insulin and transferrin or albumin, the sections were incubated overnight with antibodies against insulin and transferrin (goat polyclonal; Santa Cruz Biotechnology) or albumin (rabbit polyclonal; Biogenesis, Kingston, New Hampshire) at 4 °C. Antibodies against insulin, transferrin, and albumin were diluted 1:1000, 1:5000, and 1:5000, respectively, in PBS. For double staining of insulin and transferrin, the sections were then incubated for 1 h at room temperature in a mixture of TRITC-conjugated sheep anti-mouse IgG and FITC-conjugated donkey anti-goat IgG (Jackson Immuno Research, West Grove, PA) diluted 1:1000 in PBS. For double staining of insulin and albumin, the sections were incubated in a mixture of Alexa Fluor 488 goat anti-mose IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 546 goat anti-rabbit IgG diluted 1:1000 in PBS. Sections were observed under a fluorescence microscope (Leica DM RXA, Leica Microsystems, Wetzlar, Germany). The image was analyzed with a Q-fluoro analyzing system (Leica).

## Results

To express a PDX1 mutant, in the liver, which is constitutively active without association with protein partners, we prepared a recombinant adenovirus encoding the VP16 activation domain from herpes simplex virus [19,20] fused to the C-terminus of murine PDX1 (PDX1-VP16). For comparison, we also prepared recombinant adenoviruses encoding the wild-type PDX1 (wt-PDX1) and LacZ. These recombinant adenoviruses, at $2 \times 10^8$ pfu, were injected intravenously 6 days after STZ administration, when hyperglycemia had already developed; blood glucose levels after a 10 h fast were approximately 400 mg/dl (Fig. 1B). Mice given the LacZ adenovirus were used as controls (LacZ-mice). Systemic infusion of recombinant adenoviruses into mice through the tail vein caused transgene expression primarily in the liver, with no detectable expression in peripheral tissues such as muscle, fat, kidney or brain (data not shown), as reported previously [21].

As shown in Fig. 1A, immunoblotting of hepatic lysates on day 3 after adenoviral administration with anti-PDX1 antibody revealed that ectopic expression of wt-PDX1 or PDX1-VP16 was obtained in the liver. Administration of recombinant adenoviruses at the same titer induced similar levels of PDX1 protein expression.

We next examined the effects of treatment with these adenoviruses on STZ-induced hyperglycemia (Fig. 1B). Administration of wt-PDX1 adenovirus did not significantly decrease fasting blood glucose levels through day 20. Although, interestingly, fasting blood glucose levels were slightly but significantly decreased after day 30 as compared with those in STZ-treated LacZ-mice, administration of wt-PDX1 adenovirus at such a low titer exerted only very small effects in terms of reversal of hyperglycemia.

In contrast, administration of PDX1-VP16 adenovirus more effectively reversed STZ-induced hyperglycemia (Fig. 1B). Hepatic expression of PDX1-VP16 induced significant, profound decreases in fasting blood glucose levels. Although fasting blood glucose levels rose slightly between day 10 and day 15, the therapeutic effects were sustained throughout the experiments. As shown in Table 1, some variation in results was observed. Thirteen percent of PDX1-VP16-mice exhibited almost no decrease in blood glucose levels, although the proportion of these mice was significantly lower than that of wt-PDX1-mice. In contrast, in 27% of PDX1-VP16-mice, fasting blood glucose levels were lower than 200 mg/dl. No such normalization of glucose levels was obtained by wt-PDX1 adenovirus administration (Table 1). Thus, PDX1-VP16 expression in the liver more effectively lowered blood glucose levels and these effects persisted even after adenoviral-mediated gene expression had declined.

To examine the mechanism whereby administration of PDX1-VP16 adenovirus efficiently and persistently lowered blood glucose levels in STZ-treated mice, liver sections from these mice on day 40 after adenoviral administration were immunostained with anti-insulin antibody (Fig. 1C). No insulin staining was detectable in the livers of LacZ-mice. In wt-PDX1-mice, very faint staining with anti-insulin antibody was detected in the liver. In contrast, in PDX1-VP16 mice, strong insulin staining was detected in the cytoplasm of hepatocytes in scattered portions of the liver. The insulin positive cells were seen mostly around vessels. The scant residual insulin-positive cells in the pancreas did not differ significantly among these mice (data not shown). Thus, insulin secretion from hepatocytes is likely to contribute to lowering blood glucose levels in PDX1-VP16-mice.

To confirm that the hepatocytes were secreting insulin, serum levels of immunoreactive insulin in these mice on day 40 after adenoviral administration were measured. In LacZ-mice, STZ treatment induced severe insulinopenia: fasting serum insulin levels were less than 40 pg/ml (Fig. 1D), resulting in severe hyperglycemia. Adenoviral administration of the wt-PDX1 gene slightly increased serum insulin levels. In contrast, PDX1-VP16 adenoviral administration resulted in a substantial increase in serum insulin levels, i.e., more than 6-fold (Fig. 1D). On the other hand, fasting serum insulin levels in the control C57Bl/6N mice of the same age, without STZ treatment, were $340.7 \pm 29.9$ pg/ml ($n = 6$). Thus, hepatic PDX1-VP16 expression improved fasting serum insulin levels to approximately two-thirds those in normal mice. These data suggest that transient PDX1-VP16 expression in the liver exerted sustained and stronger effects in terms of production and secretion of insulin as compared with wt-PDX1 expression, resulting in the reversal of STZ-induced hyperglycemia.

Oral glucose tolerance tests were performed using LacZ-mice, wt-PDX1-mice, and PDX1-VP16-mice on day 40 (Fig. 2A). STZ-treated LacZ-mice exhibited hyperglycemia: more than 450 mg/dl throughout the

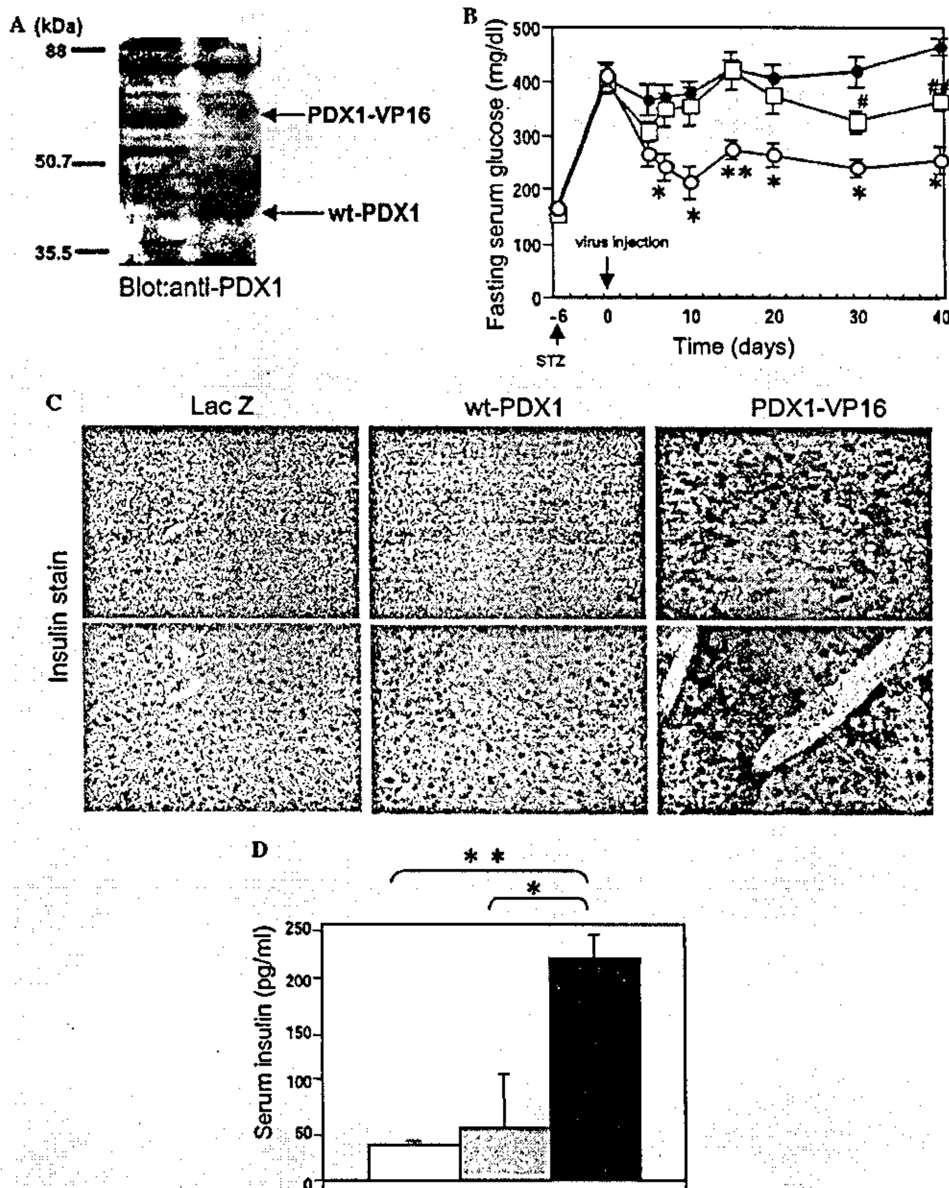


Fig. 1. Effects of wt-PDX1 and PDX1-VP16 adenoviral gene therapy on STZ-induced diabetic mice. (A) Liver lysates from STZ-mice infused with $2 \times 10^8$ pfu/body of adenovirus containing wt-PDX1 (left lane) or PDX1-VP16 (right lane) were immunoblotted with anti-PDX1 antibody. (B) Fasting blood glucose levels of STZ-mice treated with LacZ adenovirus (closed circle; $n = 13$), wt-PDX1 adenovirus (open square; $n = 8$) or PDX1-VP16 adenovirus (open circle; $n = 15$). Amount of injected adenoviruses was $2 \times 10^8$pfu/body in all experiments. (C) Liver sections from LacZ-mice (left panels), wt-PDX1-mice (middle panels), and PDX1-VP16-mice (right panels) on day 40 after adenoviral treatment were immunostained with anti-insulin antibody. Original magnification 100× (upper panels) and 200× (lower panels). (D) Fasting serum insulin levels 40 days after adenoviral treatment with LacZ (open bar; $n = 7$), wt-PDX1 (gray bar; $n = 8$), or PDX1-VP16 (black bar; $n = 7$) adenovirus. Data are presented as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ versus wt-PDX1, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus LacZ, assessed by unpaired $t$ test.

tests. In PDX1-VP16-mice, glucose levels throughout the tests were significantly lower than those in wt-PDX1-mice. The blood glucose levels peaked at 30 min after glucose load and thereafter tended to fall, although the reversal was incomplete at 120 min. These findings suggest that, in PDX1-VP16-mice, glucose-responsive insulin secretion from the liver is involved in lowering post-prandial blood glucose levels but is not enough to rapidly reverse a rise in blood glucose levels after a glucose load, in contrast to that from the pancreas by β cells.

