascular diseases. Recent studies have shown that microinflammation is involved in the pathogenesis of diabetic nephropathy as well as of cardiovascular diseases. This study aimed to test the hypothesis that the serum level of IL-18 is a common predictor of nephropathy and atherosclerosis in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — Eighty-two Japanese patients with type 2 diabetes and 55 age- and sex-matched healthy control subjects were enrolled. Patients with renal dysfunction (creatinine clearance <1 ml/s) were excluded. We assessed clinical parameters and measured serum and urinary IL-18 levels, serum IL-6 levels, carotid intima-media thickness (IMT), and brachial-ankle pulse wave velocity (baPWV) in all patients. Further, we evaluated changes of urinary albumin excretion rate (AER) after 6 months in 76 diabetic patients.

RESULTS — Serum and urinary IL-18 levels were significantly elevated in patients with type 2 diabetes as compared with control subjects (serum IL-18 179 ± 62 vs. 121 ± 55 pg/ml, $P < 0.001$; urinary IL-18 97 ± 159 vs. 47 ± 54 pg/ml, $P = 0.035$). Univariate linear regression analysis showed significant positive correlations between serum IL-18 and AER (r [correlation coefficient] = 0.525, $P < 0.001$), HbA_{1c} ($r = 0.242$, $P = 0.029$), high-sensitivity C-reactive protein (hs-CRP) ($r = 0.240$, $P = 0.031$), and urinary β-2 microglobulin ($r = 0.235$, $P = 0.036$). Serum IL-18 levels also correlated positively with carotid IMT ($r = 0.225$, $P = 0.042$) and baPWV ($r = 0.232$, $P = 0.040$). We also found a significant correlation between urinary IL-18 and AER ($r = 0.309$, $P = 0.005$). Multivariate linear regression analysis showed that AER (standard correlation coefficients [B] = 0.405, $P < 0.001$) and hs-CRP ($B = 0.207$, $P = 0.033$) were independently associated with serum IL-18 levels. AER was also independently associated with urinary IL-18 levels ($B = 0.295$, $P = 0.005$). Moreover, serum and urinary IL-18 levels correlated positively with AER after 6 months ($r = 0.489$, $P < 0.001$ and $r = 0.320$, $P = 0.005$) and changes in AER during the follow-up period ($r = 0.268$, $P = 0.018$ and $r = 0.234$, $P = 0.042$).

CONCLUSIONS — Serum levels of IL-18 might be a predictor of progression of diabetic nephropathy as well as cardiovascular diseases.

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Abbreviations: ACEI, ACE inhibitor; AER, albumin excretion rate; ARB, angiotensin II type 1 receptor blocker; baPWV, brachial-ankle pulse wave velocity; DBP, diastolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; ICAM, intercellular adhesion molecule; IL, interleukin; IMT, intima-media thickness; SBP, systolic blood pressure; TNF, tumor necrosis factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Low-grade inflammation (microinflammation) occurs in diabetic patients as well as those with cardiovascular diseases (1,2). Several reports indicate that high-sensitivity C-reactive protein (hs-CRP) (3) and proinflammatory cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and IL-18 are elevated in patients with type 2 diabetes (4–7). The mechanisms for elevation of serum IL-18 levels in type 2 diabetes remain unclear, although oxidative stress is a candidate (8). Activation of nuclear factor-κB through oxidative stress induced by hyperglycemia increases concentrations of circulating proinflammatory cytokines (2).

High serum IL-18 concentrations have recently been identified as a strong predictor of death in patients with coronary artery disease (9) and acute ischemic stroke (10). A major mechanism of cardiovascular events mediated by IL-18 is decreased stability of plaque. Carotid intima-media thickness (IMT) measured by carotid ultrasound is a useful tool for assessing cardiovascular diseases in diabetes (11), and a clinical study demonstrated that carotid IMT in patients with high IL-18 shows a greater thickness than in patients with normal IL-18 (12).

Microalbuminuria is a predictor of cardiovascular and renal risk in diabetes (13) and nondiabetes (13,14). Patients with diabetic nephropathy, especially in the context of type 2 diabetes, have a high incidence of cardiovascular disease, which leads to increased mortality (15). Indeed, worldwide, diabetic nephropathy is the major reason for dialysis, and survival of type 2 diabetes undergoing dialysis therapy is very poor due to cardiovascular events. However, the precise mechanisms underlying the relationship between microalbuminuria and cardiovascular disease remain unclear.

Recent studies, including ours, suggest that an inflammatory mechanism mediated by macrophages may play important roles in the pathogenesis of diabetic nephropathy. We previously demonstrated that the intercellular adhesion

molecule (ICAM)-1 is upregulated and mediates infiltration of macrophages in kidneys of patients with diabetic nephropathy and in diabetic animals (16–18). Moreover, we have reported that ICAM-1–deficient mice are resistant to renal injuries after induction of diabetes, suggesting that inflammatory processes contribute to the development of diabetic nephropathy. IL-18 is a proinflammatory cytokine produced from activated macrophages. Recently, serum IL-18 levels have been reported elevated in patients with diabetic nephropathy (19). IL-18 is known to lead to production of other proinflammatory cytokines (20), endothelial apoptosis (21), upregulation of ICAM-1 (22), and hyperhomocysteinemia (12). Thus, IL-18 might be an important factor not only in the process of atherosclerosis but also in the development and progression of diabetic nephropathy.

This study aims to investigate whether serum and urinary IL-18 levels are predictors of diabetic nephropathy as well as of atherosclerosis in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

— A total of 82 patients (48 females and 34 males) with type 2 diabetes who had been referred to the diabetes outpatient department at the Okayama Saiseikai General Hospital were enrolled. The diagnosis of type 2 diabetes was made in accordance to the criteria of the World Health Organization. All patients who fulfilled the following inclusion criteria were considered for the study: no episodes of ketoacidosis, initial diagnosis of diabetes at >40 years of age, no demonstrable antibodies to GAD and renal dysfunction (creatinine clearance <1.00 ml/s). Age was 62.5 ± 7.5 years and diabetes duration 10.8 ± 6.3 years (means ± SD). BMI was 23.8 ± 3.0 kg/m^2. Past history of cardiovascular disease was defined as a clinical attack of stroke, ischemic heart disease, and arteriosclerosis obliterance.

