

Asp905Tyr Polymorphism of the Gene for the Skeletal Muscle-Specific Glycogen-Targeting Subunit of Protein Phosphatase 1 in NIDDM

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OBJECTIVE— To clarify the contribution of the Asp905Tyr polymorphism of the muscle-specific glycogen-targeting subunit of protein phosphatase 1 (PP1G) to insulin resistance and related diseases.

RESEARCH DESIGN AND METHODS— We investigated the Asp905Tyr polymorphism of the *PPP1R3* gene, which encodes the muscle-specific glycogen-targeting subunit of PP1G, in 259 Japanese patients with NIDDM and 194 healthy control subjects.

RESULTS— No significant difference was found in the genotype distribution between NIDDM patients ($n = 259$; Asp/Asp = 0.10, Asp/Tyr = 0.44, Tyr/Tyr = 0.46) and healthy control subjects ($n = 194$; Asp/Asp = 0.13, Asp/Tyr = 0.37, Tyr/Tyr = 0.50) or between patient groups subdivided by the mode of treatment: NIDDM patients with insulin therapy (Asp/Asp = 0.14, Asp/Tyr = 0.46, Tyr/Tyr = 0.40) and those without insulin therapy (Asp/Asp = 0.07, Asp/Tyr = 0.43, Tyr/Tyr = 0.50). However, NIDDM patients with the Tyr allele, which was previously reported to be associated with insulin resistance, tended to have lower BMIs than those without this allele (Asp/Asp: 24.5 ± 1.1 kg/m², Asp/Tyr: 22.6 ± 0.4 kg/m², Tyr/Tyr: 22.8 ± 0.3 kg/m², $P = 0.06$ by analysis of variance).

CONCLUSIONS— These data suggest that the Asp905Tyr polymorphism of the *PPP1R3* gene is not associated with NIDDM or high BMI, both of which are known to be insulin-resistant states, in the Japanese population.

NIDDM is a polygenic and heterogeneous disease whose etiology is based on the interplay between genetic and environmental factors (1). It is characterized by at least two major defects: impaired insulin secretion and insulin resistance (2). Several lines of evidence suggest that insulin resistance and/or hyperinsulinemia are fundamental components of the pathogenesis of obesity, essential hypertension, and hyperlipidemia, as well as NIDDM

(3–5). Skeletal muscle glycogen synthesis has been found to represent an important site of insulin resistance in these conditions, and activation of glycogen synthase by insulin is reported to be impaired in these patients (6).

Protein phosphatase 1 is one of the major classes of serine and threonine phosphatases and has been found in all eukaryotic cells. Glycogen-bound protein phosphatase 1 (PP1G) is a key protein in

glycogen synthesis and regulation of nonoxidative glucose disposal (7,8). Both basal and insulin-stimulated PP1G activities in skeletal muscle have been reported to be reduced in insulin-resistant Pima Indians (9). PP1G in skeletal muscle is a heterodimer consisting of a catalytic subunit and a muscle-specific glycogen-targeting regulatory subunit encoded by the *PPP1R3* gene (10,11), can bind to muscle glycogen with high affinity, and plays an important role in the control of glycogen metabolism regulated by insulin and other hormones (7). Therefore, molecular abnormalities of PP1G may lead to insulin resistance and NIDDM. Recently, an amino acid substitution at codon 905 causing an Asp to Tyr change (Asp905Tyr) in the *PPP1R3* gene has been reported (12). Danish subjects who carry the Tyr allele were reported to exhibit insulin resistance and hypersecretion of insulin. This mutation, therefore, is a strong candidate to be involved in the pathogenesis of insulin resistance in hypertension and NIDDM.

Our recent study, however, suggested that Asp905Tyr polymorphism of *PPP1R3* is not associated with essential hypertension or with insulin resistance in patients with essential hypertension (13). To further clarify the contribution of this polymorphism to genetic susceptibility to insulin resistance and related diseases, we studied Asp905Tyr polymorphism of *PPP1R3* in a large number of Japanese patients with NIDDM.