Using HDAD, PDX1 expression in the liver reportedly induces expression of exocrine enzymes in insulin-producing cells in the liver and causes severe hepatitis. It has also been reported that, in transgenic mice expressing PDX1 ectopically in the liver, not only insulin but

Table 1
Distribution of blood glucose levels in each treatment group

| Blood glucose (mg/dl) | 100–200 | 200–300 | 300–400 | 400–500 | 500–600 |
|---|---|---|---|---|---|
| LacZ (%) | 0 | 0 | 8 | 69 | 23 |
| wt-PDX1 (%) | 0 | 12 | 50 | 38 | 0 |
| PDX1-VP16 (%) | 27 | 47 | 13 | 13 | 0 |

Blood glucose levels were determined 40 days after each adenoviral treatment. Blood glucose levels of mice before the adenoviral treatment (6 days after STZ injection) were all above 300 mg/dl. (Lac Z; $n = 13$, wt-PDX1; $n = 8$, and PDX1-VP16; $n = 15$.)

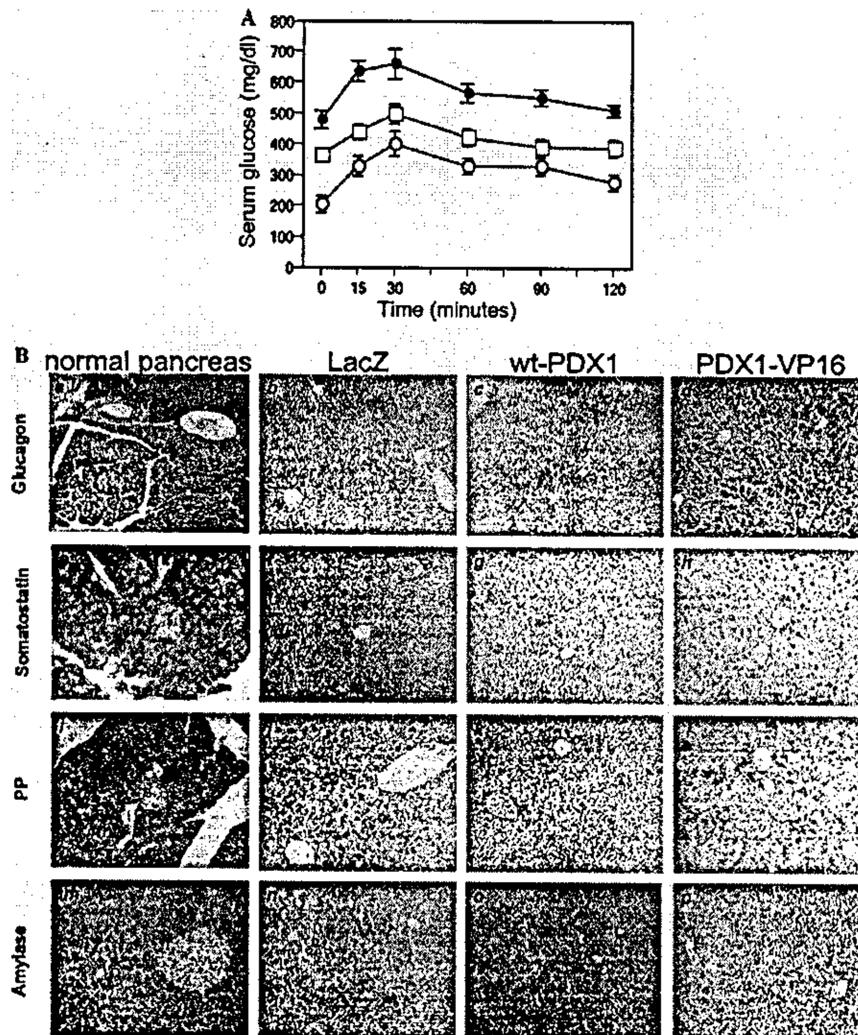


Fig. 2. Effects of wt-PDX1 and PDX1-VP16 adenoviral gene therapy on blood glucose levels after a glucose load, and glucagon, somatostatin, pancreatic polypeptide, and amylase expressions. (A) Blood glucose levels during oral glucose tolerance testing (1 g/kg body weight) in LacZ-mice (closed circle; $n = 7$), wt-PDX1-mice (open square; $n = 8$), and PDX1-VP16-mice (open circle; $n = 7$) on day 40 after adenovirus administration. Data are presented as means ± SEM. (B) immunohistochemical staining of livers from LacZ-mice (b,f,j,n), wt-PDX1-mice (c,g,k,o), and PDX1-VP16-mice (d,h,l,p) with glucagon (b–d), somatostatin (f–h), pancreatic polypeptide (j–l) or amylase (n–p) antibody. Sections of normal pancreas were used as positive controls for each staining procedure (a,e,i,m). Original magnification 100×.

also other endocrine hormones as well as pancreatic exocrine genes are expressed, resulting in dysmorphogenesis and hepatic failure [10]. In contrast, in the present study, adenovirus-mediated transduction of the wt-PDX1 or the PDX1-VP16 gene into the liver did not induce lobe structural abnormalities or substantial infiltration of inflammatory cells (Fig. 2B). Furthermore, using immunohistochemistry, no immunoreactivity against glucagon

or somatostatin was detected in livers from wt-PDX1-mice and PDX1-VP16-mice. In addition, in these livers there was no detectable production of amylase, a pancreatic exocrine enzyme (Fig. 2B), which may explain the normal morphogenesis in our experimental animals. On the other hand, pancreatic polypeptide was expressed in livers from PDX1-VP16-mice, and in those from wt-PDX1-mice though to a lesser extent. These results demonstrate that transient expression of PDX1-VP16 alters the character of hepatocytes to preferentially produce insulin and pancreatic polypeptide, but not other endocrine hormones or exocrine enzymes.

Adenoviral gene transfer induced gene expression for 1 week but, after 2 weeks, this expression reportedly disappeared [22]. However, in the present study, the blood glucose lowering effects and hepatic insulin expression persisted for at least 40 days. Therefore, the time course of PDX1 protein expression levels was examined. As shown in Fig. 3A, immunoblotting using anti-VP16 activation domain antibody revealed PDX1-VP16 protein to be expressed on day 3 but expression was markedly decreased on day 7, and undetectable on day 21. Thus, even after disappearance of VP16-PDX1 expression, hepatocytes expressed insulin, resulting in lowering of blood glucose levels. Interestingly, immunoblotting using anti-PDX1 antibody showed that endogenous PDX1 protein, which had the same molecular weight as wt-PDX1, came to be expressed on day 21. Thus, transient expression of PDX1-VP16 endowed hepatocytes with certain pancreatic β cell features and endogenous PDX1 expression is likely to maintain the insulin-producing function of these cells.

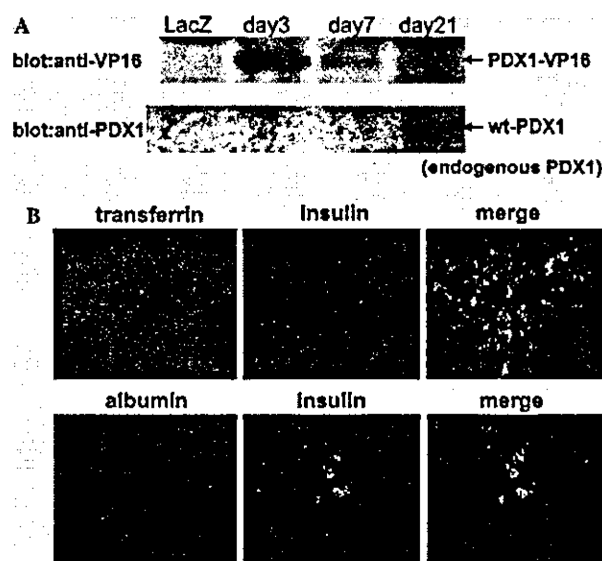


Fig. 3. Treatment with PDX1-VP16 adenovirus induced persistent expression of endogenous PDX1 but albumin and transferrin were co-expressed in insulin-expressing cells. (A) Liver lysates from PDX1-VP16 mice at different time points after adenoviral treatment were immunoblotted with anti-VP16 (upper panel) or anti-PDX1 (lower panel) antibody. (B) Liver sections from PDX1-VP16 mice on day 40 were double-immunostained with insulin (middle panels) and transferrin (upper-left panels) or albumin (lower-left panels) antibodies. Right panels represent the merged images.

To determine whether the insulin-producing cells in the liver had completely transdifferentiated and lost their hepatocytic character, liver sections from PDX1-VP16 mice on day 40 were immunostained with insulin and transferrin (upper panels in Fig. 3B) or albumin (lower panels in Fig. 3B). Fluorescence immunohistochemistry revealed that insulin-producing cells in the liver also expressed transferrin and albumin. Expression levels of these liver-specific proteins were not substantially decreased as compared with non-insulin-producing cells around the insulin-producing cells. These findings suggest functional hepatocyte-specific characteristics are maintained in insulin-producing cells in the liver. Thus, these hepatocytes were not completely converted to pancreatic cells.

## Discussion

In the present study, administration of recombinant adenovirus containing an activated form of PDX1 efficiently induced insulin production in hepatocytes, resulting in reversal of STZ-induced hyperglycemia. The effects were sustained even when exogenous protein expression was no longer detectable. In turn, endogenous PDX1 protein came to be expressed in hepatocytes, which is likely to be the mechanism underlying the sustained effects. On the other hand, albumin and transferrin expressions were observed in insulin-producing cells, suggesting the maintenance of hepatocyte-specific characteristics.

