SASプログラムを作成し、hierarchical regressionの手法を合わせて使用すれば、中間指標が治療効果をどの程度説明できるかを評価できることを示した。本年度研究により、治療効果解析のための生物統計学的方法論が準備できた。これらの解析において欠損値をどう扱うかについて検討が必要である。

F．健康危機情報

該当なし。

G．研究発表

1．論文発表

なし

2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

I．引用文献

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厚生労働科学研究費補助金（循環器疾患等総合研究事業）
分担研究報告書

糖尿病性腎症の寛解を目指したチーム医療による集約的治療 ―遺伝子解析研究―

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研究要旨　糖尿病性腎症の発症進展には遺伝因子の関与が重要である事から本研究においても、集約的治療あるいは標準的治療反応性に関わる遺伝因子の同定を試みる。本年度は解析する候補遺伝子を決定するために分担研究者が行ってきたゲノムワイドな糖尿病性腎症関連遺伝子検索の結果の検証ならびにすでに候補遺伝子として報告のあるレニン・アンジオテンシン系遺伝子に関する検証を行った。その結果ゲノムワイドな検索から112箇所の候補領域を選出し、ACE, AGT, AGTR1遺伝子と合わせ現時点での解析候補とした。

A．研究目的

糖尿病性腎症の発症進展をより効率的に阻止するために、腎症の発症進展、および治療応答性に関わる遺伝因子を同定する。

B．研究方法

日本人の１塩基多型（SNP）データベースより無作為に抽出した約８万箇所のSNPの解析結果から、糖尿病性腎症との関連をみとめた領域の検証を行った。また、候補遺伝子としてアンジオテンシン変換酵素（ACE)、アンジオテンシノーゲン（AGT)、１型アンジオテンシン２受容体（AGTR1）遺伝子を選択し、各遺伝子内のSNPと糖尿病性腎症との関連を検討した。各SNPの遺伝子型はインベーダー法により決定し、解析はカイ２条検定で行った。

（倫理面への配慮）

DNA試料はインフォームドコンセントを得たのちに提供を受け、匿名化された状態で理化学研究所において解析が行われる。本研究はヒトゲノム・遺伝子解析研究に関する倫理指針（文部科学省、厚生労働省、経済産業省、平成13年３月29日、平成16年12月28日全部改正）に準拠し、理化学研究所横浜研究所倫理委員会の承認のもとに行われている。

C．研究結果

８万箇所のSNP座を解析した結果、カイ２乗検定でP値0.01未満となったSNP座を131箇所みとめ、その中でHerdy-Weinberg平衡試験による検証により112箇所のSNP座を現時点での解析候補領域とした。また最も強い相関を示したSLC12A3内のSNPに関して10年間の後ろ向きコホート研究を行い腎症との関連を再確認した(Nishiyama et al. Diabetologia)。ELMO1遺伝子に関しては遺伝子機能解析により、糖尿病状態でELMO1遺伝子が増加する事、ELMO1過剰が細胞外基質産生を助長することを明らかにした。

一方、ACE, AGT, AGTR1内の各10, 6,

26SNPsを解析しACE内の4SNPs, AGT内の5SNPs, AGTR1内の1SNPと腎症との有意な相関を認めた（P<0.05）。

D．考　察

SLC12A3遺伝子は腎におけるNa再吸収を調節し血圧との関連が示唆され、またELMO1に関してもその機能から、腎症の治療応答性に関わるものと推察される。またACE, AGT, AGTR1遺伝子はACE阻害薬、アンジオテンシン２受容体拮抗薬などの効果に影響をおよぼす可能性が考えられる。

E．結　論

現時点でSLC12A3, ELMO1, ACE, AGT, AGTR1を含む116のSNP座を解析候補とした。

F．研究発表

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神山真澄他、大規模SNP解析による糖尿病性腎症関連遺伝子の同定とその機能解析

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厚生労働科学研究費補助金（循環器疾患等総合研究事業）
分担研究報告書

