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A missense mutation of the muscle glycogen synthase gene (M416V) is associated with insulin resistance in the Japanese population

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Summary Muscle glycogen synthase (GYS1) is a key enzyme of non-oxidative pathway of glucose metabolism that has been reported to be related to insulin resistance in non-insulin-dependent diabetic (NIDDM) patients. We scanned the GYS1 gene for mutation by single strand conformational polymorphism in 244 non-obese Japanese NIDDM patients and 181 non-diabetic control subjects, and found two missense mutations; Met to Val at position 416 in the exon 10 (M416V) and Pro to Ala at position 442 in the exon 11 (P442A). The P442A mutation was found in only one NIDDM patient treated with sulfonylureas. On the other hand, the M416V mutation was widely found in the Japanese population. The mutant allele frequency in the NIDDM patients (13.7%) was slightly higher but not statistically significant compared with that in non-diabetic subjects (9.7%). However, the insulin sensitivity index [SI: $\times 10^{-4} \times \mathrm{min}^{-1} \times (\mu\mathrm{U/ml})^{-1}$] estimated by Minimal Model analysis in the NIDDM patients carrying the M416V mutation was significantly lower than that in those without the mutation (1.18 ± 0.27, $n = 21$ vs 2.20 ± 0.20, $n = 60$, mean ± SEM, $p < 0.01$). Glucose effectiveness, age, body mass index, and levels of glycated haemoglobin and serum lipids were not significantly different between the two groups. The same trend could be seen in non-diabetic subjects (SI: 3.70 ± 0.46, 9 subjects with the mutation vs 5.94 ± 0.66, 19 subjects without the mutation, $p < 0.05$). These findings indicate that the M416V mutation of the GYS1 gene is one of the factors contributing to the insulin resistance in the Japanese population and may play some role in the pathogenesis of NIDDM. [Diabetologia (1997) 40: 947–952]

Keywords Muscle glycogen synthase, insulin resistance, NIDDM, genetics.

Non-insulin-dependent diabetes mellitus (NIDDM) is thought to be a multifactorial disease [1], and both inherited and acquired factors contribute to its pathogenesis. NIDDM is also characterized by insulin resistance as well as reduced insulin secretion.

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Abbreviations: GYS1, Muscle glycogen synthase; NIDDM, non-insulin-dependent diabetes mellitus; SSCP, single strand conformational polymorphism; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SI, insulin sensitivity; SG, glucose effectiveness.

Muscle glycogen synthase (GYS1) is a key enzyme of non-oxidative glucose metabolism which is a major pathway of glucose disposal during insulin stimulation [2]. Several recent studies have shown reduced insulin-stimulated GYS1 activity in skeletal muscle of NIDDM patients [2–6] and their first-degree relatives with normal glucose tolerance [3, 6, 7]. Decreased levels of GYS1 mRNA in skeletal muscle of NIDDM patients have also been reported [8, 9]. These findings suggest that impaired GYS1 activity is an inherited trait in patients with NIDDM. In fact, positive associations between GYS1 gene polymorphism [10, 11] and NIDDM have been reported in Finns [10], French [12], Pima Indians [13], and Japanese [14]. Recently, the human GYS1 gene was isolated and characterized, and a missense mutation in the

exon 11 (Gly464Ser: G464S) was found in 2 of 228 Finnish patients with NIDDM in relation to severe insulin resistance and arteriosclerosis [15]. This study makes it possible to study the role of GYS1 in the development of insulin resistance and NIDDM in other populations. We therefore scanned the GYS1 gene for mutation in Japanese NIDDM patients by single strand conformational polymorphism (SSCP) analysis.

Subjects and methods

Subjects. For the screening of mutations of the GYS1 gene, we selected 244 unrelated (within third-degree relatives from interview) Japanese NIDDM patients diagnosed by World Health Organization (WHO) criteria and 181 unrelated non-diabetic control subjects without a family history of diabetes from interview. The presumed onset age of the NIDDM patients was between 35 and 60 years old. The clinical characteristics of the subjects are shown in Table 1. Severely obese subjects with body mass index (BMI) over 30 kg/m^2 were excluded. All of the non-diabetic subjects were over 60 years old, and their glycated haemoglobin levels were below 6.0%.