RESEARCH DESIGN AND METHODS

A total of 453 Japanese subjects (259 patients with NIDDM and 194 healthy control subjects with no family history of NIDDM) were studied. All the subjects were judged as unrelated by interview on annual health checkup in the case of control subjects and on clinic visit in the case of patients. The clinical characteristics of the subjects are shown in Table 1. All the patients were recruited from the Osaka University Hospital and an affiliated hospital. NIDDM was diagnosed according to World Health Organization (WHO) criteria. Informed consent was obtained from all

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Received for publication 13 October 1997 and accepted in revised form 9 March 1998.

Abbreviations: ANOVA, analysis of variance; PCR, polymerase chain reaction; PP1G, glycogen-bound protein phosphatase 1; WHO, World Health Organization.

Table 1—Clinical characteristics of subjects

	NIDDM patients	Control subjects	P value
n	259	194	—
Male (%)	49	57	NS
Age (years)	61 ± 9.5	53.8 ± 9.9	NS
BMI (kg/m ²)	24.3 ± 0.3	22.5 ± 0.2	0.0001
Fasting plasma glucose (mmol/l)	9.2 ± 0.3	5.4 ± 0.1	0.0001

Data are n, %, or means ± SEM.

subjects. The NIDDM patients were divided into two subgroups according to the mode of treatment: insulin-treated patients and patients without insulin therapy (treated with diet only or diet plus oral hypoglycemic agents). BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Age at onset of diabetes was estimated from the time of the first symptoms attributable to the disease and/or the time of first detection of glycosuria based on medical records. All of the control subjects had normal glucose tolerances by WHO criteria on a 75-g oral glucose tolerance test at an annual health checkup and had no family history of diabetes in their first-degree relatives. There were 46 control subjects newly genotyped for this study, and the rest of the control subjects were the same as those in our previous study (13). Because the distribution of genotypes of the control subjects newly typed in this study was not significantly different from that in the previous study, these were combined for further analysis. Statistical analyses were performed with both newly typed data and combined data, with the same results for both data sets.

Genomic DNA was extracted from peripheral blood leukocytes. The genotypes were determined by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method, according to the method of Hansen et al. (12), except that 20 ng genomic DNA was used as a template, total volume was reduced to 10 µl, and amplification was performed as follows: annealing at 55°C for 2 min, extension at 72°C for 2 min, and denaturation at 94°C for 15 sec. The PCR products were digested with restriction enzyme *Dde* I and were separated on 9% polyacrylamide gel for genotype determinations.

Results are given as means ± SEM. Hardy-Weinberg equilibrium was checked by χ^2 goodness-of-fit test. The χ^2 test for 3 by 2 contingency table was used to compare frequencies of genotypes. For comparison of frequencies of alleles between the Japan-

ese population in the current study and the Danish population reported previously (12), the χ^2 test for 2 by 2 contingency table was used. Subjects subdivided by diabetes status were compared with respect to BMI, blood pressure, and age at onset of NIDDM using Student's *t* test for unpaired data. Subjects subdivided by *PPP1R3* genotypes were compared with respect to clinical variables using analysis of variance (ANOVA).

RESULTS — The Asp905Tyr substitution of the *PPP1R3* gene was found in 234 (90%) of 259 NIDDM subjects and in 169 (87%) of 194 normal control subjects (Table 2). The genotype frequencies of the *PPP1R3* gene were in agreement with Hardy-Weinberg equilibrium in both the NIDDM patients and the control subjects. The distribution of the genotypes in the NIDDM patients (Asp/Asp = 0.10, Asp/Tyr = 0.44, Tyr/Tyr = 0.46) was not significantly different from that in healthy control subjects (Asp/Asp = 0.13, Asp/Tyr = 0.37, Tyr/Tyr = 0.50). Because *PPP1R3* is a candidate gene for insulin resistance, we further studied the association of the polymorphism with NIDDM in a subgroup of patients who were more likely to be insulin resistant by excluding insulin-treated