「糖尿病性腎症の寛解を目指したチーム医療による集約的治療」に関する研究（事務局）糖尿病性腎症の予後予測因子としての炎症マーカーに関する研究

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研究要旨　1.「糖尿病性腎症の寛解を目指したチーム医療による集約的治療に関する研究」の事務局を担当した。平成17年7月15日、東京においてKick off meetingを開催した。倫理委員会の承認を受け、全国公募により128施設の参加を得て症例登録を開始した。2．糖尿病性腎症の新しい予後予測因子としての炎症マーカーを探索するために、糖尿病患者における炎症性サイトカイン、ケモカイン、可溶性接着分子濃度を測定した。その結果、血清IL-18濃度が糖尿病性腎症の新しい予後予測因子となる可能性が示された。

A．研究目的

1．「糖尿病性腎症の寛解を目指したチーム医療による集約的治療に関する研究」の事務局を担当し、本研究を円滑に推進することを目的とした。

2．昨年度に引き続き、糖尿病性腎症の新しい予後予測因子としての炎症マーカーを探索することを目的とした。

B．研究方法

1．平成17年度7月15日国際研究交流会館（東京都中央区築地）において、発足説明会（Kick off Meeting）を行った。日本糖尿病学会、日本腎臓学会、糖尿病性腎症合同委員会を通して、参加施設の公募を行い、全国から131施設（その後3施設が参加取り止めとなり現在128施設）の参加を得て、症例登録を開始した。岡山大学医学部歯学部附属病院治験審査委員会と岡山大学大学院医歯学総合研究科倫理委員会の承認を受けた。

2．2型糖尿病患者82例（腎症1期：41例、2期：31例、3期：10例）と健常者55例の血清中IL-6, IL-18, TNF-α, ICAM-1, VCAM-1, MCP-1, IP-10濃度、血清中高感度CRP（hsCRP）を測定して比較するとともに、腎症の指標として尿中アルブミン濃度、動脈硬化の指標としてABI、baPWVおよび内頚動脈の内膜肥厚度（IMT）を測定して相関を検討した。

C．研究結果

現在60症例が観察期間に入っている。

症例は、Web site上で登録して、管理している。さらに、腎症の進行に関連する遺伝子を探索するために、理化学研究所横浜研究所遺伝子多型研究センターの糖尿病性腎症関連遺伝子研究チームとの共同研究で、本研究の症例の遺伝子解析を開始した。

1．2型糖尿病患者では、健常人に比較して血

中IL-18，IL-6，MCP-1,IP-10，TNF-α濃度の上昇が見られた。血清IL-18濃度と尿中アルブミン排泄率（AER）との間に強い相関を認めた。さらに、血清IL-18濃度とbaPWVおよびIMTとの間にも正の相関を認めた。さらに、血清IL-18高値群では6ヵ月後のAERが有意に増加したが、低値群では増加を認めなかった。

D．考　察

1．「糖尿病性腎症の寛解を目指したチーム医療による集約的治療に関する研究」の症例登録を開始した。今後は、定期的に全体会議を行いながら症例の登録を速やかに進めていく予定である。
2．IL-18をはじめとする炎症性サイトカインおよびケモカインが、動脈硬化のみならず腎症の進展を予測する鋭敏な指標となりうる可能性が示されるとともに、糖尿病性腎症の成因に微小な炎症が関与するという我々の仮説が支持された。

E．結　論

糖尿病性腎症の寛解を目指したチーム医療による集約的治療に関する研究の事務局を担当し、治験審査委員会、倫理委員会の承認を得るとともに、症例登録を開始した。

糖尿病性腎症の成因に炎症が関与しており、IL-18をはじめとするサイトカインおよびケモカインの血清中濃度は、腎症の進展を予測する鋭敏な指標になりうることが示唆された。

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H．知的財産権の出願・登録状況

なし

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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.

Diabetes Care 28:2890–2895, 2005

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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.

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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