Ferber et al. [7] reported that administration of wt-PDX1 adenovirus at $2 \times 10^9$ pfu/mouse ameliorates STZ-induced hyperglycemia but the observed period was very short (no more than 10 days). The same research group also reported the long-term effects of PDX1 gene transfer but the titer of recombinant adenovirus used was relatively high ($1\text{–}5 \times 10^{10}$ pfu/mouse) [12]. Such high titers may result in liver damage due to adenoviral toxicity. In the present study, to avoid adenoviral toxicity, recombinant adenoviruses were injected at a titer as low as $2 \times 10^8$ pfu. With such a small adenoviral delivery, the wt-PDX1 adenovirus exerted very small effects on insulin and glucose levels, whereas PDX1-VP16 adenovirus substantially increased insulin levels and reversed STZ-induced hyperglycemia. These findings suggest that constitutive activation of PDX1 overcomes the inefficiency associated with low expression levels of PDX1 proteins. Thus, adenoviral transfer of the PDX1-VP16 gene into the liver would presumably be safer than wt-PDX1 gene therapy.

HDAD-mediated PDX1 expression in the liver reportedly causes severe hepatitis including marked inflammatory cell infiltration with focal necrosis associated with expression of pancreatic exocrine genes [10]. In addition, conditional transgenic mice generated by crossing CAG-CAT-PDX1 mice with alb-Cre recombinase-mice also displayed functional liver failure with hepatic expression of exocrine enzymes [11]. In these two models, exogenous PDX1 expression is persistent. Transgenes delivered by HDADs are expressed for long periods exceeding several months. In conditional transgenic mice [11], cells, in which the albumin promoter had once been activated, permanently expressed PDX1 driven by the CAG promoter. These findings suggest that high and persistent expression of PDX1 induces exocrine enzyme expression and thereby liver failure. In the present study, exogenous gene expressions of wt-PDX1 and PDX1-VP16 were transient and expression levels were relatively low on day 7 (Fig. 3A). Thus, transient expression appears to be important for endowing hepatocytes with certain features of pancreatic β cells, but not of exocrine cells.

It is noteworthy that exogenous, transient expression of PDX1-VP16 induced prolonged expression of endogenous PDX1 which apparently contributed to persistent insulin production with hepatocytic features. Ber et al. also reported that rat PDX1 gene transduction using first-generation adenovirus induced persistent endogenous (murine) PDX1 expression. Thus, transient expression of wt-PDX1, and more efficiently PDX1-VP16, may induce persistent and low-level expression of endogenous PDX1. In the adult pancreas, persistent but low-level expression of PDX1 is detected only in β cells [3] and PDX1 expression is required for maintaining normal pancreatic β cell function [6]. These observations suggest that persistent, low-level expression of PDX1 is involved in preferential production of insulin and pancreatic popypeptide in hepatocytes.

In transgenic *Xenopus* tadpoles expressing Xlhbox8 (*Xenopus* homolog of PDX1) carrying the VP16 activation domain under a transthyretin promoter, part or all of the liver is reportedly converted to pancreatic tissue without expression of liver-specific gene products, suggesting complete conversion of hepatocytes to pancreatic cells [14]. In contrast, in the present study, insulin-producing cells in the liver in PDX1-VP16 mice also expressed albumin and transferrin, which suggests preservation of hepatocytic functions. This discrepancy may be explained by the differences between amphibian and mammalian cells. Alternatively, the conversion may occur during embryonic differentiation, while, in adult and differentiated hepatocytes, complete transdifferentiation into pancreatic endocrine or exocrine cells would be difficult to achieve even with PDX1-VP16 expression. Although intensive research is necessary to unravel the precise mechanisms underlying transdifferentiation, the partial conversion induced by PDX1-VP16 expression in adult hepatocytes has practical applications, since loss of hepatocytic functions may result in liver failure. Furthermore, incomplete transdifferentiation could prevent the generated insulin-producing cells from being attacked by a destructive autoimmune response in type 1 diabetics.

## Acknowledgments

We thank Dr. H. Kanamori (University of Tokyo) for the generous gift of the VP16 gene. We also thank Ms. I. Sato, K. Kawamura, and M. Hoshi for technical support. This work was supported by a Grant-in-Aid for Scientific Research (B2, 15390282), a Grant-in-Aid for Exploratory Research (15659214) to H. Katagiri, and a Grant-in-Aid for Scientific Research (13204062) to Y. Oka from the Ministry of Education, Science, Sports and Culture of Japan. This work was also supported by Tohoku University 21st Century COE Program "CRESCENDO" to J. Imai, J. Gao, and H. Katagiri.

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# Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus: association with progress of diabetic nephropathy

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Received 23 March 2004; accepted 8 June 2004
Available online 11 September 2004

**Abstract**

Urotensin II (UII) is the most potent vasoconstrictor peptide ever identified. In order to clarify the pathophysiological role of UII in diabetes mellitus, we examined plasma immunoreactive UII levels and urinary excretion of immunoreactive UII in 10 control subjects and 48 patients with Type 2 diabetes mellitus. The patients were divided into three groups according to the renal function: Group I with Ccr ≥ 70 ml/min, group II with 30 ≤ Ccr < 70 ml/min and group III with Ccr < 30 ml/min. Plasma immunoreactive UII levels were elevated in the three diabetic groups compared with normal controls ($P < 0.05$). Group III patients had significantly higher plasma immunoreactive UII levels (15.9 ± 2.2 fmol/ml, mean ± S.E.M., $n = 6$) by approximately 1.6-fold than did group I (10.9 ± 0.9 fmol/ml, $n = 17$) and group II (10.8 ± 0.8 fmol/ml, $n = 25$) ($P < 0.05$). Urinary excretion of immunoreactive UII was significantly increased in group III patients (52.4 ± 14.8 pmol/day) by more than 1.8-fold compared with control subjects, groups I and II ($P < 0.005$). Fractional excretion of immunoreactive UII significantly increased as renal function decreased. Presence of diabetic retinopathy or neuropathy had negligible effects on plasma immunoreactive UII levels and urinary immunoreactive UII excretion. Reverse phase HPLC analyses showed three immunoreactive peaks in normal plasma extracts and multiple immunoreactive peaks in normal urine extracts. Thus, Type 2 diabetes mellitus itself is a factor to elevate plasma immunoreactive UII levels, and accompanying renal failure is another independent factor for the increased plasma immunoreactive UII levels in Type 2 diabetic patients. Increased urinary immunoreactive UII excretion in Type 2 diabetic patients with advanced diabetic nephropathy may be due not only to the elevated plasma immunoreactive UII levels but also to increased UII production and/or decreased UII degradation in the diseased kidney.
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*Keywords:* Urotensin II; Diabetes; Plasma; Urinary excretion; Radioimmunoassay

## 1. Introduction

Urotensin II (UII) is an 11 amino acid polypeptide initially isolated from the caudal neurosecretory system of teleost fish [1,3,17]. UII is the most potent vasoconstrictor peptide. The

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0196-9781/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2004.06.024

potency of the vasoconstriction of UII is an order of magnitude greater than that of endothelin-1 [1,4]. UII also has vasodilatory effect in rats [2] and human [15] through release of endothelium-derived hyperpolarizing factor and nitric oxide. This peptide has a positive inotropic action [10] and stimulates proliferation of vascular smooth muscle cells [12,23] and tumor cells [18]. Moreover, it inhibited insulin release from the perfused rat pancreas [14].

We reported elevated plasma immunoreactive (IR)-UII levels in patients with chronic renal failure [20]. Plasma IR-UII levels were also elevated in patients with heart failure [5,8,9] and in patients with liver cirrhosis [6]. Furthermore, we have recently reported that plasma IR-UII levels are elevated in diabetic patients with normal renal function [21]. Since UII and UII receptor (UT receptor) mRNAs are expressed in various peripheral organs including heart, kidney and vascular tissues [1,20], it is likely that UII acts as a circulating vasoactive hormone as well as a paracrine/autocrine factor, and may have important roles in the pathophysiology of these diseases.

Wenyi et al. showed that S89N polymorphism in the UII gene was associated with development of Type 2 diabetes via insulin sensitivity in the Japanese population [24]. Pathophysiological roles of UII in Type 2 diabetes mellitus have not been clarified yet. In the present study, we examined plasma UII levels and urinary UII excretion in patients with Type 2 diabetes mellitus and analyzed the relation of UII to diabetic complications.

## 2. Methods

### 2.1. Subjects

Ten control subjects (8 men and 2 women, 18–69 years old) and 48 patients with Type 2 diabetes mellitus (22 men and 26 women, 52.2 ± 15.2 years old, mean ± S.D.) were enrolled in the study. The control subjects had normal blood pressure and fasting blood sugar. Patients undergoing hemodialysis due to renal failure were excluded from this study. Informed consent was obtained from each subject. The study has been performed in accordance with the principles expressed in the Declaration of Helsinki, and has been approved by the Ethics Committee of Tohoku University School of Medicine.

Blood samples were collected from a forearm subcutaneous vein into tubes containing aprotinin (Trasylol; 500 kallikrein inhibitory units/ml of blood; Bayer, Germany) and EDTA (1 mg/ml of blood), and centrifuged at 4 °C. The plasma samples were stored at −30 °C until assayed. 24 h urine samples were collected at room temperature. After urine volume was measured, 10 ml of each sample was stored at −30 °C until extraction. The stability of UII in urine at room temperature was confirmed by incubating synthetic UII in normal urine. After 24 h incubation at room temperature, the urine was extracted with a Sep-Pak $C_{18}$ cartridge (Waters Associates, Milford, MA, USA) and assayed. No significant decrease in the IR-UII concentration was observed (over 96% of added peptide).

The Type 2 diabetic patients were divided into three groups according to their renal function, as in our previous study [11] (Table 1): Group I, creatinine clearance (Ccr) ≥ 70 ml/min; group II, 30 ≤ Ccr < 70 ml/min; group III, Ccr < 30 ml/min. Then, the patients in the group I were subdivided into two groups according to their urinary microalbumin levels (Ualb): group Ia, Ualb < 20 mg/day; group Ib, Ualb > 20 mg/day. The patients in the group II were similarly subdivided into three groups according to their urinary microalbumin levels and the presence of proteinuria: group IIa, Ualb < 20 mg/day; group IIb, Ualb > 20 mg/day without overt proteinuria; group IIc, Ualb > 20 mg/day with positive proteinuria.