Venous blood and urine were obtained in the early morning after an overnight fast. Urinary albumin excretion rate (AER) was measured with an immunoturbidimetric assay Micro Alb (Nitto Boseki, Tokyo, Japan). Normoalbuminuria was defined as AER <30 mg/gCr (n = 41), microalbuminuria as AER 30–299 mg/gCr (n = 31), and macroalbuminuria as AER >300 mg/gCr (n = 10).

Thirty-two patients received insulin therapy, 49 received oral antidiabetic agents, and 9 received only diet therapy. Twenty-two patients had hypertension, defined as systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg or, alternatively, as having treatment with one or more antihypertensive agents. The latter included ACE inhibitor (ACEI) or angiotensin II type 1 receptor blocker (ARB) (n = 34), combination therapy with ACEI and ARB (n = 7), and statins (n = 20). No patients included in this study received hormone replacement therapy.

As control subjects, 55 nondiabetic subjects (32 females and 23 males), without any medical treatment, were selected to match the overall age and sex distribution of the patients with type 2 diabetes. The control subjects also fulfilled the following inclusion criteria: normal blood pressure (SBP <140 mmHg and DBP <90 mmHg), normal glucose tolerance (fasting plasma glucose <6.11 mmol/l and HbA_{1c} (A1C) <5.8%), AER <30 mg/gCr, creatinine clearance >1.00 ml/s, no clinical history of cardiovascular disease, and no symptoms of acute inflammatory disease. The mean age of healthy control subjects was 59.5 ± 8.7 years. Informed consent was obtained from all participants, and the study was approved by the ethical committee of Okayama Saiseikai General Hospital.

Measurement of serum and urinary levels of IL-18 and serum levels of hs-CRP and IL-6

Serum levels of hs-CRP were measured using an immunonephelometric assay kit (Dade Behring, Marburg, Germany). Serum levels of IL-6 were measured using a chemiluminescent enzyme assay (CLEIA kit; Fujirebio, Tokyo, Japan). Serum and urinary IL-18 levels were measured using a commercially available enzyme-linked immunosorbent assay (MBL, Nagoya, Japan). Urinary IL-18 levels were divided by urinary creatinine levels (pg/mlCr).

Measurements of carotid IMT and brachial-ankle pulse wave velocity

IMT of the common carotid artery was determined using duplex ultrasonography with a 7.5-MHz linear transducer (SSD-5500; Aloka, Tokyo, Japan). Carotid IMT was defined as the distance from the leading edge of the first echogenic line to the leading edge of the second on a sonographic image. Measurements of IMT were made at each of the three sites of the greatest thickness on both sides. Carotid IMT was defined as the mean of these maximal IMT measurements. SBP and DBP were measured twice with the patient in a sitting position after 5 min rest. ABI-form (BP-203RPE II; Nippon Colin, Komaki, Japan) allows an automated multiple pulse wave measurement and was used to measure left and right brachial-ankle pulse wave velocity (baPWV). A trained physician at our institution performed all scans. In this study, the highest values of SBP, DBP, IMT, and baPWV from the left and the right sides were used for the evaluation of each patient.

AER follow-up after 6 months

After the initial assessment of baseline AER (pre-AER), measurement of AER was repeated after 6 months in patients continuing with the same treatment during follow-up. AER after 6 months (post-AER) and changes in AER over the 6 months (post-AER–to–pre-AER ratio) were assessed.

Statistics

Statistical analyses were performed with the SPSS for Windows statistical software system. Data are presented as means ± SD or actual numbers. Variables of serum or urinary IL-18 levels, serum IL-6 levels, hs-CRP, pre-AER, post-AER, and post-AER–to–pre-AER ratio did not show a Gaussian distribution (Shapiro-Wilks test), and natural logarithmic transformation was used to render the distribution of these variables normal (ln). Comparisons of data between control subjects and patients with type 2 diabetes were analyzed by Student's unpaired t test or χ^2 test for sex (female). Correlation was determined by univariate or multivariate linear regression analysis. Differences between mean in pre- and post-AER were assessed with Student's paired t test. A P value <0.05 was accepted as indicating statistical significance.

RESULTS

Association of serum and urinary IL-18 levels with clinical data

Characteristics of control subjects and patients with type 2 diabetes and univariate analysis of relationships between serum or urinary IL-18 and characteristics of patients with type 2 diabetes are shown in Table 1. Serum IL-18 levels, urinary IL-18 levels, and serum IL-6 levels were significantly higher in patients with type 2 diabetes than in age- and sex-matched control subjects (serum IL-18 179 ± 62

Table 1—*Characteristics of control and type 2 diabetic subjects, and univariate analysis of relationships between logarithmic serum or urinary IL-18 levels and characteristics of type 2 diabetes*