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

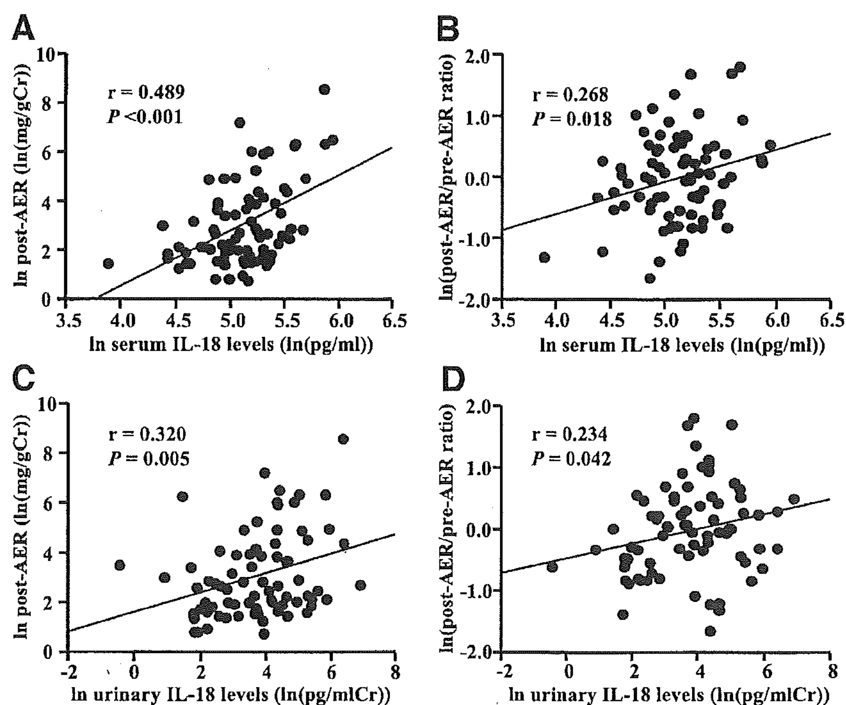

Figure 1—*Serum (A, B) and urinary (C, D) IL-18 levels correlate positively with post-AER or post-AER–to–pre-AER ratio. Variables of serum or urinary IL-18 levels, post-AER, and the post-AER–to–pre-AER ratio were naturally transformed logarithmically.*

(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.

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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

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

	In 1993 In 2003	Group N Normo →Normo	Micro →Micro	Micro →Normo	Group P 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	156/27/10	49/13/9
(%)	(78/15/7)	(81/14/5)	(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$_1$c (%)			
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$_1$c 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$_1$c, 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$_1$c (%)	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*

gene (accession number: NT_007592), we designed PCR primers to amplify appropriate fragments of genomic DNA. Repetitive elements were excluded from the search by invoking the REPEAT MASKER computer program, in the manner described previously (Seki et al. 2000). PCR reactions and DNA sequencing were carried out, as previously described (Saito et al. 2001). The SNPs in this region were genotyped by means of Invader (Japanese samples) or Amplifluor assays (UK samples) (Bengra et al. 2002), and VNTR loci were analyzed with respect to allele sizes using the Applied Biosystems ABI PRISM 3700 Automated DNA Sequencer and Gene-Scan software (GenoTyper program).

Reverse transcription and polymerase chain reactions

First-strand cDNA was prepared by reverse transcription (RT) of total RNA extracted from the murine 3T3-L1 cells by oligo-dT priming using Superscript II reverse transcriptase (Invitrogen). Human cDNAs from multiple tissues were obtained from CLONTECH Inc. (Palo Allto, CA, USA). The first-strand or double-strand cDNAs were amplified by PCR experiments using primers mAP2RT-F (5′-GCG TCC TCA GAA GAG CCA AAT C-3′) and mAP2RT-R (5′-GTG CGT GAT GAG ACT GAA GTG C-3′) for murine *TFAP2B* and hAP2RT-F (5′-CCA AAT CTG TGA CTT CTC TAA TGA-3′) and hAP2RT-R (5′-GTA ACG TGA CAT TTG CTG CTT TG-3′) for human *TFAP2B*. Real time quantitative RT-PCR for human *TFAP2B* was performed by TaqMan assay using primers hAP2BTM-F (5′-TTG AAC CGG CAG CAC ACA-3′), hAP2BTM-R (5′-CTT GGT GGC CAA CAG CAT ATT-3′) and probe hAP2BTM-P (5′(FAM)-CCG AGT GAC CTG CAC TCC CGA AA-(TAMRA)3′).

Statistical analysis

Statistical methods for determining associations, haplotype frequencies and to calculate linkage disequilibrium (LD) coefficients (Δ) were described previously (Yamada et al. 2001). Analysis of haplotype structure was carried out by estimating haplotype phasing using the EM algorithm (Excoffier and Slatkin 1995) and by constructing haplotype blocks, as previously described (Ozaki et al. 2002; Daly et al.2001).