The presence of diabetic retinopathy was assessed by diabetic ophthalmologists. The patients were divided into three groups: patients without retinopathy, patients with simple diabetic retinopathy and patients with pre-proliferative retinopathy or proliferative retinopathy (Table 2). The presence of diabetic peripheral neuropathy was assessed by at least one abnormality in the vibration sensation threshold, the Achilles tendon reflex, the knee tendon reflex, or the presence of abnormal sensation (Table 2).

Table 1
Clinical profiles of 48 patients with Type 2 diabetes mellitus classified by their renal function

| Group | Ccr (ml/min) | Sex | | Age (years) | Duration (years) | FBS (mmol/l) | HbA1c (%) | Ccr (ml/min) | Hb (g/100 ml) | BMI |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M | F | | | | | | | |
| I | ≥70 | 8 | 9 | 42.4 (15.7) | 6.5 (5.2) | 8.44 (3.02) | 8.4 (2.5) | 84.5 (11.4) | 13.4 (1.7) | 26.3 (6.4) |
| II | 30 ≤ Ccr < 70 | 12 | 13 | 57.4* (12.6) | 10.6 (7.7) | 8.64 (3.46) | 8.8 (2.3) | 52.2** (12.2) | 13.1 (2.4) | 22.2***** (3.4) |
| III | <30 | 2 | 4 | 56.8 (11.2) | 12.7 (11.0) | 8.11 (2.38) | 9.3 (2.7) | 18.6**,*** (3.8) | 10.4**** (1.3) | 23.8 (4.3) |
| Total | | 22 | 26 | 52.2 (15.2) | 9.4 (7.6) | 8.51 (3.14) | 8.7 (2.4) | 59.4 (24.3) | 12.9 (2.3) | 23.9 (5.1) |

Data are shown as the mean (S.D.). M, male; F, female; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; Ccr, creatinine clearance; Hb, hemoglobin levels; BMI, body mass index.

* $P < 0.005$ vs. GI.
** $P < 0.0001$ vs. GI.
*** $P < 0.0001$ vs. GII.
**** $P < 0.01$ vs. GI and GII.
***** $P < 0.05$ vs. GI.

Table 2
Clinical characteristics of 48 patients with Type 2 diabetes mellitus: incidence of retinopathy, peripheral neuropathy and hypertension

| Group | Retinopathy | | | Peripheral neuropathy | | Hypertension | |
|---|---|---|---|---|---|---|---|
| | Absent | Simple | Pre-proliferative + proliferative | Absent | Present | Absent | Present |
| Ia | 11 | 0 | 2 | 5 | 8 | 8 | 5 |
| Ib | 3 | 0 | 1 | 3 | 1 | 3 | 1 |
| IIa | 8 | 1 | 2 | 7 | 4 | 8 | 3 |
| IIb | 2 | 1 | 3 | 4 | 2 | 2 | 4 |
| IIc | 1 | 0 | 7 | 3 | 5 | 1 | 7 |
| III | 1 | 0 | 5 | 1 | 5 | 1 | 5 |
| Total | 26 | 2 | 20 | 23 | 25 | 23 | 25 |

Number of patients in each group is shown. Simple, simple diabetic retinopathy; pre-proliferative, pre-proliferative diabetic retinopathy; proliferative, proliferative diabetic retinopathy.

The patients on anti-hypertensive drugs, or with systolic blood pressure >160 mmHg, or diastolic blood pressure >95 mmHg were considered to have hypertension (25 out of 48 Type 2 diabetic patients) (Table 2).

### 2.2. Radioimmunoassay

Plasma IR-UII concentrations were measured by radioimmunoassay following the extraction using Sep-Pak $C_{18}$ cartridges (Waters Associates, Milford, MA, USA), as previously reported [20,21]. Urine samples were extracted with Sep-Pak $C_{18}$ cartridges. Three ml urine was acidified with 6 ml of 0.75 mol/l acetic acid and loaded onto the cartridge, which was pretreated with 10 ml acetonitrile, 10 ml methanol and then 10 ml of 0.75 mol/l acetic acid. After washing the cartridge with 10 ml of 0.75 mol/l acetic acid, the peptide was eluted from the cartridge with 2 ml of 60% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. The eluate was air-dried, reconstituted with assay buffer and assayed as previously reported [20,21]. The recovery, which was determined by adding UII to the urine prior to the extraction, was more than 96% ($n$ = 5). The cross reaction was about 1% with urotensin II-related peptide [16] (Peptide Institute, Osaka, Japan), but less than 0.001% with other peptides including endothelin-1, neuropeptide Y, urocortin 1, and somatostatin [19].

### 2.3. Chromatography

Chromatographic characterization of IR-UII in the plasma and urine was performed by reverse phase high performance liquid chromatography (HPLC) using a μBondapak C18 column (3.9 mm × 300 mm, Waters). The pooled plasma and urine obtained from normal subjects were extracted by Sep-Pak C18 cartridges and reconstituted in 0.1% (v/v) trifluoroacetic acid (TFA). The HPLC analysis was performed with a linear gradient of acetonitrile containing 0.1% TFA from 10% to 60% at a flow rate of 1 ml/min per fraction over 50 min. Each fraction (1 ml) was collected, dried by the air, reconstituted with assay buffer and assayed.

### 2.4. Statistics

Data are shown as mean ± S.E.M. unless otherwise stated. The statistical analysis was performed by one-way analysis of variance and Scheffe's multiple comparison test. Correlation was examined with Pearson's correlation coefficient.

## 3. Results

### 3.1. IR-UII in plasma and urine of patients with Type 2 diabetes mellitus: relationship with renal function

IR-UII was detectable in all plasma and urine samples. IR-UII concentrations were distributed from 2.5 to 24.0 fmol/ml in plasma and from 4.5 to 63.6 fmol/ml in urine. In 48 patients with Type 2 diabetes mellitus, plasma IR-UII concentrations showed a significant positive correlation with urinary IR-UII excretion ($r = 0.385$, $P = 0.0065$) and a significant negative correlation with IR-UII clearance ($r = -0.378$, $P = 0.0075$), but no significant correlation with fractional excretion of IR-UII (FE-UII) ($P = 0.937$). The plasma IR-UII concentrations, urinary IR-UII excretion and IR-UII clearance in control subjects and Type 2 diabetic patients are shown in Fig. 1 and fractional excretion of IR-UII (FE-UII) in Type 2 diabetic patients is in Fig. 2.

Plasma IR-UII concentrations were significantly elevated in patients with Type 2 diabetes mellitus when compared with control subjects (5.2 ± 0.4 fmol/ml, $P < 0.005$) ($F(3,54) = 10.618$, $P < 0.0001$). Plasma IR-UII levels were much higher in group III with Ccr < 30 ml/min (15.9 ± 2.2 fmol/ml) than in groups I and II (group I, 10.9 ± 0.9 fmol/ml; and group II, 10.8 ± 0.8 fmol/ml, $P < 0.05$) (Fig. 1A).

Urinary IR-UII excretion was significantly increased in group III (52.4 ± 14.8 pmol/day) by more than 1.8-fold compared with control subjects, groups I and II ($P < 0.005$) ($F(3,54) = 6.431$, $P < 0.001$) (Fig. 1B). There was no significant difference in urinary IR-UII excretion among these three groups (Fig. 1B). There was no significant difference in IR-UII clearance among the control subjects and three groups of Type 2 diabetic patients ($F(3,54) = 3.005$, $P > 0.05$) (Fig. 1C).

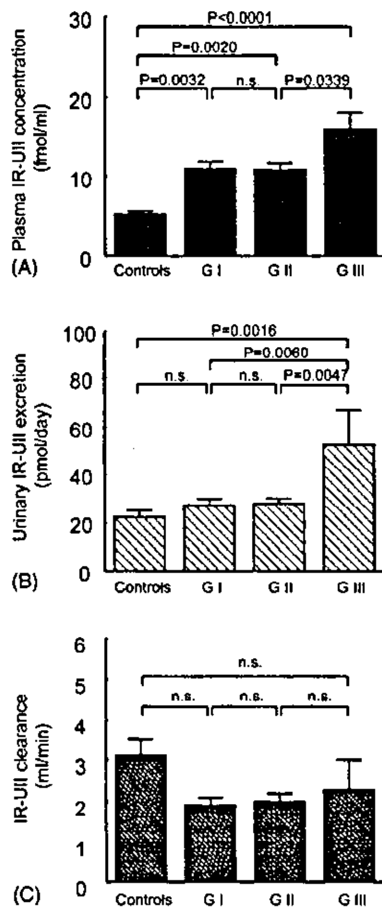


Fig. 1. (A) Plasma immunoreactive-urotensin II (UII) concentrations, (B) urinary excretion of immunoreactive-UII and (C) immunoreactive-UII clearance in 48 patients with Type 2 diabetes mellitus and 10 control subjects. The Type 2 diabetic patients were divided into three groups (group I, GI; group II, G2; and group III, GIII) according to their renal function (Table 1). Data are shown as the mean ± S.E.M.

FE-UII significantly increased as renal function decreases ($F(2,45) = 23.428$, $P < 0.0001$) (Fig. 2). Group III showed the highest FE-UII (0.13 ± 0.04 %), more than three-fold compared with groups I and II ($P < 0.0001$). The presence of microalbuminuria or proteinuria had a negligible effect on the plasma IR-UII concentration or urinary IR-UII excretion in patients with Ccr ≥ 30 ml/min (groups I and II) ($P > 0.1$)(data not shown).

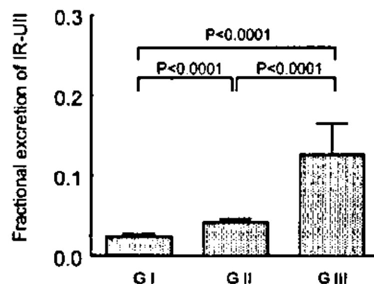


Fig. 2. Fractional excretion of immunoreactive-urotensin II (UII) in 48 patients with Type 2 diabetes mellitus. group I, GI; group II, GII; and group III, GIII. Data are shown as the mean ± S.E.M.

### *3.2. Relationship with other diabetic complications and clinical parameters*

The presence of pre-proliferative or proliferative diabetic retinopathy had a negligible effect on the plasma IR-UII concentration ($F(2,45) = 0.745$, $P > 0.4$) or urinary IR-UII excretion ($F(2,45) = 1.644$, $P > 0.2$) in Type 2 diabetic patients. Fractional excretion of UII in patients with pre-proliferative or proliferative diabetic retinopathy, however, was significantly higher than those in patients without diabetic retinopathy (0.066 ± 0.015 and 0.031 ± 0.004%, respectively) ($F(2,45) = 3.468$, $P < 0.05$), which may reflect the accompanying renal dysfunction in these patients.