For Minimal Model analysis, we selected 81 NIDDM patients not on insulin treatment from among the 244 NIDDM patients, and an additional 28 healthy control subjects [non-severely obese, normotensive and non-diabetic (glycated haemoglobin < 6.0%)] without family history of diabetes and hypertension from interview. Their clinical characteristics are shown in Table 2.

PCR/SSCP analysis. Genomic DNA was extracted from peripheral leukocytes. At blood sampling, informed consent was obtained from each individual according to the protocol established by the human studies committee. Each exon and 847 bp upstream from the transcriptional initiation site of the GYS1 gene was amplified by polymerase chain reaction (PCR) according to the methods described by Orho et al. [15] using fluorescence-labelled primers. The denatured PCR products were separated with a 6% polyacrylamide gel at DNA fragment specific temperatures using an ALFred autosequencer (Pharmacia, Uppsala, Sweden), and the results were analysed using Fragment Manager software (Pharmacia).

Nucleotide sequencing and NlaIII RFLP. The DNA fragment which showed abnormal migrating bands on SSCP was subcloned into pGEM4Z(+) (Promega, Madison, Wis, USA) and was sequenced by the dideoxy chain termination method. For the restriction fragment length polymorphism (RFLP) analysis, the PCR products of the exon 10 were ethanol precipitated and were then digested by restriction enzyme NlaIII at 37°C for 3 h. The digested DNA fragments were analysed by 2.5% agarose gel.

Biochemical studies and evaluation for insulin sensitivity. We employed Minimal Model analysis [16, 17] to estimate the insulin sensitivity in vivo. After overnight fasting, frequent sampling intravenous glucose tolerance tests combined with intravenous insulin injections were performed. After insertion of a cannula for blood sampling into the anticubital vein, blood samples for biochemical analysis [glycated haemoglobin, and serum lipids (triglyceride, total cholesterol and HDL cholesterol)] were drawn. Baseline values of plasma glucose, serum insulin, and serum C-peptide were taken in duplicate at 5 min intervals. Glucose (0.3 g/kg body weight) was injected intravenously into the contralateral antecubital vein over a period of 2 min. At 20 min after the start of the glucose injection, a bolus of human insulin (Novolin R, Novo Nordisk Pharma A/S, Copenhagen, Denmark: 0.05 U/kg in NIDDM patients and 0.02 U/kg in non-diabetic subjects) was injected through the same vein over a period of 5 s. Venous blood was sampled at 4, 6, 8, 10, 15, 19, 22, 24, 30, 40, 70, 90, and 180 min, timed from the start of the glucose injection to measure serum insulin and plasma glucose. The insulin sensitivity index (SI) and glucose effectiveness (SG) were calculated using MINMOD software [18].

Statistical analysis. Data are shown as mean ± SEM. The statistical analysis was performed by Student's *t*-test or chi-square analysis, and *p* values less than 0.05 are considered significant.

Table 1. Clinical characteristics of the subjects studied for SSCP analysis

	NIDDM (n = 244)	Control subjects (n = 181)	P value
Age (years)	64.4 ± 0.6	67.5 ± 0.9	NS
Sex (male/female)	128/122	93/88	NS
Body mass index (kg/m^2)	23.2 ± 0.2	22.0 ± 0.5	NS
HbA_{1c} (%)	8.1 ± 0.1	5.5 ± 0.1	$p < 0.01$
Frequency of positive family history of diabetes (%)	54.2	0	$p < 0.01$

NS: not significant

Table 2. Clinical characteristics of the NIDDM patients studied for minimal model analysis

	M416V(+) (n = 21)	M416V(−) (n = 60)	P value
Age (years)	64.4 ± 2.3	61.0 ± 1.4	NS
Body mass index (kg/m^2)	23.4 ± 0.7	23.3 ± 0.4	NS
HbA_{1c} (%)	7.9 ± 0.5	7.5 ± 0.2	NS
Fasting plasma glucose (mmol/l)	8.09 ± 0.67	7.95 ± 0.26	NS
Total cholesterol (mmol/l)	5.39 ± 0.17	4.96 ± 0.12	NS
Triglyceride (mmol/l)	1.38 ± 0.20	1.11 ± 0.08	NS
HDL cholesterol (mmol/l)	1.22 ± 0.10	1.28 ± 0.05	NS
SI [$10^{-4} \times \min^{-1} \times (\mu U/ml)^{-1}$]	1.18 ± 0.27	2.20 ± 0.20	$p < 0.01$
SG (10^{-2}/min)	2.11 ± 0.18	1.84 ± 0.10	NS
Insulin (pmol/l)	68.6 ± 10.4	61.5 ± 6.0	NS
C-peptide (ng/ml)	2.16 ± 0.31	1.92 ± 0.10	NS