For the simulation approach, to calculate the actual type 1 error rate in our study, we simulated exactly the process of the third tests (first test: case 1 versus control 1; second test: case 2 versus control 1; third test: case 2 versus control 2) using the Monte-Carlo method. Since the two sets of cases (cases 1 and 2) and the two sets of controls (controls 1 and 2) were all collected independently, two alleles were independently drawn for each subject, assuming that the frequency of the minor allele was the same for all the four groups. This means that the simulations were performed under the null hypothesis. For the simulations, the frequency of the minor alleles was changed from 0.02 to 0.5. After the genotypes of all subjects were determined, the cases and controls were compared in four different ways (first and second tests). Thus, (1) the differences in allele frequencies were tested using the allele frequency 2×2 contingency tables; (2) the differences in the frequencies of the subjects with minor alleles were then tested using the 2×2 contingency tables; (3) the differences in the frequencies of the subjects with major alleles were tested; and (4) the frequencies of three genotypes were tested using 3×2 contingency tables. All tests were done using the Pearson's chi-square test. The Monte-Carlo simulation was performed using Mersenne Twister uniform pseudo-random number generator (Matsumoto and Nishimura 1998).

For step-wise logistic regression analysis, the probability Pc of an individual of being a case rather than a control was assumed to be affected by a set of SNPs according to the logistic model: $\text{logit}(Pc) = a_0 + a_1 x_1 + a_2 x_2$ for single SNP, for example. Here, we used a coding scheme $x_1 = -1, 0, 1$, and $x_2 = -0.5, 0.5, -0.5$ for genotypes 1/1, 1/2 and 2/2, respectively, for representing an additive effect by x_1 and a dominance/recessive effect by x_2 (Cordell and Clayton 2002). The weights were estimated by the maximum-likelihood method and tested by comparison with the null-hypothesis logit $(Pc) = a_0$ (constant). For multiple SNPs, interaction effects were added further in addition to the main effects of additional SNPs and tested step-wise whether their effects were significant or not. The tests were performed using *R*. We applied both strategies of forward selection (starting from one SNP) and backward selection (starting from all SNPs) until the most significant SNP set were obtained (Cordell and Clayton 2002).

Results

Association study

We first genotyped 188 Japanese patients with type 2 diabetes (case 1) at 58,266 SNP loci and compared their allelic or genotype frequencies at these loci with those in the general population (control 1) (first test). At each locus, we tested the differences between the two populations by the four ways described in the Methods section; 1,496 SNP loci revealed a P value of <0.01 by at least one of the four tests (Table 1). We then analyzed these 1,496 loci for the second test using another larger group of patients (case 2). When case 2 and control 1 were compared by the four ways described in the Methods section, we found that the distribution of genotypes at a landmark SNP locus in the second intron of the *TFAP2B* gene on chromosome 6p12 was most strongly associated with type 2 diabetes [GG versus GC + CC: $\chi^2 = 15.9$, $P = 0.00007$, odds ratio = 1.65, 95% CI 1.29–2.11, G versus C: $\chi^2 = 11.3$, $P = 0.0007$, odds ratio = 1.38, 95% CI 1.14–1.66, (Tables 2 and 3)]. Furthermore, we compared the frequency of the alleles

Table 1 Distribution of P values in the first test[a]