				(ln)serum IL-18		(ln)urinary IL-18	
	Control subjects	Type 2 diabetic subjects	P*	r	P	r	P
n	55	82					
Sex (female) (*n*)	32	48	0.076	0.175	0.123	0.255	0.022†
Age (years)	59.5 ± 8.7	62.5 ± 7.5	0.054	−0.172	0.118	0.152	0.117
Duration of diabetes (years)	—	10.8 ± 6.3	—	0.120	0.282	0.073	0.517
History of cardiovascular events (yes) (*n*)	—	17	—	0.174	0.117	0.136	0.229
BMI (kg/m^2)	22.4 ± 2.5	23.8 ± 3.0	0.005‡	0.142	0.204	−0.130	0.251
SBP (mmHg)	124 ± 14	131 ± 16	0.004‡	0.147	0.187	0.011	0.925
DBP (mmHg)	75 ± 10	77 ± 10	0.215	0.140	0.208	−0.920	0.418
Total cholesterol (mmol/l)	5.59 ± 0.98	5.28 ± 0.80	0.044†	−0.025	0.824	0.088	0.437
HDL cholesterol (mmol/l)	1.60 ± 0.44	1.34 ± 0.41	0.001‡	−0.138	0.218	0.041	0.718
LDL cholesterol (mmol/1)	3.31 ± 0.85	3.21 ± 0.75	0.428	−0.060	0.590	0.123	0.275
Lipoprotein-α (g/l)	0.25 ± 0.20	0.22 ± 0.18	0.478	−0.460	0.687	0.076	0.515
Fasting plasma glucose (mmol/l)	5.47 ± 0.51	8.44 ± 3.28	<0.001§	0.213	0.055	−0.018	0.877
A1C (%)	5.1 ± 0.5	7.3 ± 1.1	<0.001§	0.242	0.029†	0.128	0.259
Creatinine clearance (ml/s)	1.55 ± 0.37	1.53 ± 0.49	0.766	0.097	0.384	−0.003	0.982
Serum β-2 microglobulin (μg/ml)	1.66 ± 0.28	1.83 ± 0.47	0.022†	0.088	0.435	−0.017	0.883
Urinary β-2 microglobulin (μg/ml)	0.06 ± 0.07	0.14 ± 0.20	0.009‡	0.235	0.036†	0.136	0.233
Fibrinogen (g/l)	2.85 ± 0.61	3.00 ± 0.53	0.144	0.054	0.630	0.172	0.130
AER (mg/gCr)	8 ± 7	103 ± 432	—	—	—	—	—
(ln)AER (1n[mg/gCr])	1.85 ± 0.74	2.91 ± 1.55	<0.001§	0.525	<0.001§	0.309	0.005‡
hs-CRP (mg/l)	1.02 ± 3.24	1.05 ± 1.43	—	—	—	—	—
(ln)hs-CRP (1n[mg/l])	−0.93 ± 1.12	−0.45 ± 0.97	0.009‡	0.240	0.031†	0.087	0.441
Patient with ACEI or ARB (yes) (*n*)	—	34	—	0.227	0.041†	−0.031	0.782
Patient with statins (yes) (*n*)	—	20	—	0.275	0.021†	−0.028	0.804
Carotid IMT (mm)	0.70 ± 0.14	0.86 ± 0.18	<0.001§	0.225	0.042†	0.034	0.768
baPWV (m/s)	14.2 ± 3.5	17.1 ± 3.4	<0.001§	0.232	0.040†	0.208	0.068
Serum IL-6 (pg/ml)	1.50 ± 1.24	1.95 ± 1.16	—	—	—	—	—
(ln)serum IL-6 (1n[pg/ml])	0.20 ± 0.62	0.49 ± 0.60	0.006‡	0.032	0.779	0.029	0.798
Serum IL-18 (pg/ml)	121 ± 55	179 ± 62	—	—	—	—	—
(ln)serum IL-18 (1n[pg/ml])	4.69 ± 0.48	5.14 ± 0.34	<0.001§	—	—	−0.019	0.866
Urinary IL-18 (pg/mlCr)	47 ± 54	97 ± 159	—	—	—	—	—
(ln)urinary IL-18 (1n[pg/mlCr])	3.28 ± 1.11	5.14 ± 0.34	0.035†	−0.019	0.866	—	—

Data are means ± SD. *P for type 2 diabetic versus control subjects. †$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$. *r*, correlation coefficient.

vs. 121 ± 55 pg/ml, $P < 0.001$; urinary IL-18 97 ± 159 vs. 47 ± 54 pg/ml, $P = 0.035$; IL-6 1.95 ± 1.16 vs. 1.50 ± 1.24 pg/ml, $P = 0.006$; age 62.5 ± 7.5 vs. 59.5 ± 8.7 years, $P = 0.054$). By univariate linear regression analysis, we found a significant correlation between serum IL-18 and A1C (r [correlation coefficient] = 0.242, $P = 0.029$), urinary β-2 microglobulin ($r = 0.235$, $P = 0.036$), patients with ACEI or ARB (yes; $r = 0.227$, $P = 0.041$), patients with statins (yes; $r = 0.275$, $P = 0.021$), urinary AER ($r = 0.525$, $P < 0.001$), or hs-CRP ($r = 0.240$, $P = 0.031$) in patients with type 2 diabetes. On the other hand, we found no significant correlation between serum IL-18 levels and A1C, urinary β-2 microglobulin, or hs-CRP in control subjects. Moreover, we found a significant correlation between urinary IL-18 and sex (female: $r = 0.255$, $P = 0.022$) or AER ($r = 0.309$, $P = 0.005$) in patients with type 2 diabetes. However, we found no significant correlation between serum IL-18 levels and urinary IL-18 levels or serum IL-6 levels in control subjects and in patients with type 2 diabetes.

Independent factors of serum and urinary IL-18 levels in patients with type 2 diabetes

We next performed multivariate linear regression analysis for factors significantly correlated with serum and urinary IL-18 levels (Table 2). AER (standard correlation coefficients [B] = 0.405, $P < 0.001$) and hs-CRP ($B = 207$, $P = 0.033$) were independently associated with serum IL-18 levels. AER was also independently associated with urinary IL-18 levels ($B = 0.295$, $P = 0.005$).

Association of serum and urinary IL-18 levels with parameters of atherosclerosis

We performed univariate analysis of the relationships between the parameters of atherosclerosis and IL-18 levels in patients with type 2 diabetes (Table 1). Serum IL-18 levels correlated positively with carotid IMT and baPWV ($r = 0.225$; $P = 0.042$ and $r = 0.232$, $P = 0.040$). Urinary IL-18 levels were not related to IMT and baPWV. We also found no significant correlation between serum IL-18 levels and carotid IMT or baPWV in control subjects.