There were no significant differences in plasma IR-UII concentrations ($P = 0.0738$) or urinary IR-UII excretion ($P > 0.8$) between patients with and without diabetic peripheral neuropathy. The presence of hypertension had a negligible effect on the plasma IR-UII concentration or urinary IR-UII excretion in patients with Ccr ≥ 30 ml/min (groups I and II) ($P > 0.3830$).

We then examinated correlation of plasma IR-UII concentrations, urinary IR-UII excretion, IR-UII clearance and FE-UII to other clinical parameters in 48 patients with Type 2 diabetes mellitus. Creatinine clearance (Ccr) had significant negative correlations to urinary IR-UII excretion ($r = -0.336$, $P = 0.0191$) and FE-UII ($r = -0.625$, $P < 0.0001$). No significant correlation, however, was observed between plasma IR-UII concentrations and Ccr ($P = 0.1293$), fasting blood sugar level (FBS), hemoglobin A1c, body mass index (BMI), cardiothoracic ratio (CTR) on chest rentogenogram, urinary excretion of *N*-acetyl-glucosaminidase, ages or duration of diabetes mellitus in the 48 Type 2 diabetic patients ($P > 0.05$). There was also no significant relation between urinary excretion of IR-UII and FBS, hemoglobin Alc, BMI, CTR, urinary excretion of *N*-acetyl-glucosaminidase or duration of diabetes mellitus in these patients ($P > 0.05$). No significant relation was found again when these correlations were examined within each group with similar renal functions (groups I, II and III).

### *3.3. Chromatography*

HPLC analysis of pooled normal plasma showed three immunoreactive peaks, one of which was eluted in the position of synthetic human UII (Fig. 3A). Urinary IR-UII consisted of at least six components; one of them was eluted in the position of synthetic human UII and two of them were eluted in the similar positions of immunoreactive peaks shown in the plasma (Fig. 3B).

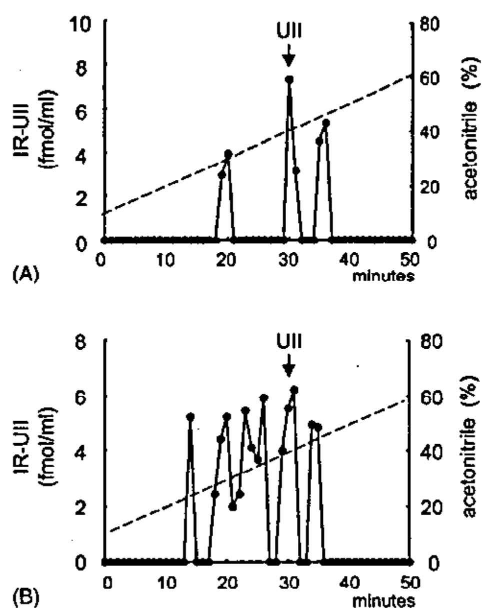


Fig. 3. Reverse phase high performance liquid chromatography of (A) the pooled plasma and (B) the urine extracts obtained from normal subjects. An arrow (UII) indicates the elution position of synthetic human urotensin II. A dotted line indicates a gradient of acetonitrile.

## 4. Discussion

The present study has shown high plasma IR-UII levels in Type 2 diabetic patients (Fig. 1A). The finding is compatible with our previous report on high plasma IR-UII levels in diabetic patients without renal failure [21]. In addition to diabetes mellitus and renal failure, heart failure [5,8,9] and liver cirrhosis [6] have been reported as the diseases having the elevated plasma IR-UII levels. Though increased production of UII in these pathological states were supposed, the precise mechanism of the elevation has not been clarified yet. The present study also has shown that Type 2 diabetic patients with advanced renal dysfunction had higher plasma IR-UII levels than those with normal or moderately decreased renal function. These results suggest that pathological state of Type 2 diabetes mellitus itself is a factor to elevate plasma IR-UII levels, and accompanying renal failure is another independent factor for the increased plasma IR-UII levels in Type 2 diabetic patients.

Two possible explanations are supposed for the elevated plasma IR-UII levels in the renal dysfunction: an increase of UII production in various organs, and a decrease of IR-UII excretion to urine. We therefore examined whether urinary excretion of IR-UII was decreased in diabetic patients with the renal dysfunction. Contrary to our expectation, diabetic patients with advanced renal dysfunction had increased urinary IR-UII excretion compared to those with normal to moderately decreased renal function. Increased urinary excretion of IR-UII was accompanied by increased levels of the fractional excretion of IR-UII in these patients. We previously reported increases in urinary excretion and fractional excretion of brain natriuretic peptide in patients with chronic renal failure [22]. Matsushita et al. reported that urinary IR-UII concentrations per gram creatinine were significantly increased in patients with renal tubular abnormality, but not with glomerular diseases, when compared with normal individuals [7]. Possible explanation for increased urinary excretion of IR-UII in group III may be an increased glomerular UII filtration due to the elevated plasma IR-UII levels and a decreased degradation of UII derived from plasma by the kidney and its excretion into the urine. Another possibility is increased production of UII by the kidneys with diabetic nephropathy, possibly by renal tubular cells.

The kidney is one of the most important organs for the degradation of peptide hormones. Urinary IR-UII levels were similar to plasma IR-UII levels, and IR-UII clearance was much lower than creatinine clearance both in control and Type 2 diabetic patients. These findings suggest that a large part of IR-UII filtered in glomerulus from plasma is metabolized in the renal tubules and only a very small part of IR-UII is excreted in the urine. On the other hand, renal tubular cells may synthesize and secrete UII into the urine. UII and UT receptor mRNAs are expressed in the kidney [7,20]. Immunocytochemistry showed that UII was localized in the renal tubular cells [13]. Urinary excretion of UII, therefore, may not be an indicator representing the UII production in the body, but rather reflect the renal production and secretion of UII, which could be stimulated by renal damage, such as diabetic nephropathy.

Reverse phase HPLC of normal plasma showed three immunoreactive peaks, suggesting that the IR-UII in the plasma consisted of at least three molecular forms. This result is in contrast with that of Ng et al. [8], who reported a single immunoreactive peak in plasma extracts by size-exclusion chromatography. Reverse phase HPLC analysis of normal urine showed multiple immunoreactive peaks. Three of the immunoreactive peaks in the urine extract were co-eluted with those shown in the plasma extract. This finding is different from that of Matsushita et al. [7], who showed a single major immunoreactive peak in the urine extract. The discrepancy in these results may be due to the difference of chromatographic methods or assay methods used. Immunoreactive peaks eluting in the positions other than authentic UII may represent the UII precursor or UII precursor fragments. We could not deny the possibility, however, that these immunoreactive materials were generated during the extraction procedure.

It also remains to be answered why plasma IR-UII levels were elevated in Type 2 diabetic patients without renal dysfunction. Plasma IR-UII concentrations and urinary excretion of IR-UII showed no significant relations to fasting blood sugar levels or hemoglobin A1c, suggesting that high levels of blood sugar are unlikely to affect plasma IR-UII concentrations or urinary excretion of IR-UII in Type 2 diabetic patients. The recent intriguing report by Wenyi et al. showed that certain polymorphism in the UII gene was associated with Type 2 diabetes mellitus [24]. UII may therefore be related not only to endothelial cell damage in diabetes but

also to glucose metabolism, for example, in liver and skeletal muscles. Actually UII mRNA is expressed in the liver together with UT receptor mRNA [21].

The present study has shown that Type 2 diabetes mellitus itself is a factor to elevate plasma IR-UII levels, and accompanying renal failure is another independent factor for the increased plasma IR-UII levels in Type 2 diabetic patients. Furthermore, urinary excretion of IR-UII may not be an indicator representing the UII production in the body, but rather reflect the renal production and secretion of UII, which may be enhanced in advanced diabetic nephropathy.

## Acknowledgments

We are grateful to Ms. Kikuchi for her secretarial and technical assistance. This study has been supported partly by Grant-in-aid for Scientific Research (C) (No. 13671094) (to KT) and (B) (No. 14370217) (to YI) from the Ministry of Education, Science, Sports and Culture of Japan.

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VI. ウロテンシン

基礎研究の進展

# ウロテンシン測定法

Measurement of urotensin

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Key words：ウロテンシン，ラジオイムノアッセイ，腎不全，糖尿病，心不全

## はじめに

ウロテンシン(urotensin)は，大部分の読者にとってなじみの薄い物質であろうと思われるが，実は古くて新しいペプチドホルモンである．30年前，硬骨魚の脊髄尾部にある神経内分泌器官urophysisから分離同定された[1]．当初からウロテンシンIとウロテンシンIIの存在が知られており，ウロテンシンIはcorticotropin releasing factor(CRF)様の作用をもつペプチドとされてきた．1995年Vaughanら[2]によりヒトを含む哺乳類でのウロテンシンIホモローグとしてウロコルチン(urocortin：UCN)が同定された．ウロコルチンは血管拡張作用および心臓保護作用などを有し，ストレスを下げる物質と理解されている．ウロコルチンは更にウロコルチンIIおよびウロコルチンIIIが同定されている．

一方，ウロテンシンIIは永らく塩類の排泄調節に関係する魚のホルモンとして理解され，臨床医学の世界では注目されていなかったが，1999年にAmesら[3]がreverse pharmacologyの手法を用いて，オルファンレセプターであるGPR14のリガンドがウロテンシンIIであることを発見し，同時にウロテンシンIIがラット胸部大動脈に対してエンドセリンよりも強力な血管収縮作用を示すこと，またヒトの動脈硬化病変部に発現を認めたことを報告し，注目されることとなった．更にウロテンシンIIには細胞増殖を促すgrowth factorとしての作用があること，心臓に対してinotropic作用があることなどが報告され，ウロテンシンIIはエンドセリンやアンジオテンシンIIなどと同じく，心血管・腎臓の病態と障害の進展に深くかかわっているのではないかと考えられるようになった．