patients who are characterized by impaired insulin secretion rather than insulin resistance. No statistically significant difference was observed between insulin-treated NIDDM patients (Asp/Asp = 0.14, Asp/Tyr = 0.46, Tyr/Tyr = 0.40), NIDDM patients without insulin therapy (Asp/Asp = 0.07, Asp/Tyr = 0.43, Tyr/Tyr = 0.50), and control subjects (Table 2). The frequency of Tyr allele in Japanese subjects (68.3%) (Table 2) was significantly higher (odds ratio 21.2, $P < 1 \times 10^{-10}$) than that reported in the Danish population (9.3%) (12).

Among the three groups of NIDDM patients with different *PPP1R3* genotypes, BMI of the subjects with Asp/Asp genotype (24.5 ± 1.1 kg/m²) tended to be higher than BMI of those with other genotypes (Asp/Tyr: 22.6 ± 0.4 kg/m², Tyr/Tyr: 22.8 ± 0.3 kg/m², $P = 0.06$ by ANOVA) (Table 3), suggesting that the Asp/Asp genotype is associated with a higher BMI and the Tyr allele is associated with a lower BMI. When the patients were divided into two groups according to their mode of treatment, higher BMI was observed for the Asp/Asp genotype than for the other genotypes in patients without insulin therapy, but not in patients treated with insulin. No significant difference in BMI was observed in control subjects with different *PPP1R3* genotypes (Asp/Asp: 22.5 ± 0.9 kg/m², Asp/Tyr: 22.9 ± 0.5 kg/m², Tyr/Tyr: 22.5 ± 0.4 kg/m²). No significant difference was observed in age at onset of NIDDM, blood pressure, or mode of treatment among the three groups of patients with different *PPP1R3* genotypes (Tables 3 and 4).

CONCLUSIONS — Several lines of evidence suggest that insulin resistance in NIDDM is genetically determined. PPIG is

Table 2—Genotype and allele frequencies of the *PPP1R3* gene polymorphism Asp905Tyr in NIDDM patients and control subjects

	Control subjects	NIDDM patients		Total
		With insulin	Without insulin	
Genotype				
n	194	95	164	259
Asp/Asp	25 (12.9)	13 (13.7)	12 (7.3)	25 (9.7)
Asp/Tyr	72 (37.1)	44 (46.3)	70 (42.7)	114 (44.0)
Tyr/Tyr	97 (50.0)	38 (40.0)	82 (50.0)	120 (46.3)
Allele				
n	388	190	328	518
Asp	122 (31.4)	70 (36.8)	94 (28.7)	164(31.7)
Tyr	266 (68.6)	120 (63.2)	234 (71.3)	354(68.3)

Data are n or n (% of subjects).

Table 3—Clinical characteristics of NIDDM patients with different genotypes of the Asp905Tyr polymorphism in the PPP1R3 gene

	Asp/Asp	Asp/Tyr	Tyr/Tyr
n	22	110	118
Age (years)	62.5 ± 7.3	60.9 ± 3.6	61 ± 7.5
Age at onset of NIDDM (years)	39.5 ± 3.5	42 ± 4.8	42.5 ± 6.4
BMI (kg/m ²)	24.5 ± 1.1	22.6 ± 0.4	22.8 ± 0.3
Family history of diabetes (%)	48 (12/25)	43.9 (50/114)	53.3 (64/120)
Fasting plasma glucose (mmol/l)	10.3 ± 0.8	8.9 ± 0.4	9.2 ± 0.3
HbA _{1c} (%)	8.6 ± 2.1	7.9 ± 0.7	8.0 ± 1.1
Prevalence of hypertension (%)	48.0 (12/25)	46.4 (52/112)	41.7 (50/120)
Systolic blood pressure (mmHg)	137.3 ± 3.3	143.0 ± 2.4	138.4 ± 1.9
Diastolic blood pressure (mmHg)	76.0 ± 2.4	78.9 ± 1.4	75.7 ± 1.1