SI, Insulin sensitivity estimated by minimal model analysis;
SG, glucose effectiveness estimated by minimal model analysis;
NS, not significant

Results

PCR/SSCP analysis. On PCR/SSCP analysis, abnormal conformers were found in the PCR products corresponding to exon 10 (Fig. 1) and 11. DNA sequencing of these exons revealed a single missense

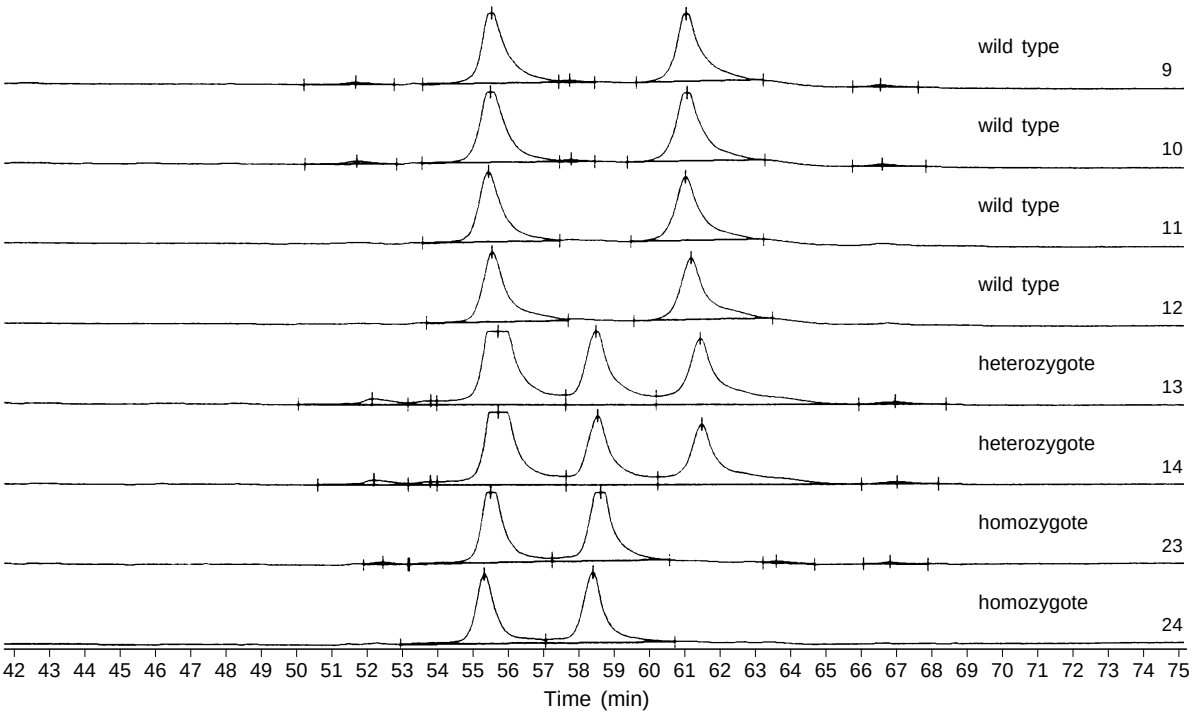

Fig. 1. PCR/SSCP analysis in the exon 10 of human muscle glycogen synthase gene

mutation in amino acid 416 [Met (ATG) to Val (GTG): M416V mutation] and 442 [Pro (CCT) to Ala (GCT): P442A mutation], respectively. The M416V mutation destroys the recognition site of restriction enzyme NlaIII. Using RFLP, all PCR products with abnormally migrating bands were confirmed to have the same M416V mutation (Fig. 2). The genotype and mutant allele frequencies are shown in Table 3. The genotype frequency in both NIDDM patients and control subjects was statistically consistent in Hardy-Weinberg equilibrium. Although the mutant allele frequency was slightly higher in NIDDM patients compared with those in non-diabetic subjects, there were no significant differences in either the genotype or the mutant allele frequencies of the M416V mutation between these two groups (Table 3). The P442A mutation (heterozygote) was found in only one NIDDM patient treated with sulfonylureas. She had hypertension and ischaemic heart disease, her mother also had NIDDM. We could not find the G464S mutation [15] in the exon 11, C to G change at position + 42 [15], or silent mutation in the exon 7 (Phe342Phe) [15] by SSCP analysis. We also performed an HaeIII RFLP study using the PCR products of exon 11 of 35 randomly chosen NIDDM patients to search for the G464S mutation, but no aberrant bands could be seen. Unlike in previous studies [15, 19], no abnormal conformers were found in the promoter region in our system.