著者らはラジオイムノアッセイ(RIA)法によるウロコルチンIIIおよびヒトウロテンシンIIの測定法を開発し，ヒト血液中および組織中にウロコルチンIII様免疫活性(UCNIII-LI)およびウロテンシンII様免疫活性(UII-LI)が存在するか否かを検討した．その結果，ヒト血液中にUCNIII-LIが存在することを見いだした[4]．更に，ウロテンシンIIはヒト血液中に循環ホルモンとして存在すること，腎不全(**図1**)[5]，糖尿病[6]などの病態で血中濃度が上昇することを見いだし，報告してきた．現在では，重症な心疾患[7-10]や門脈圧亢進を伴う肝硬変患者[11]などでも血中濃度が上昇することが報告されている．また，ウロテンシンIIがヒトの尿中に排出されていることが報告されている[12]．中枢および末梢組織での検討では，ウロテンシンIIのmRNA

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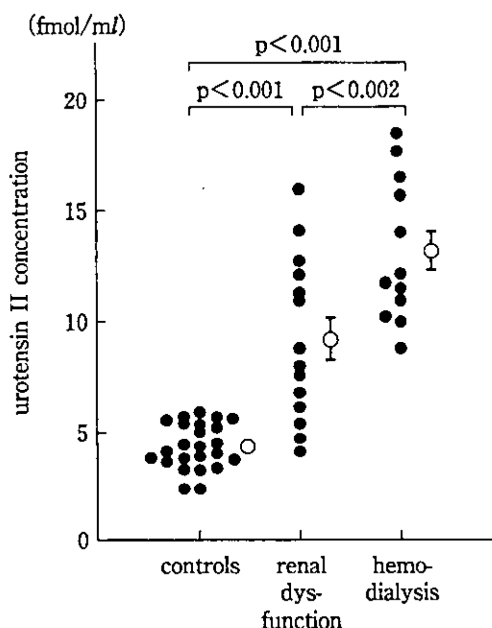


**図1 血中ウロテンシンII濃度**(文献[5]より引用改変)
controls: 健常者, renal dysfunction: 非透析慢性腎不全患者, hemodialysis: 透析患者.

の発現がほとんどすべての組織, 臓器にみられる[5]のに対し, 蛋白レベルでの発現は免疫染色法による検討で, 脳, 心臓[13], 腎臓[12], 腎腫瘍組織で発現が認められるも, RIA法を用いた検討では, 各組織でのウロテンシンIIの濃度は非常に低く[14], SW-13副腎皮質癌細胞の培養液中からUII-LIが検出されているのみである[15].

本稿では, ウロテンシンIIの測定法を主体に概説する.

## 1. ウロテンシンIIの測定法

ウロテンシンIIの測定にはRIA法とELISA法の2種類での測定報告がみられる.

### a. RIA法

RIA法は各研究者が独自に開発したものが主に使われているが, キットとしても市販されている. 本稿では, 著者らが使用している一般に第二抗体法と呼ばれる方法について述べる.

#### 1) サンプルの採取

血液サンプルは静脈血を採血し, EDTA・アプロチニン加チューブに移し4℃にて冷却遠心し, 血漿を分離して凍結保存する. 組織は摘出後すぐに抽出するか, ドライアイスまたは液体窒素で凍結し, −80℃にて保存する.

#### 2) 抽　出

血漿, 尿および培養液は夾雑物の除去と濃縮を目的として, セパックC18カートリッジ(Sep-Pak C18 cartridge, Waters社)を用いて抽出する.

組織は1M酢酸中で100℃煮沸後, ポリトロンまたはガラス・テフロン管を用いてホモジネートし, メタノールを加えて超遠心する. 上清を分離し空気流下に乾固する. BSA加酢酸液で再溶解し, セパックC18カートリッジを用いて抽出する.

セパックC18カートリッジは前処置としてメタノール, アセトニトリルおよび4%酢酸各10 mlで洗浄する. サンプル3 mlに対し4%酢酸6 mlを加えて酸性化しカートリッジにロードする. 4%酢酸10 mlで洗浄後2 mlの溶出液(アセトニトリル:水:トリフルオロ酢酸=60:40:0.1)で溶出し, 乾固する. 500 μlのアッセイバッファーで再溶解しRIAに供する. ここで用いた溶液の詳細は文献[5,6]を参照されたい.

尿, 組織抽出液および細胞培養液なども同様に抽出する. 本方法での回収率は血液サンプルで95%以上である.

#### 3) RIA

著者らが用いているRIAの手順を概説する. アッセイバッファーで再溶解したサンプル200 μlに対し抗ウロテンシンIIウサギ抗体(ペプチド研究所, ロットNo.992-500601)の希釈溶液(1,500倍)100 μlを加え4℃で48時間インキュベートする. $^{125}$I-urotensin II(アマシャム社)溶液100 μl(3,000 cpm/100 μl)を加え, 更に48時間のインキュベーションを行い, 100 μlの5%抗ウサギIgGヤギ抗体と500 μlの10%ポリエチレングリコール水溶液を加える. 5時間後4℃, 3,000×gで30分遠心して上清を除去し, 沈殿の放射線量をガンマカウンターで測定する.

本RIA法で検出可能な最小量は2 fmol/tubeである.

### b. ELISA法

Ngら[8]は, ペニンスラ社製のELISAキットを用いて心不全患者の血中ウロテンシンII濃度を

測定して報告している．著者らはELISA法での測定経験はないので文献[8]を参照されたい．

## 2. UII-LIの組成

RIA法であれELISA法であれ抗体を用いる方法では，本来のウロテンシンII以外にもUIIの前駆体やその分解産物など，UIIと構造上類似する物質も含めてウロテンシンIIとしてカウントしてしまう性質をもつ．したがってUII抗体を用いて測定している場合，測定している物質をウロテンシンII様免疫活性(UII-LI)と呼ぶことが多い．

UII-LIがどのような分子型から構成されているかは，Gelクロマトグラフィーまたは高速液体クロマトグラフィー(HPLC)により検討されている．Ngら[8]はヒト血液中のUII-LIをGelクロマトグラフィーで検討し，ヒトウロテンシンIIに一致する単一ピークを報告している．Takahashiら[15]は，SW-13副腎皮質癌細胞の培養液中に含まれるUII-LIをHPLC法で検討し，3つの成分からなることを見いだし，Gelクロマトグラフィーでの検討から，そのほとんどが11個のアミノ酸からなるヒトUIIよりも大分子量の物質であることを報告している．

## 3. ウロテンシンIIの濃度

ウロテンシンIIの臨床的意義を明確にするには，各種疾患での血液・組織中のUII濃度測定が必要となる．前述のRIA法を用いた著者らの測定では，健常成人のUII濃度は平均4.4fmol/m*l*であり，Richardsら[7]の報告では1.9fmol/m*l*，Ngら[8]の報告では6.6fmol/m*l*，Dschietzigら[9]の報告では60-80fmol/m*l*と測定者により差がみられる．Hellerら[11]は2.6pmol/m*l*と報告しているが，他の報告者に比して1,000倍もの高値であり，測定法または抽出法などに問題があるものと思われる．

著者ら[5]は非透析慢性腎不全患者の血中ウロテンシンII濃度が2倍，透析患者で3倍に上昇していることを報告した(図1)．透析の前後で濃度は変化せず，心房性ナトリウム利尿ペプチド(ANP)とは異なり，血中ウロテンシンII濃度は短期での体液量の変化は反映しないと考えられた．腎不全のない糖尿病患者でも血中ウロテンシンII濃度の上昇が認められ[6]，ウロテンシンIIの血中濃度の上昇は腎臓からの排泄の低下によるのではなく，これらの病態において産生が増加していることが示唆される．心不全患者でも2-3倍に上昇すると報告されている[7,8]が，上昇しないとの報告もみられる[9]．心不全患者の血中ウロテンシンII濃度は重症度に相関するとの報告がある一方で，重症度と関係しないとの報告もあり[8,9]，明確な答えは将来の詳細な研究を待たねばならない．

また，Hellerら[11]は肝硬変患者で約3倍の濃度上昇を報告している．Matsushitaら[12]は高血圧患者でウロテンシンIIの尿中排泄が増加していることを報告している．また，Thompsonら[16]は脊髄液中のウロテンシンII濃度は血中濃度に比して約15%低いことを報告している．

ヒト血中に存在するウロテンシンIIの産生部位は，エンドセリンと同様に血管内皮細胞が考えられるが，いまだ確定していない．ヒトの組織内ウロテンシンII濃度は，脳皮質，心臓，腎臓で検出限界以下であり，一部の副腎腫瘍組織でウロテンシンIIが検出されているのみである．

## 4. ウロコルチンの測定

血液および組織中のウロコルチンの測定は2つのグループから報告されている．Watanabeら[17]はRIA法にて血液中ウロコルチン濃度を測定し，健常成人男性で平均3.6fmol/m*l*，女性で2.7fmol/m*l*，妊娠女性で2.7-3.7fmol/m*l*であったと報告している．また，Ngら[18]は化学発光を用いたELISA法にて心不全患者の血中濃度を検討し，健常者17.3fmol/m*l*に対し，心不全患者は43.6fmol/m*l*と有意な高値を示したことを報告している．各方法の詳細については文献[17,18]を参照されたい．

## おわりに

ウロテンシンIIは多彩な生理作用を有するペプチドホルモンであり，これまで述べてきたように，心不全，腎不全，動脈硬化，糖尿病やあ

る種の腫瘍の病態に関与している可能性が示唆される．ウロテンシンIIの血中濃度の上昇がこれらの病態の原因であるのか結果であるのか，今後更なる検討が必要である．ウロテンシンIIの病態生理学的役割をより詳細に解明するには，多くの症例で血中濃度を測定し，結果を集積していく必要がある．しかしながら，血液中に含まれるウロテンシンIIの濃度は非常に低く，現在の測定法では感度の面から，なお大量のサンプル容量を必要としており，負荷テスト時などでの頻回の採血，測定などには困難が多い．少量のサンプルで測定が可能な，より簡便で高感度な測定法の開発が待たれる．今回は簡単に触れただけであるが，ウロコルチンについても全く同じ状況にあり，これからの研究課題といえよう．

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J Hum Genet (2004) 49:115–122
DOI 10.1007/s10038-003-0118-z

ORIGINAL ARTICLE

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# Linkage and association of childhood asthma with the chromosome 12 genes

Received: 1 September 2003 / Accepted: 19 November 2003 / Published online: 7 February 2004
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**Abstract** Several studies have shown linkage of chromosome region 12q13–24 to bronchial asthma and related phenotypes in ethnically diverse populations. In the Japanese population, a genome-wide study failed to show strong evidence of linkage of this region. Chromosome 12 genes that showed association with the disease in at least one report include: the signal transducer and activator of transcription 6 gene (*STAT6*), the nitrogen oxide synthetase 1 gene (*NOS1*), the interferon γ gene (*IFNG*), and the activation-induced cytidine deaminase gene (*AICDA*). To evaluate the linkage between chromosome 12 and childhood asthma in the Japanese population, we performed sib-pair linkage analysis on childhood asthma families using 18 microsatellite markers on chromosome 12. To investigate association between chromosome 12 candidate genes and asthma, distributions of alleles and genotypes of repeat polymorphisms of *STAT6*, *NOS1*, and *IFNG* were compared between controls and patients. Single nucleotide polymorphism of *AICDA* was also investigated. Chromosome region 12q24.23–q24.33 showed suggestive linkage to asthma. The *NOS1* intron 2 GT repeat and *STAT6* exon 1 GT repeat were associated with asthma. Neither the *IFNG* intron 1 CA repeat nor 465C/T of *AICDA* showed any association with asthma. Our results suggest that *NOS1* and *STAT6* are asthma-susceptibility genes and that chromosome region 12q24.23–q24.33 contains other susceptibility gene(s).