Data are n, means ± SEM, or % (ratio).

a key protein involved in glycogen synthesis and regulation of nonoxidative glucose disposal whose defect is a major characteristic of insulin resistance in several diseases, including NIDDM (7). One form of PP1G, consisting of a catalytic subunit and a skeletal muscle-specific glycogen-targeting subunit, is expressed in skeletal muscle, which is a major site of insulin resistance in NIDDM and plays an important role in the regulation of glycogen metabolism by insulin. Therefore, mutation in the skeletal muscle-specific glycogen-targeting subunit, which is encoded by the PPP1R3 gene, may cause insulin resistance. In fact, Asp905Tyr substitution of the PPP1R3 gene has recently been reported to be associated with insulin resistance and hypersecretion of insulin in the Danish population. In our recent study in Japanese patients with essential hypertension, however, Asp905Tyr polymorphism of PPP1R3 was not associated with essential hypertension or with insulin resistance in essential hypertension (13). To further clarify the contribution of this polymorphism to insulin resistance, we examined the genotypes of PPP1R3 in Japanese patients with NIDDM. The results in NIDDM patients were quite similar to those in patients with essential hypertension. In the Danish population, the frequency of the Tyr/Tyr genotype is very low (1% in both normal control subjects and NIDDM patients) (12). In the Japanese population, in contrast, the Tyr/Tyr genotype was identified in 97 (50%) of 194 normal control subjects, in 120 (46.3%) of 259 NIDDM subjects, and in 56 (51.4%) of 109 hypertensive subjects (13), which were much higher frequencies than those reported in the Danish population. The large number of individuals homozygous

for the Tyr allele in the Japanese population, including healthy control subjects, hypertensive patients, and NIDDM patients, allows us to assess the functional alteration related to the Tyr allele in the homozygous form. The data of the present study indicated that the Tyr allele is not associated with NIDDM even after exclusion of insulin-treated patients, who are usually characterized by impaired insulin secretion and are thought to be less insulin resistant than patients without insulin therapy. These data, together with those of our previous study on hypertension (13), suggest that the Tyr allele of PPP1R3 plays little, if any, role in insulin resistance and its related diseases, such as NIDDM and hypertension.

The distribution of genotypes of PPP1R3 in this study was markedly different from that reported in the Danish population

(12). Most Japanese patients with NIDDM are known to be less obese and less insulin resistant than Caucasian patients. The marked difference in the distribution of genotypes of PPP1R3 may therefore be related to the difference in the degree of obesity and insulin resistance between Japanese and Caucasian populations. However, the frequency of the Tyr allele, which is reported to be associated with insulin resistance in the Danish population, was much higher in the Japanese population than in the Danish population, making it unlikely that the difference in genotype distribution of the polymorphism is directly related to the difference in the degree of obesity and/or insulin resistance. A marked difference in the frequencies of genotypes in Japanese from those reported in Caucasians was previously reported for other candidate genes for NIDDM, such as the genes for glycogen synthase (14) and glucagon receptor (15). The difference may reflect the differences in the genetic backgrounds of the populations and emphasizes the importance of studies on genetic susceptibility to NIDDM in different ethnic groups.

Although the Asp905Tyr polymorphism was not associated with NIDDM or hypertension, BMI tended to be higher in NIDDM patients with the Asp/Asp genotype than in those with other genotypes. The association of a higher BMI with insulin resistance is well known. It is, therefore, conceivable that the Asp/Asp homozygous genotype may be associated with insulin resistance through its effect on BMI. In the Danish population, however, the Tyr allele,

Table 4—Clinical characteristics of NIDDM patients subdivided by mode of treatment according to genotypes of the PPP1R3 gene polymorphism Asp905Tyr