Clinical characteristics of subjects with the M416V mutation. Eighty-one NIDDM patients not on insulin treatment and in stable metabolic control were subjected to Minimal Model analysis to estimate their insulin sensitivity in vivo. They were divided into two subgroups according to the presence or absence of the M416V mutation, and their SI, SG and clinical characteristics are compared in Table 2. The SI of the subgroup with the M416V mutation was significantly lower than that of those without the mutation. There were no significant differences in SG, age, BMI, blood pressure, and glycated haemoglobin and serum lipid levels between the two subgroups. Fasting serum insulin and C-peptide levels in the subgroup with the mutation were slightly higher than those in the subgroup without the mutation, although the difference was not statistically significant. The correlation between individual SI and BMI values is shown in Figure 3. The regression line of the subgroup with the mutation situated below that of those without the mutation indicates that the SI of the patients with the mutation was reduced regardless of their BMI. We also performed the same analysis on 28 healthy control subjects, and the results are shown in Table 4. The SI of the subjects with the mutation was also significantly lower than that of those without the mutation. The SG, age, BMI, blood pressure, glycated haemoglobin and serum lipids were not significantly different between the two subgroups. Fasting serum insulin ($p < 0.05$) and C-peptide levels of the subjects with the mutation were also higher than those of the subjects without the mutation.

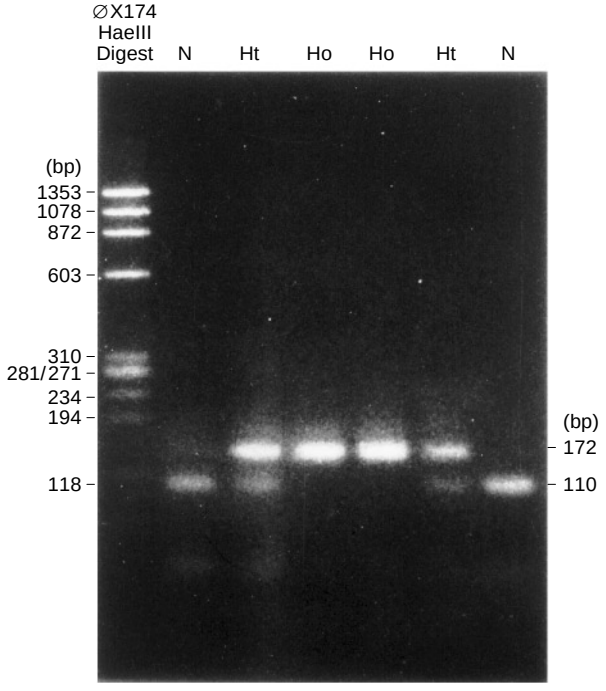

Fig. 2. NlaIII restriction fragment length polymorphism in the exon 10 of the human muscle glycogen synthase gene. N: wild type, Ht: heterozygote, Ho: homozygote

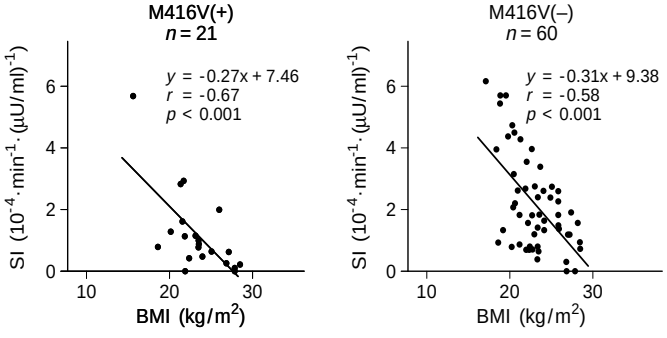

Fig. 3. Correlation of individual insulin sensitivity index (SI) estimated by minimal model analysis and body mass index in NIDDM patients. Left panel: M416V mutation (+); Right panel: M416V mutation (–)