Table 2—*Multivariate analysis of relationships between logarithmic serum or urinary IL-18 levels and characteristics of type 2 diabetes*

Variables	*B*	*P*
Dependent variable: (ln)serum IL-18, R^2 = 0.378, P <0.001		
Independent variable		
(ln)AER	0.405	<0.001
(ln)hs-CRP	0.207	0.033
Patient with statins (yes)	0.158	0.108
Urinary β-2 microglobulin	0.157	0.118
A1C	0.114	0.242
Patient with ACEI or ARB (yes)	0.021	0.838
Dependent variable: (ln)urinary IL-18, R^2 = 0.138, P = 0.003		
Independent variable		
(ln)AER	0.295	0.005
Sex (female)	0.208	0.053

B, standard correlation coefficients; R^2, multiple coefficients of determination.

Relationships between serum or urinary IL-18 levels and AER after 6 months or changes in AER during the follow-up period

During the following-up period, two patients dropped out and four (two with hyperglycemia, two with cardiovascular disease) were admitted to hospitals. Consequently, post-AER was assessed in 76 patients (Fig. 1). Pre-AER in 76 patients was 107 ± 440 mg/gCr [(ln)pre-AER 3.03 ± 1.55 (ln)mg/gCr]. Changes in AER were revealed as post-AER–to–pre-AER ratio. The mean of AER showed no significant change during the follow-up period [post-AER 151 ± 601 mg/gCr, (ln) post-AER 3.03 ± 1.71 (ln)mg/gCr, P = 0.958]. Serum and urinary IL-18 levels correlated positively with post-AER (r = 0.489, P < 0.001 and r = 0.320, P = 0.005). Moreover, serum and urinary IL-18 levels correlated positively with the post-AER–to–pre-AER ratio (r = 0.268, P = 0.018 and r = 0.234, P = 0.042).

CONCLUSIONS — In the present study, we found that serum IL-18 levels were closely correlated with AER as well as with carotid IMT and baPWV in patients with type 2 diabetes. AER was an independent determinant of serum and urinary IL-18 levels. Moreover, serum and urinary IL-18 levels correlated positively with AER after 6 months and changes in AER during the follow-up period. These results provide the first evidence of a close association of serum and urinary IL-18 levels with AER. The serum IL-18 level might be a predictor not only of cardiovascular diseases but also of diabetic nephropathy in patients with type 2 diabetes.

A1C and hs-CRP were also positively correlated with serum IL-18 levels, with hs-CRP being an independent determinant of serum IL-18 levels. However, there was no significant correlation between serum IL-18 and serum IL-6 levels. In our present study, serum IL-6 levels were not correlated with AER. While there have been several studies suggesting that IL-6 is involved in the pathogenesis of diabetic nephropathy in vivo and in vitro (23–26), Moriwaki et al. (19) reported that serum IL-18 and tumor necrosis factor (TNF)-α levels were significantly elevated in diabetic patients with microalbunimuria as compared with normoalbuminuria, whereas serum IL-6 levels were not elevated in diabetic patients with microalbuminuria. It remains unclear why we could not find an association of serum IL-6 levels with AER; however, it is possible that serum levels of IL-6 are less sensitive to renal injury than urinary IL-6 levels. Absence of a correlation between serum IL-18 and IL-6 levels might indicate that IL-18 is involved in the pathogenesis of diabetic nephropathy through a different mechanism than IL-6.

The close correlation between serum and urinary IL-18 levels and AER strongly suggest a relationship between low-grade inflammation and albuminuria in patients with type 2 diabetes, as recently described (15,27). IL-18 is a potent proinflammatory cytokine that induces interferon-γ (28), which in turn induces functional chemokine receptor expressions in human mesangial cells (29). Furthermore, IL-18 leads to production of other proinflammatory molecules, including IL-8, IL-1β, TNF-α, and intercellular adhesion molecule-1 (20,22), from mononuclear cells and macrophages. These molecules are known to increase in type 2 diabetes

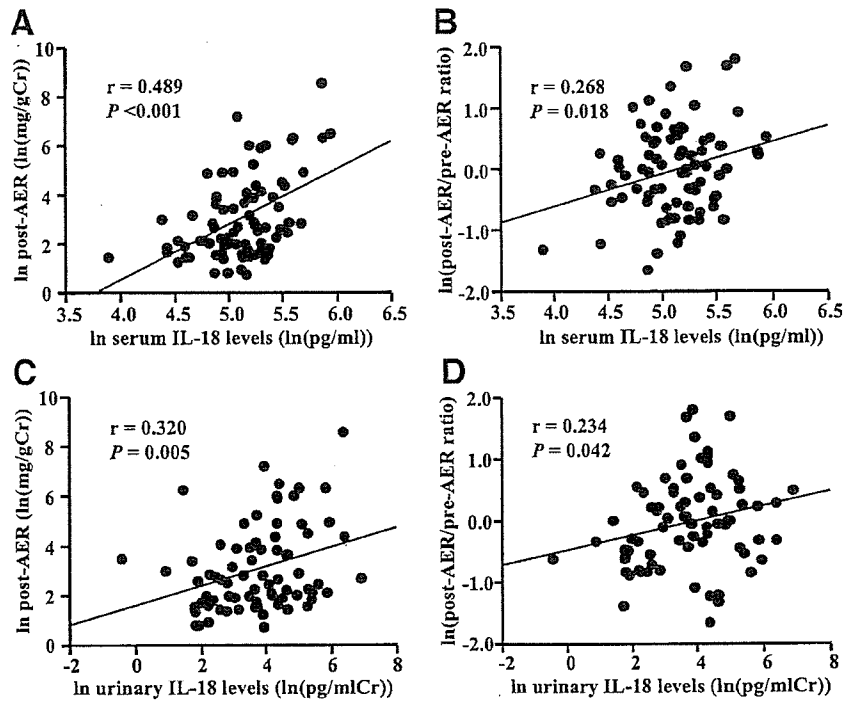

Figure 1—*Serum (A, B) and urinary (C, D) IL-18 levels correlate positively with post-AER or post-AER–to–pre-AER ratio. Variables of serum or urinary IL-18 levels, post-AER, and the post-AER–to–pre-AER ratio were naturally transformed logarithmically.*

(4,5,30) and may contribute to maintain microinflammation in renal tissues of patients with type 2 diabetes.