	Asp/Asp	Asp/Tyr	Tyr/Tyr
NIDDM patients with insulin therapy			
n	10	35	30
Age (years)	65.1 ± 3.4	60.4 ± 1.6	59.9 ± 1.4
BMI (kg/m ²)	22.9 ± 0.9	22.8 ± 0.6	22.4 ± 0.5
HbA _{1c} (%)	8.4 ± 0.9	8.1 ± 0.3	7.7 ± 0.2
Systolic blood pressure (mmHg)	145.1 ± 10.3	143.1 ± 2.8	138.2 ± 3.7
Diastolic blood pressure (mmHg)	81.6 ± 8.5	76.3 ± 1.7	76.8 ± 2.3
NIDDM patients without insulin therapy			
n	12	70	82
Age (years)	57.2 ± 2.9	62.5 ± 1.0	61.2 ± 1.1
BMI (kg/m ²)	24.9 ± 0.8	22.5 ± 0.4	22.7 ± 0.5
HbA _{1c} (%)	7.9 ± 0.5	8.0 ± 2.9	8.1 ± 0.2
Systolic blood pressure (mmHg)	135.7 ± 6.8	142.1 ± 3.3	138.4 ± 2.2
Diastolic blood pressure (mmHg)	77.2 ± 4.8	76.0 ± 1.6	78.2 ± 1.3

Data are n or means ± SEM.

but not the Asp allele, was associated with insulin resistance and hyperinsulinemia. These data suggest that Asp905Tyr polymorphism may not contribute to insulin resistance in itself, and that association of this gene with insulin resistance and BMI, if it exists, may be due to another nearby mutation that is in linkage disequilibrium with the Asp allele in the Japanese population and with the Tyr allele in the Danish population.

High BMI in subjects with the Asp/Asp genotype was observed in NIDDM patients but not in control subjects. These data suggest that the Asp/Asp genotype may not be directly associated with body weight gain, but that it may lead to resistance to weight reduction during therapy in NIDDM patients with obesity, which is caused by factors other than the Asp905Tyr polymorphism, such as overeating, decreased physical activity, and other genetic factors. Further studies on the effect of the polymorphism on gain and reduction of body weight are necessary to clarify the relationship between PPP1R3 polymorphism and body weight.

Regarding the statistical power of this study, the probability of detecting significant ($P < 0.05$) results using the samples in the current study was estimated by the Monte Carlo method under the assumption that the Asp905Tyr polymorphism is the polymorphism responsible for variation of BMI and has a moderate effect (heritability $h^2 = 0.05$). The probability was estimated as 0.92, 0.91, and 0.90 for dominant, additive, and recessive models, respectively, indicating that the number of samples analyzed in the current study may be enough to detect significant results if the effect of the polymorphism is marked.

Stimulation of glycogen synthesis by insulin is thought to be mediated by the activation of PPIG as a result of the phosphorylation of Ser at position 46 of the regulatory subunit (16). Other potential phosphorylation sites are Ser at positions 38 and 42, although the relation to insulin-stimulated activation of glycogen synthesis is not fully clarified. The Asp905Tyr polymorphism is located far from these phosphorylation sites in the primary structure, and therefore may not be directly related to

the functional alteration of the molecule, although it may have functional significance through its three-dimensional structure.

In summary, the data of the present study, together with those of the previous study, suggest that the Asp905Tyr polymorphism in PPP1R3 does not play a critical role in the pathogenesis of NIDDM and essential hypertension. Rather, the Tyr allele, which is associated with insulin resistance in the Danish population, tended to be associated with lower BMI, which is known to be an insulin-sensitive, rather than an insulin-resistant, state in Japanese patients with NIDDM. These data suggest that the Tyr allele of the polymorphism is not associated with insulin resistance in itself, and that association of this gene with insulin resistance, if it exists, may be due to linkage disequilibrium with nearby mutations in the same gene or nearby genes.

Acknowledgments— This work was supported in part by a Grant for Diabetes Research from the Ministry of Health and Welfare; Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture; and a Grant from the Kato Memorial Trust for Nambyo Research.

We thank Yumiko Ueno for her skillful technical assistance.

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