	Genotype: n (%)	Allele: n (%)	Dominant model: n (%)	Recessive model: n (%)	Overall: n (%)
$P < 0.01$	626 (1.0)	755 (1.3)	605 (1.0)	630 (1.1)	1,496 (2.6)
$0.01 \leq P < 0.05$	2,435 (4.1)	2,691 (4.5)	2,323 (4.0)	2,380 (4.1)	4,931 (8.5)
$0.05 \leq P < 0.1$	2,899 (5.0)	3,121 (5.4)	3,146 (5.4)	3,105 (5.3)	5,816 (10.0)
$0.1 \leq P < 0.2$	5,784 (9.9)	6,138 (10.5)	5,671 (9.7)	5,752 (9.9)	9,421 (16.2)
$0.2 \leq P < 0.3$	5,762 (9.8)	5,725 (9.8)	5,573 (9.6)	5,836 (10.0)	8,224 (14.1)
$0.3 \leq P < 0.4$	5,562 (9.5)	5,926 (10.2)	5,405 (9.3)	5,460 (9.4)	6,945 (11.9)
$0.4 \leq P < 0.5$	5,696 (9.8)	5,935 (10.2)	5,873 (10.0)	5,993 (10.3)	6,504 (11.2)
$0.5 \leq P < 0.6$	5,810 (10.0)	5,703 (9.8)	5,909 (10.1)	5,842 (10.0)	5,347 (9.2)
$0.6 \leq P < 0.7$	5,707 (9.8)	5,584 (9.6)	5,175 (8.9)	5,255 (9.0)	3,789 (6.5)
$0.7 \leq P < 0.8$	5,798 (10.0)	5,623 (9.7)	5,316 (9.1)	5,478 (9.4)	2,943 (5.1)
$0.8 \leq P < 0.9$	5,760 (9.9)	5,592 (9.6)	5,157 (8.9)	5,356 (9.2)	1,891 (3.2)
$0.9 \leq P \leq 1$	6,427 (11.0)	5,523 (9.5)	8,113 (13.9)	7,179 (12.3)	959 (1.6)
Total	58,266	58,266	58,266	58,266	58,266

[a] Data are presented as number of SNPs

Table 2 Distribution of P values in the second test[a]

	Genotype	Allele	Dominant	Recessive	Overall
$P < 0.0001$	0	0	1[b]	0	1[b]
$0.0001 \leq P < 0.001$	5	15	6	4	17
$0.001 \leq P < 0.01$	43	44	46	32	85
$0.01 \leq P$	1,448	1,437	1,443	1,460	1,393

[a] Data are presented as number of SNPs
[b] SNP at *TFAP2B* locus

Table 3 Association of landmark SNP in *TFAP2B* (intron 2+58 G/C) during genome-wide screening[a]

	GG: n (%)	GC: n (%)	CC: n (%)	G	C
Case 1	73 (44)	60 (37)	31 (19)	0.63	0.37
Case 2	267 (42)	285 (45)	79 (13)	0.65	0.35
Case 3	121 (41)	132 (45)	41 (14)	0.64	0.36
Control 1	237 (55)	148 (34)	48 (11)	0.72	0.28
Control 2	423 (48)	373 (42)	93 (10)	0.69	0.31
Control 3	285 (48)	251 (42)	61 (10)	0.69	0.31

P values		Genotype (2x3)	Allele	GG versus others	CC versus others
First test	Case 1 versus control 1	0.02	0.003	0.03	0.01
Second test	Case 2 versus control 1	0.0002	0.0007	0.00007	0.48
Third test	Case 2 versus control 2	0.1	0.03	0.04	0.21
Fourth test	Case 3 versus control 3	0.09	0.03	0.06	0.1
Total		0.0004	0.00005	0.0002	0.006

[a] Genotype data are presented as number of subjects

at this locus in type 2 diabetic subjects (case 2) with that in a different set of controls (control 2) (third test) and identified significant association with type 2 diabetes (GG versus GC+CC: $P=0.04$, odds ratio = 1.21, 95% CI: 1.00–1.48, G versus C: $P=0.03$, odds ratio = 1.18, 95% CI: 1.01–1.37).

We further examined the difference in the allele frequencies at this locus using 349 cases (case 3) and 598 controls (control 3), both of which were unused for the first three tests (fourth test) and confirmed a significant difference with the P value of 0.03.

Since the above three tests (first through third) used the overlapped materials, the P values from the three tests might not be correct. Therefore, the overall empirical type I error rate of these three tests was calculated by the simulation, as described in the Methods section. The simulation was iterated two hundred million times for each given minor allele frequency. As shown in Fig. 1, the type I error rate increased according to the increase of the minor allele frequency from 0.02 to 0.5. Under all conditions tested, the upper limits of the 95% CI of the type I error rates were lower than 1.61×10^{-5} (Fig. 1). This empirical P value obtained by the simulations was multiplied by the P value for the fourth test ($P=0.03$) because the later test was independent from the former. The resulting P value of $< 4.67 \times 10^{-7}$ is considered to be the probability of the SNP association to pass the four tests. When we test 58,266 SNPs in this way, the probability to judge at least one of the SNPs to be significant was $P < 0.028$, as