Microalbuminuria, hs-CRP, and other proinflammatory markers are known to be associated with cardiovascular diseases. In the present study, we also found that serum IL-18 levels were positively correlated with carotid IMT and baPWV in patients with type 2 diabetes. Several studies have reported that carotid IMT (11,12) and baPWV (31) are useful markers for evaluation of atherosclerosis in type 2 diabetes. A clinical study reported that decrease in glomerular filtration rate is linked to atherosclerosis (32). We showed that urinary β-2 microglobulin, a marker for tubulo-interstitial injuries, is positively correlated with serum IL-18 levels. Recently, several studies have demonstrated that proximal tubular cells are potential sources of IL-18 as well as monocytes/macrophages and T cells in ischemic acute tubular necrosis in mice (33,34). Thus, increase in serum IL-18 levels might be provoked by tubulo-interstitial injuries in patients with diabetic nephropathy.

We assessed the changes of AER at 6 months to test the hypothesis that the IL-18 level is a predictor of the progression of diabetic nephropathy in patients with type 2 diabetes. Serum and urinary IL-18 levels correlated positively with AER after 6 months and with changes in AER during the follow-up period. These results suggest that elevation of serum and urinary IL-18 levels may be a risk factor for development of diabetic nephropathy. In our study, 34 patients were prescribed an ACEI or ARB. Some studies have reported that angiotensin II blockade reduced production of inflammation molecules, including CRP (35), TNF-α, and IL-18 (36). ARBs suppress the expansion of reactive oxygen species generation and nuclear factor-κB with decreasing concentration of CRP (35). ACEIs inhibit lipopolysaccharide-induced production of TNF-α, IL-1β, IL-10, IL-12, and IL-18 in human monocyte-derived dendritic cells (36). Contrary to these reports, Tan et al. (37) report that ARB reduces AER in diabetic nephropathy with no significant change of hs-CRP. On the other hand, statins are known to have anti-inflammatory effects independent of their lipid-lowering effect (38). However, patients with ACEI/ARB or statins were positively correlated with serum IL-18 levels in our study. The mechanism underlying these discrepancies remains unclear, although patients with higher AER might have been administered these drugs. Because a cross-sectional study is not suitable to assess the effects of drugs, a prospective study will be required to resolve this question.

In the present study, we showed cross-sectional data associated with serum or urinary IL-18 levels. This makes it difficult to prove causal relationships. Prospective studies or in vitro studies are needed to clarify the causal relationships between IL-18 and both atherosclerosis and diabetic nephropathy in patients with type 2 diabetes.

In conclusion, the present results indicate that serum and urinary IL-18 levels are elevated and closely correlated with AER in patients with type 2 diabetes. Serum IL-18 levels may be a predictor of the progression of diabetic nephropathy as well as of cardiovascular diseases. Moreover, IL-18 might be a crucial molecule that connects albuminuria and cardiovascular disease in patients with type 2 diabetes.

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SHORT COMMUNICATION

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Polymorphism of the solute carrier family 12 (sodium/chloride transporters) member 3, *SLC12A3*, gene at exon 23 (+78G/A: Arg913Gln) is associated with elevation of urinary albumin excretion in Japanese patients with type 2 diabetes: a 10-year longitudinal study

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Abstract *Aims/hypothesis:* We have shown previously that the *SLC12A3* +78G/A polymorphism in exon 23 (Arg913Gln) was a new candidate for conferring susceptibility to diabetic nephropathy. The aim of this study was to confirm the effect of this polymorphism on the elevation of urinary albumin excretion in type 2 diabetic patients. *Methods:* We retrospectively studied 264 Japanese patients with type 2 diabetes over a ten-year period. The subjects were classified into two groups: (1) persistent normoalbuminuria or microalbuminuria, or improvement from microalbuminuria to normoalbuminuria (group N); and (2) progression from normoalbuminuria to microalbuminuria or overt proteinuria, or progression from microalbuminuria to overt proteinuria (group P). They were assessed for association with the +78G/A polymorphism. *Results:* The frequency of the +78A allele was significantly higher in group N than in group P (10% vs 1%, p=0.021). By logistic regression analysis and discriminant analysis, the substituted allele was shown to be an independent factor correlating negatively to the elevation of albumin excretion (p=0.043 and 0.022, respectively). *Conclusions/interpretation:* The *SLC12A3* +78A(+) genotype may have a protective effect against the development and/or progression of diabetic nephropathy in Japanese type 2 diabetic patients.