**Keywords** Childhood asthma · Linkage · Association · Polymorphism · NOS1 · STAT6 · IFNG · AICDA

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## Introduction

Bronchial asthma is an inflammatory disease of the airways characterized by airway obstruction and increased airway responsiveness. Asthma is an etiologically complex disease and develops by the interaction of multiple genes and environmental factors. Genome-wide

linkage studies have identified a number of autosomal regions providing evidence of linkage to asthma, atopy, eosinophilia, and/or other associated phenotypes (CSGA 1997; Daniels et al. 1996; Dizier et al. 2000; Haagerup et al. 2002; Hakonarson et al. 2002; Laitinen et al. 2001; Ober et al. 2000; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Xu et al. 2001b; Yokouchi et al. 2000). Some of these studies (CSGA 1997; Dizier et al. 2000; Haagerup et al. 2002; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Yokouchi et al. 2000) and those focused on a single chromosome (Barnes et al. 1999; Barnes et al. 1996; Kruglyak et al. 1996; Malerba et al. 2000; Nickel et al. 1997; Wilkinson et al. 1998; Raby et al. 2003) suggested linkage of chromosome 12q regions to asthma or related phenotypes in diverse populations.

In a genome-wide linkage analysis of mite-sensitive Japanese childhood asthma, the 110–145 cM region from the pter (the telomere of the short arm) showed maximum logarithm of odds score (MLS) more than 1.0 with the highest MLS of 1.92 at 111.9–125.3 cM (Yokouchi et al. 2000). The highest MLS did not reach the value of "significant" (MLS = 3.6) or "suggestive" (MLS = 2.2) linkage to the disease (Lander and Kruglyak 1995). The region in which MLS exceeded 1.0 was roughly overlapped by those of studies on Afro-Caribbean, French, and British populations (Barnes et al. 1996; Dizier et al. 2000; Wilkinson et al. 1998). To establish the linkage between asthma and chromosome 12 region, evidence of the suggestive linkage must be replicated using a different set of samples from the same population (Lander and Kruglyak 1995).

Candidate genes of chromosome 12q15–q24 include the signal transducer and activator of transcription 6 gene (*STAT6*), interferon-γ (*IFNG*), stem cell factor (*SFC*), leukotriene A4 hydrolase (*LTA4H*), insulin-like growth factor (*IGF1*), β-subunit of nuclear factor-Y (*NFYB*), B-cell translocation gene 1 (*BTG1*), and nitrogen oxide synthetase 1 (*NOS1*) (Barnes et al. 1996; Dizier et al. 2000; Wjst et al. 1999). Of these, *STAT6*, *NOS1*, and *IFNG* were investigated with case-control studies and showed positive association with asthma in at least one study. Gao et al. (2000b) demonstrated the association of the single nucleotide polymorphism (SNP) 2964G/A of *STAT6* with adult asthma in Japanese populations. However, this association was not replicated in later studies on German/Swedish (Duetsch et al. 2002) or Japanese populations (Tamura et al. 2001). Instead, a GT repeat polymorphism in exon 1 was associated with eosinophil count in the German/Swedish study and with allergic diseases in the Japanese study. A dinucleotide repeat marker in *NOS1* was also reported to be associated with the disease in the British population (Gao et al. 2000a). An association between a SNP in *NOS1* and eosinophil count was also shown in German/Swedish patients (Immervoll et al. 2001). Hyden et al. (1997) reported that no polymorphism in the *IFNG* was associated with atopic asthma, whereas an association between the GT repeat in intron 1 of *IFNG* and childhood asthma was suggested in the Japanese population (Nakao et al. 2001). Heinzmann et al. (2000a) screened polymorphisms in *SCF*, *STAT6*, *TR2* (thyroid receptor 2), and *LTA4H* and found two polymorphisms in *SCF* and one in *TR2* in the German population. They found no evidence of linkage or association of these genes with atopy.

All of the above-mentioned studies were based on case-control design. On the other hand, using the transmission disequilibrium test (TDT), Noguchi et al. (2001) reported that the activation-induced cytidine deaminase gene (*AICDA*) was associated with childhood asthma in the Japanese population. The *AICDA* gene is located in the short arm of chromosome 12, where linkage has never been suggested. They selected this gene as a candidate gene for asthma because deficiency of *AICDA* resulted in low IgE production, thereby the variations of the gene might be responsive to atopy. More recently, Isidoro-García et al. (2003) reported a case-control study on the same SNP of *AICDA* gene in the Spanish population. They failed to show the association of this SNP with the disease.

Among candidate genes of chromosome region 12q15–q24, *STAT6*, *IFNG*, and *NOS1* have been suggested to be associated with asthma in at least one study. Although the locus was not a suspected linkage to asthma, the *AICDA* gene showed a positive result in one study. As often seen in genetic analyses of a complex disease (Ioannidis et al. 2001), inconsistencies were noticed between the studies of chromosome 12 candidate genes for asthma. Studying other sets of samples in the same population is necessary to conclude whether a particular gene is truly associated with this complex disease.

In the present study, we investigated linkage of markers on chromosome 12 to childhood asthma in the Japanese population. We also investigated association of four candidate genes, *AIDCA*, *STAT6*, *NOS1*, and *IFNG*, with Japanese childhood asthma.

## Materials and methods

### Families and individuals

For linkage analysis, 18 families with affected sib pairs and one family with an affected sib trio were recruited. For the association study, 184 controls and 115 patients were genotyped. One hundred control subjects were selected in the Osaka area, Japan, as previously described (Heinzmann et al. 2000b; Mao et al. 1996), and 84 controls were selected from adult staff and student volunteers from Tohoku University School of Medicine in Sendai, Japan. Individuals with a history of treatment for asthma or eczema were excluded from controls. Forty-two patients were diagnosed at hospitals in the Sendai area, which included patients from 19 families for linkage analysis. For an association study, one patient per family was selected. Other patients were recruited as described (Heinzmann et al. 2000b; Mao et al. 1996). None of the samples were previously analyzed for chromosome 12 linkage markers, *NOS1*, *IFNG*, *STAT6*, or *AICDA*. Diagnosis of asthma of probands was made by pediatricians specializing in allergic diseases.

The criteria of asthma were two or more episodes of wheezing and shortness of breath and reversibility of the wheezing and dyspnea, either spontaneously or by bronchodilator treatment. The definition of "childhood" asthma was asthma with onset before age 15 years. Diagnosis of other family members was based on the modified ATS-DLD questionnaire (Ferris 1978). Total serum IgE was regarded as high when the level was 250 IU/ml or higher. The specific IgE against house dust mite [*Dermatophagoides pteronyssinus* (Dp)] was judged positive when the RAST score against Dp was 2 (0.70 $U_A$/ml) or higher. "Atopy" was defined as either having high total IgE and/or positive Dp-specific IgE. Eighteen families had an affected sibpair and one family had an affected sib trio. All patients with childhood asthma were atopic. All affected sibs and their parents were genotyped.

This study was approved by the ethics committee of Tohoku University School of Medicine.

### Genotyping

DNA was extracted from peripheral blood leukocytes using the Genomic DNA purification kit (Promega, Madison, WI, USA). Chromosome 12 microsatellite markers of the Human GenePairs Primers version 9 (Invitrogen, Carlsland, CA, USA) were used for the linkage analysis. The 18 markers genotyped in this study are shown in Table 1. Information on marker order and position was obtained from LDB2000: Sequence-based Integrated Maps of the Human Genome (http://cedar.genetics.soton.ac.uk/public_html/LDB2000.html) (Wilkinson et al. 1998). The location of *AICDA* was not cited in this database and was estimated using the NCBI Human Map Viewer.

In chromosome 12 microsatellite marker analyses, PCR mixtures contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of deoxynucleotide triphosphate (dNTPs), 0.25 U of rTaq DNA polymerase (TAKARA, Tokyo, Japan), 5 μM of each primer, and 10 ng of template DNA in a total volume of 10 μl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The size of the PCR products was estimated using a 373XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). GeneScan 500XL TAMRA labeled standard (Applied Biosystems) was used for estimation of fragment lengths.

Primers for the *NOS1* intron 2 GT repeat were as described previously (Gao et al. 2000a). One of the primers, 5'-ATAGAGCCTGTGCTGAGCCTTC, was 6-FAM labeled. The PCR mixture contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 250 μM each of dNTPs, 0.5 U of rTaq DNA polymerase, 200 μM of each primer, and 10 ng of template DNA in a total volume of 15 μl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s, with a final extension of 72°C for 10 min.

The primers for the *STAT6* exon 1 GT repeat were 5'-GGAGAAGCCGGAAACAGCGG and 5'-GTTCAAGGCTGGCCCTGCTAGC (6-FAM labeled). The PCR mixture was the same as for of *NOS1*. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min.

Primers for the *IFNG* intron 2 CA repeat were as previously described (Nakao et al. 2001). The PCR mixture was the same as for *NOS1*, except that 0.25 U of rTaq and 600 μM of each primer were used. The cycle conditions were 95°C for 5 min, followed by 25–34 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 5 min.