Keywords Genotype · Japanese · Longitudinal study · Macroalbuminuria · Microalbuminuria · Nephropathy · Polymorphism · *SLC12A3* · Solute carrier family 12 · Type 2 diabetes

Abbreviations ADA: American Diabetes Association · ACR: urinary albumin/creatinine ratio · NCCT: thiazide-sensitive NaCl cotransporter · *SLC12A3*: solute carrier family 12 (sodium/chloride transporters) member 3 · SNP: single nucleotide polymorphism

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Introduction

Multiple genetic factors are assumed to influence the development or progression of diabetic nephropathy based on previous reports [1–3]. Using a genome-wide, gene-based single nucleotide polymorphism (SNP) approach, we found recently the gene encoding solute carrier family 12 (sodium/chloride transporters) member 3, *SLC12A3*, as a new candidate for conferring susceptibility to diabetic nephropathy in a Japanese population [4]. *SLC12A3* is located on chromosome 16q13 and is expressed specifically in the kidneys, where it encodes a thiazide-sensitive Na–Cl cotransporter (NCCT) that mediates reabsorption of Na^+ and of Cl^- in the distal convoluted tubule. Mutation of *SLC12A3* is known to be responsible for Gitelman syndrome, an autosomal recessive renal tubular disorder characterised by hypokalaemic metabolic alkalosis, hypomagnesaemia, and low urinary calcium [5, 6]. We have identified several SNPs of this gene associated with nephropathy. In particular, +78G/A polymorphism in exon 23 (Arg913Gln) shows the strongest association among them. In the present study, to clarify whether polymor-

Table 1 Changes of urinary albumin excretion in the two groups

		Group N			Group P		
	In 1993 In 2003	Normo →Normo	Micro →Micro	Micro →Normo	Normo →Micro	Normo →Overt	Micro →Overt
Number	264	168	16	9	38	11	22
Male/female	177/87	103/65	13/3	8/1	28/10	10/1	15/7

Micro microalbuminuria, *Normo* normoalbuminuria, *Overt* overt proteinuria

phism of the +78G/A actually had an influence on the elevation of urinary albumin excretion, we examined the effect of this polymorphism in the patients with type 2 diabetes in a 10-year retrospective longitudinal study.

Subjects, materials and methods

Subjects and research design Two hundred and sixty-four unrelated Japanese patients with type 2 diabetes diagnosed by the criteria recommended by the American Diabetic Association (ADA) [7], who showed normoalbuminuria or microalbuminuria at baseline (in 1993) and could be completely followed for 10 years (177 men and 87 women aged 53.2±0.7 years, mean±SEM), were recruited from the diabetic outpatients at Juntendo University Hospital or Saiseikai Central Hospital (Tokyo, Japan). Diabetic nephropathy is clinically characterised by persistent proteinuria, decline of GFR and hypertension. The elevated urinary excretion of albumin may not be a specific marker for nephropathy, but the earliest clinical evidence of nephropathy is the appearance of microalbuminuria. Thus, patients with microalbuminuria are referred to as having incipient nephropathy [8]. To specifically evaluate the associations of genotypes with albuminuria referred to nephropathy, we excluded patients with microscopic or macroscopic haematuria, abnormal urinary sediment, or a past history of glomerulonephritis or nephroureterolithiasis, renal pelvic dilation, or severe renal atrophy. All subjects gave written informed consent before enrolment in the study, which was approved by the Ethics Committees of Juntendo University or that of Saiseikai Central Hospital. Each year, the grade of albuminuria was determined from the average of at least two measurements of the urinary albumin:creatinine ratio (ACR) or AER, and was categorised as normoalbuminuria (ACR<30 mg/g Cre or AER<20 μg/min), microalbuminuria (30≦ACR<300 mg/g Cre or 20≦AER<200 μg/min), and overt proteinuria (ACR≧300 mg/g Cre or AER≧200 μg/min) by ADA recommendations [8]. BP, serum lipids, HbA_1c and BMI values were calculated as the average over the 10-year period. The subjects were classified into two groups: group N showed persistent normoalbuminuria or microalbuminuria for 10 years, or regression from microalbuminuria to normoalbuminuria, while group P showed progression from normoalbuminuria to microalbuminuria or overt proteinuria over 10 years, or progression from microalbuminuria to overt proteinuria.

Genotyping of SNPs in the SLC12A3 gene Genomic DNA was extracted from peripheral blood cells using a DNA extraction kit (QIAamp DNA Blood Kit, Qiagen, Tokyo, Japan). On the basis of the GenBank information about the sequence containing the *SLC12A3* gene (accession no. AC012181.6), we designed PCR primers to amplify target fragments. Genomic DNA was amplified using a forward primer (5′-TCCATGTGTCCTCCAGGATCATTTC-3′) and a reverse primer (5′-GATGCTAGATGGGGTCTGTATG TTGC-3′). The PCR products were purified and used for direct sequencing by the fluorescent dye-terminator cycle sequencing method (ABI) with the same primers as those for PCR reactions.

Table 2 Clinical characteristics of groups N and P

	All subjects	Group N	Group P
Number	264	193	71
Male/female (% male)	177/87 (67)	124/69 (64)	53/18 (75)
Age (years)	53.2±0.7	53.1±0.8	53.6±1.3
Duration of diabetes (years)	7.8±0.5	7.6±0.6	8.5±0.9
Retinopathy none/simple/preproliferative or proliferative (%)	205/40/19 (78/15/7)	156/27/10 (81/14/5)	49/13/9 (69/18/13)
Smoker (%)	79 (30)	46 (24)	33 (46)*
Antihypertensive therapy (%)	78 (30)	52 (27)	26 (37)
ACEIs or ARBs	57 (73)	38 (73)	19 (73)
Ca^{2+} channel blockers	38 (49)	25 (48)	13 (50)
Alpha, beta-blockers, diuretics, and others	20 (26)	15 (29)	5 (19)
SLC12A3 +78G/A			
A(−) genotype (%)	244 (92)	174 (90)	70 (99)#
A(+) genotype (%)	20 (8)	19 (10)	1 (1)#

Data are the mean±SE or *n* (%) *$p<0.05$ and #$p<0.02$ vs group N. ACEIs, angiotensin converting enzyme inhibitors; ARBs, angiotensin II type 1 receptor blockers

Table 3 Initial, final and 10-year mean characteristics of group N and group P

	All subjects	Group N	Group P
BMI (kg/m^2)			
Initial	22.0±0.2	22.0±0.2	22.3±0.4
Final	22.6±0.2	22.4±0.2	23.2±0.4
10-year mean	22.3±0.2	22.2±0.2	22.7±0.4
Systolic BP (mmHg)			
Initial	129.2±1.2	129.5±1.5	128.4±2.3
Final	134.4±1.0	133.0±1.1	138.5±2.4*
10-year mean	133.9±0.8	131.8±0.9	135.9±1.6*
Diastolic BP (mmHg)			
Initial	77.1±0.8	77.4±0.9	76.1±1.5
Final	78.4±0.7	77.7±0.7	80.3±1.3
10-year mean	78.5±0.5	77.9±0.6	80.3±0.9*
HbA_1c (%)			
Initial	8.10±0.13	7.81±0.13	8.89±0.30*
Final	7.40±0.08	7.27±0.09	7.78±0.18*
10-year mean	7.57±0.07	7.39±0.07	8.08±0.13*
Total cholesterol (mg/dl)			
Initial	193.7±2.5	194.2±3.0	192.1±4.9
Final	201.4±2.1	202.3±2.5	198.8±3.7
10-year mean	200.0±1.7	200.8±1.9	197.6±3.2
HDL cholesterol (mg/dl)			
Initial	52.4±1.1	53.7±1.4	48.6±1.7*
Final	57.1±1.0	58.0±1.2	54.9±1.9
10-year mean	56.6±0.9	57.8±1.1	53.5±1.7*
Triglycerides (mg/dl)			
Initial	110.5±4.9	106.9±5.4	120.9±11.0
Final	114.7±4.4	114.1±5.3	116.4±7.6
10-year mean	116.0±3.7	114.2±4.3	121.2±6.9

Data are the means±SE or *n* (%)
*$p<0.05$ vs group N. ACEIs, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II type 1 receptor blockers; CCBs, calcium-channel blockers

Statistical analysis Results are expressed as the mean ±SEM. The significant difference in mean values was analysed by one-way ANOVA, followed by Scheffé's multiple comparison test. The significance of differences in frequency was determined by Fisher's exact test. To assess the relationship of *SLC12A3* genotypes with albuminuria, logistic regression analysis and discriminant analysis were performed.

Results

The changes of urinary albumin excretion in each group are summarised in Table 1. Serum creatinine was elevated above 115 μmol/l in five patients from group P, but no patient progressed to endstage renal failure by the end of this study (2003).

Clinical characteristics at baseline and the +78G/A genotypes of the subjects in each group are shown in Table 2. Seventy-eight patients (30%) were being treated with antihypertensive agents; the percentage of treated patients was higher in group P than in group N, but was not significantly different. There were 20 patients with the +78A(+) genotype in the study population, and all of them were heterozygotes. The frequency of the +78A allele was 3.8%, which was consistent with the previous report and the distribution of the genotype was within Hardy–Weinberg equilibrium. None of the subjects showed electrolyte abnormalities, familial inheritance, or other symptoms of Gitelman syndrome (data not shown). As shown in Table 2, the percentage of patients with the +78A(+) genotype in group N was significantly higher than in group P, and the +78A allele frequency was also significantly higher in group N than in group P (10% vs 1%, $p=0.021$).

The baseline, final and 10-year mean values for various clinical characteristics are shown in Table 3. The final and 10-year mean systolic BP and the 10-year mean diastolic BP were significantly higher in group P than in group N. All HbA_1c values were significantly higher in group P than in group N, while the baseline and 10-year mean HDL-cholesterol levels were significantly lower in group P than in group N. The other values did not differ between the two groups.

As shown in Table 4, both logistic regression analysis and discriminant analysis using the forward selection method showed that the +78A(+) genotype and female sex were correlated negatively with the development of microalbuminuria and/or progression to macroalbuminuria, while the 10-year mean systolic BP, 10-year mean HbA_1c, and percentage of smokers showed a positive correlation.

Discussion

As shown in Table 2, the frequencies of retinopathy and prevalence of antihypertensive therapy were not different between the two groups, although the frequency of microalbuminuric patients at baseline in the group P was

Table 4 Logistic regression analysis and discriminant analysis of factors related to the development of microalbuminuria and/or the progression to macroalbuminuria

	Logistic regression analysis			Discriminant analysis		
	Adjusted OR	95% CI	*p* value	Adjusted OR	95% CI	*p* value
SLC12A3 +78A(+) genotype	0.09	0.01–0.92	0.043	0.20	0.05–0.79	0.022
Sex (female)	0.43	0.20–0.96	0.039	0.45	0.22–0.96	0.038
Systolic BP (mmHg)	1.04	1.01–1.07	0.002	1.04	1.01–1.06	0.007
HbA_1c (%)	2.03	1.46–2.81	<0.001	2.09	1.53–2.86	<0.001
Smoking	2.92	1.46–5.85	0.003	3.24	1.53–6.86	0.002

higher than that in the group N (31% vs 13%, p=0.002). However, these rates at baseline in microalbuminuric patients with retinopathy (46%) and with antihypertensive therapy (45%) were similar to those in the report for European type 2 diabetic patients [9]. Furthermore, the progression rate from microalbuminuria to overt proteinuria seen in the present study was 47% (22 out of 47 microalbuminuric patients), and this was consistent with the results from the previous prospective studies [10, 11]. Taken together, our subjects, especially microalbuminuric patients, are unlikely to be an unusual cohort, considering previous reports.

The overall frequency of the +78A allele in our patients with type 2 diabetes was 3.8%, which did not differ from that in the Japanese general population (5.0%, unpublished data). Lemmink et al. detected the substitution of Gln for Arg at codon 913 in patients with Gitelman syndrome, but two patients who were homozygous for the +78 A allele showed no symptoms of Gitelman syndrome in our previous study [12]. Therefore, this substitution may not in itself be a cause of Gitelman syndrome.