The 467C/T (His155His) polymorphism of *AICDA* (GeneBank AB040430) was the same polymorphism reported by Noguchi et al. (2001), where they designated this polymorphism as 7888C/T. This was genotyped using a modified TaqMan PCR method employing allele-specific amplification (Fujii et al. 2000). The common forward primer was 5'-GGCCCCGAGGAAATGAGAAAAT. The reverse primers were 5'-TCCCAGGCTTTGAAAGTTCTTTAG for the C allele and 5'-TCCCAGGCTTTGAAAGTTCTTTGA for the T allele. The TaqMan probe was 5'-FAM-AGAAGACAGTTCAGGTTCCAAATCGAGG- TAMRA -3'. The PCR mixture contained 7.5 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 400 μM of each PCR primer, 0.12 μM of TaqMan probe, and 5 ng of template DNA in a final volume of 15 μl. The cycle conditions were 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

**Table 1** Map locations for chromosome 12 markers and genes

| Locus | Kb from pter | Band | Male cM | Female cM | Averaged cM |
|---|---|---|---|---|---|
| ptr | 0 | p13.33 | 0.0 | 0.0 | 0.0 |
| *D12S372* | 3761 | p13.33 | 7.5 | 2.5 | 5.0 |
| AICDA | 8468 | p13.31 | 20.0 | 9.3 | 14.7 |
| GATA49D12 | 8513 | p13.31 | 20.1 | 9.4 | 14.7 |
| D12S391 | 13246 | p13.2 | 26.1 | 21.4 | 23.8 |
| D12S373 | 18347 | p12.3 | 32.8 | 35.5 | 34.1 |
| D12S1042 | 28440 | p11.23 | 36.9 | 56.2 | 46.5 |
| cen | 39000 | q11 | 38.7 | 65.0 | 51.9 |
| D12S1301 | 46378 | q12 | 39.7 | 71.3 | 55.5 |
| D12S398 | 56808 | q13.13 | 44.4 | 87.0 | 65.7 |
| STAT6 | 61349 | q13.13 | 45.9 | 92.5 | 69.2 |
| D12S1294 | 73218 | q14.2 | 50.4 | 97.7 | 74.0 |
| IFNG | 73860 | q14.2 | 51.7 | 100.3 | 76.0 |
| D12S375 | 74485 | q14.3 | 52.7 | 103.0 | 77.8 |
| D12S1052 | 80438 | q15 | 57.1 | 106.9 | 82.0 |
| D12S1064 | 96885 | q21.33 | 67.2 | 120.5 | 93.8 |
| D12S1300 | 105918 | q23.1 | 69.8 | 133.1 | 101.4 |
| PAH | 111421 | q23.3 | 73.5 | 143.5 | 108.5 |
| D12S2070 | 125845 | q24.22 | 81.8 | 167.6 | 124.7 |
| NOS1 | 127541 | q24.22 | 82.8 | 174.6 | 128.7 |
| D12S395 | 130349 | q24.23 | 84.1 | 184.3 | 134.2 |
| D12S392 | 138666 | q24.32 | 91.9 | 200.3 | 146.1 |
| D12S2078 | 140437 | q24.33 | 98.1 | 201.8 | 149.9 |
| D12S1045 | 143552 | q24.33 | 110.7 | 212.9 | 161.8 |
| qtr | 146025 | qtr | 119.2 | 218.4 | 168.8 |

### Statistical analysis

Allele frequencies of microsatellite markers were estimated from the parental chromosomes. All chromosome 12 markers were assessed by PEDCHECK (version 1.0) (O'Connell et al. 1998) for pedigree inconsistencies. There was no genotype inconsistency at any loci in all the families. Multipoint linkage analysis was conducted using GENEHUNTER 2 (Kruglyak et al. 1996). For score calculations of the sib trio, we used "all independent pairs of affected/phenotyped sibs" option of the GENEHUNTER 2 program where one sib trio yielded two pairs. In all allelic and genotypic distribution analyses, Fisher's exact *P* values were calculated using SPSS for Windows version 11.0 J (SPSS Japan, Japan). For multiallelic markers, the *P* value of association with the disease of each allele or genotype was multiplied by the number of the alleles or genotypes to compensate for multiple testing and expressed as *Pc*. The level of significance for the association studies was set at $P(Pc) = 0.05$.

## Results

### Linkage analysis of chromosome 12

Thirty-nine sibs with childhood asthma used for the linkage analysis consisted of 20 males and 19 females. Ages of the patients ranged from 1 to 14 years with the average age of 7.3 years. All affected sibs were positive

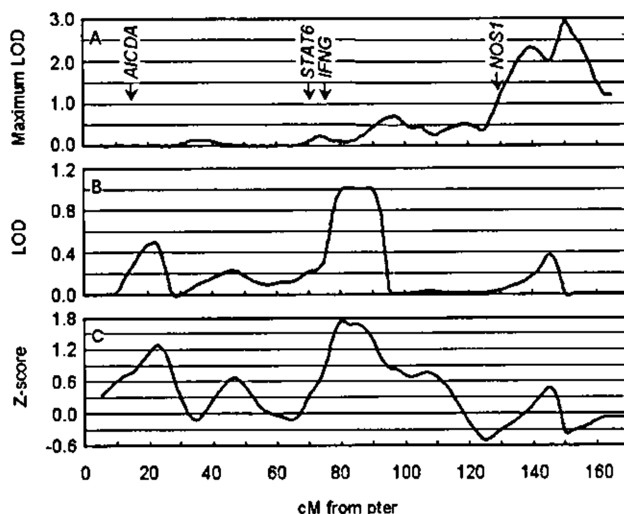


**Fig. 1A–C** Linkage results for asthma and total IgE level. All calculations were performed by GENEHUNTER 2. **A** Multipoint maximum likelihood (ML) estimate of identity by decent sharing for asthma affection status. **B** ML quantitative locus (QTL) variance estimation for $\log_{10}$ (total IgE level). ML QTL variance estimation (Kruglyak and Lander 1995) is performed instead of traditional Haseman-Elston (HE) QTL analysis because the values calculated with HE QTL analysis were affected by selection of sib pairs from the sib trio. **C** Nonparametric QTL analysis (Kruglyak and Lander 1995) for $\log_{10}$ (total IgE level)

for Dp-specific IgE. Thirty also showed atopic dermatitis. Results of the linkage analysis of the 18 microsatellite markers of chromosome 12 are shown in Fig. 1. A broad region from 135 cM to 160 cM exceeded 1.5 in MLS (Fig. 1A). Two peaks at 140 and 150 cM were observed, and the latter peak showed a MLS of 2.92, greater than the suggestive linkage level (Lander and Kruglyak 1995). Serum total IgE levels were $\log_{10}$ transformed and subjected to sib-pair quantitative locus (QTL) analysis. The result of maximum likelihood (ML) QTL variance estimation is shown in Fig. 1B. ML QTL variance estimation (Kruglyak and Lander 1995) was performed instead of the traditional Haseman-Elston (HE) QTL analysis, because the values with HE QTL analysis were affected greatly by the selection method of pairs from the sib trio. The highest peak of logarithm of odds (LOD) score (max = 1.02) was observed at 83 cM. No significant peaks were observed where linkage to asthma was observed. Nonparametric QTL analysis (Kruglyak and Lander 1995) showed the highest peak to be at 80 cM with a Z-score of 1.74 ($P$ = 0.082) (Fig. 1C). The results of QTL analyses did not suggest evidence of QTL for $\log_{10}$ (total IgE level) on chromosome 12.

### Association studies of genes on chromosome 12

Results of the case-control studies of chromosome 12 candidate genes are shown in Table 2. There were four types of alleles for the GT repeat of exon 1 of *STAT6* with the repeat number varying from 13 to 16 in our entire series. Comparison of allele frequencies between patients and controls showed a significant difference in the whole-allele distribution ($P$ = 0.0054). This difference was primarily derived from the difference in the frequency of allele 3 (15 repeats). The frequency of this allele was significantly lower in the patients ($Pc$ = 0.0044, odds ratio (OR) = 0.71, 95% confidence interval (95%CI) = 0.58–0.87). Other alleles did not show difference in frequency between controls and patients. Seven genotypes of this GT repeat were observed. The genotypic distribution differed between patients and controls ($P$ = 0.0054). Homozygotes for allele 3 had a lower risk of asthma ($Pc$ = 0.0035, OR = 0.718, 95%CI = 0.60–0.86).

We identified six alleles in the GT repeat polymorphism of intron 2 of *NOS1* (Table 2). The whole-allele distribution of this polymorphism of patients was significantly different from that of controls ($P$ = 0.0082). This difference was primarily due to the difference in frequency of allele 3 (16 repeats) ($Pc$ = 0.049, OR = 0.75, 95%CI = 0.60–0.93). Eleven genotypes of this GT repeat were observed. The whole genotypic distribution differed between patients and controls ($P$ = 0.0019). Homozygotes for allele 3 had a lower risk for asthma than the other genotypes ($Pc$ = 0.030, OR = 0.53, 95%CI = 0.33–0.84). There was a significant difference in the frequency of heterozygote for allele 4/5 between controls and patients ($Pc$ = 0.028). The numbers of this genotype is small (zero in controls and six in patients), and its biological meaning is difficult to interpret.

Association of asthma was also tested for the CA repeat of the interferon γ gene (*IFNG*). We identified seven allele types and 16 genotypes of this polymorphism in the Japanese population (Table 2). There was no evidence of association between this polymorphism and asthma in our series.

We also investigated the SNP 465C/T of *AICDA*. Frequencies of the major allele were almost the same between controls and patients. Genotype distribution of controls was in Hardy-Weinberg equilibrium, whereas that of patients was not ($\chi^2$ = 7.17, $P$ = 0.027). We re-genotyped the patients and found no evidence of typing errors. Patients showed lower CT and higher TT genotype frequencies when compared to those of controls. However, these changes did not reach statistical significance in the association study (Table 2).

## Discussion

We have found evidence of a suggestive linkage between 12q24.32–33 markers (144–155 cM) and childhood asthma in the Japanese population. The region identified was very close to the region reported by Wilkinson et al. (1998), who investigated linkage to their unique asthma score in the English population. In the genome-wide analysis of Japanese mite-sensitive childhood asthma, the highest MLS (1.92) was observed between D12S78 and D12S86 (110–132 cM) (Yokouchi et al. 2000). This