Since little is known about the clinical association between BP and the +78G/A polymorphism [13], we examined preliminarily whether +78 G/A polymorphism had an effect on 10-year mean BP in these patients. However, neither the 10-year mean systolic BP nor 10-year mean diastolic BP was significantly different between subjects with the +78A(−) and A(+) genotypes, and multiple regression analysis to investigate the association between +78G/A polymorphism and BP showed no significant correlation between them (data not shown). Therefore further research is needed to clarify mechanisms underlining the protective effect of the +78A genotype against the elevation of albumin excretion.

In conclusion, we found that the *SLC12A3* +78G/A polymorphism in exon 23 (Arg913Gln) was associated with albumin excretion, and that the +78A allele may have a protective effect on the elevation of albuminuria in patients with type 2 diabetes.

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ORIGINAL ARTICLE

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Genetic variations in the gene encoding *TFAP2B* are associated with type 2 diabetes mellitus

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Abstract To search a gene(s) conferring susceptibility to type 2 diabetes mellitus, we genotyped nearly 60,000 gene-based SNPs for Japanese patients and found evidence that the gene at chromosome 6p12 encoding transcription-factor-activating protein 2β (*TFAP2B*) was a likely candidate in view of significant association of polymorphism in this gene with type 2 diabetes. Extensive analysis of this region identified that several variations within *TFAP2B* were significantly associated with type 2 diabetes [a variable number of tandem repeat

Accession numbers and URLs for data in this article are as follows: Genbank, http://www.ncbi.nlm.nih.gov/Genbank/ [for the *TFAP2B* gene (accession number NT_007592)]. For SNPs and primers, the IMS-JST Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/). Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM [for type 2 diabetes (MIM 125853), *TFAP2B* (MIM 601601), MODY (MIM 606391), Char syndrome (MIM 169100)].

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locus: $\chi^2 = 10.9$, $P = 0.0009$; odds ratio = 1.57, 95% CI 1.20–2.06, intron 1 + 774 (G/T); $\chi^2 = 11.6$, $P = 0.0006$; odds ratio = 1.60, 95% CI 1.22–2.09, intron 1 + 2093 (A/C); $\chi^2 = 12.2$, $P = 0.0004$; odds ratio = 1.61, 95% CI 1.23–2.11]. The association of *TFAP2B* with type 2 diabetes was also observed in the UK population. These results suggest that *TFAP2B* might be a new candidate for conferring susceptibility to type 2 diabetes and contribute to the pathogenesis of type 2 diabetes.

Keywords Type 2 diabetes · Variable number of tandem repeats (VNTR) · Single nucleotide polymorphism (SNP) · Adipocytes · Association study

Introduction

Type 2 diabetes mellitus (DM) affects more than one hundred million individuals worldwide (Zimmet et al. 2001). Its pathogenesis appears to be the consequence of insulin resistance in peripheral tissues combined with dysfunction of β cells in pancreatic islets although the precise mechanism is still not well known (Kahn 1998; Saltiel 2001).

That genetic factors contribute to the onset and progression of DM is undoubted, and several genes responsible for specific forms of the disease, such as maturity-onset diabetes of the young (MODY) or mitochondrial diabetes, have been identified (Fajans et al. 2001; Kadowaki et al. 1994). However, genetic alterations associated with these specific forms of diabetes account for only a small subset of cases; the gene or genes conferring susceptibility to type 2 diabetes in most patients remain to be identified.

Worldwide efforts to sequence the entire human genome have established a nearly complete blueprint (International Human Genome Sequencing Consortium 2001), providing a large body of information regarding genes whether their functions are already known or not. Single nucleotide polymorphisms (SNPs), the type of genetic variation found most frequently throughout the sequenced genome, have become useful markers for identifying genes involved in common diseases, such as DM. We developed a high-throughput SNP genotyping system that combined the Invader assay with multiplex polymerase chain reactions (PCRs) (Ohnishi et al. 2001) and undertook genome-wide association studies using SNPs to discover loci involved in susceptibility to common diseases.

In the study presented here, we show the results of a large-scale, case-control study using nearly 60,000 gene-based SNPs as genetic markers and provide the evidence that the gene encoding *TFAP2B* at chromosome 6p12 might be a novel candidate conferring susceptibility to type 2 diabetes.

Subjects and methods

Subjects and DNA preparations

DNA samples were obtained from patients with type 2 diabetes who come regularly to the outpatient clinics of Shiga University of Medical Science, Tokyo Women's Medical University, Juntendo University, Kawasaki Medical School, Keio University School of Medicine or Iwate Medical University. Control individuals consisted of 470 members of the general population (control 1) and another set of the general population (control 2, $n = 889$) who were recruited through several medical institutes in Japan. We also used a third set of control subjects with normal plasma glucose levels (HbA1c < 5.5% or fasting plasma glucose < 100 mg/dl and no family history for diabetes, control 3, $n = 598$), for final analysis. Written informed consent was obtained from each patient, and DNA extraction was performed using a standard phenol-chloroform procedure. The UK samples comprised 590 cases with type 2 diabetes enriched for positive family history (probands from the Diabetes UK Warren 2 repository) (Wiltshire et al. 2001) and 549 UK population controls (the ECACC-HRC collection) (Groves et al. 2003).

Genotyping for gene-based SNPs

The SNPs for genotyping experiments were selected randomly from the IMS-JST Japanese SNPs database (http://snp.ims.u-tokyo.ac.jp) (Hirakawa et al. 2002; Haga et al. 2002). The genotype at each SNP locus was analyzed with the Invader assay, as previously described (Ohnishi et al. 2001). We screened 188 diabetic patients at first, and genotype and/or allele frequencies were compared with those of the general population. After evaluating the statistical data using 2×3 or 2×2 contingency tables, SNPs that showed significant differences in genotype or allele frequencies between diabetic patients and the general population were examined further in another larger set of diabetics ($n = 631$). The protocol was approved by the ethics committee of the Institute of Physical and Chemical Research.

Discovery of SNPs in the TFAP2B gene, and genotyping

On the basis of GenBank information about DNA sequences in the genomic region containing the *TFAP2B*