Discussion

Some gene mutations such as insulin receptor [20], hexokinase II [21], insulin receptor substrate-1 [22], and glycogen-associated regulatory subunit of type 1 protein phosphatase [23] have been reported in relation to insulin resistance. However, these mutations are rare. The GYS1 gene is also a candidate gene for insulin resistance in NIDDM according to many recent reports [2–9, 24]. In the present study, two novel missense mutations of the GYS1 gene were found in a Japanese population. We did not find the G464S mutation in exon 11 [15] and other variants [15, 19] in our population. These discrepancies must be the result of ethnic differences between the Finns, other Caucasians and the Japanese. The P442A mutation was found in only one NIDDM patient. As we could not get consent from her for further studies, the significance of this mutation upon the insulin resistance is unknown. On the other hand, the M416V mutation was found with high frequency in both NIDDM patients and non-diabetic subjects. The SI was significantly decreased in the subgroup carrying the mutation not only among NIDDM patients but also non-diabetic control subjects. As a reflection of insulin resistance, the fasting serum insulin level of the non-diabetic subjects with the mutation was significantly higher than that of those without the mutation. Moreover, the fasting serum C-peptide level was higher, although not significantly, in those subjects carrying the mutation which suggests hypersecretion of the pancreatic beta-cell to compensate for the reduced insulin sensitivity and to keep normal glucose tolerance in non-diabetic control subjects. These findings indicate that the M416V mutation in the GYS1 gene is closely correlated with insulin resistance in vivo. Significant differences in the genotype and allele frequency could not be found between these two groups, suggesting that the M416V mutation on its own might

Table 3. Genotype frequencies and mutant allele frequencies of the M416V mutation

Genotype	NIDDM ($n = 244$)	Control subjects ($n = 181$)
Homozygote	3 (1.2%)	1 (0.6%)
Heterozygote	58 (23.8%)	33 (18.2%)
Wild type	183 (75.0%)	147 (81.2%)
		Not significant
Allele frequency	NIDDM ($n = 488$)	Control subjects ($n = 362$)
V allele	13.7%	9.7%
		Not significant

Table 4. Clinical characteristics of the non-diabetic control subjects studied for minimal model analysis

	M416V(+) (n = 9)	M416V(–) (n = 19)	P value
Age (years)	42.6 ± 3.1	40.2 ± 2.3	NS
BMI (kg/m^2)	24.2 ± 0.8	22.4 ± 0.7	NS
HbA_{1c} (%)	5.2 ± 0.1	5.1 ± 0.1	NS
Total cholesterol (mmol/l)	5.13 ± 0.31	4.84 ± 0.17	NS
Triglyceride (mmol/l)	1.17 ± 0.16	1.17 ± 0.15	NS
HDL cholesterol (mmol/l)	1.61 ± 0.12	1.61 ± 0.06	NS
SI [$10^{-4} \times min^{-1} \times (\mu U/ml)^{-1}$]	3.70 ± 0.46	5.94 ± 0.66	$p < 0.05$
SG (10^{-2}/min)	1.91 ± 0.18	2.10 ± 0.22	NS
Insulin (pmol/l)	71.0 ± 5.2	55.8 ± 3.2	$p < 0.05$
C-peptide (ng/ml)	1.84 ± 0.15	1.59 ± 0.08	NS

SI, Insulin sensitivity estimated by minimal model analysis;
SG, glucose effectiveness estimated by minimal model analysis;
NS, not significant

cause mild insulin resistance. Thus diabetogenic power of this mutation might be weak, and other factors must be necessary for causing diabetes.

The human GYS1 consists of 737 amino acids [25], and the methionine residue at position 416 is conserved among at least four mammals [25–27], suggesting that the substitution of this amino acid reduces its function. Minimal Model analysis was not available in the four subjects carrying the homozygote form. Thus, the gene dosage effect is not known. In the case of the G464S mutation, the patient's insulin resistance in vivo estimated by glucose clamp study coexisted with reduced muscle GYS1 activity in vitro [15]. As the muscle biopsy was not available in the present case of M416V mutation, it remains uncertain whether the observed insulin resistance in vivo in the subjects with M416V mutation reflects the reduced muscle GYS1 activity. The possibility that this mutation or variant is due to a disequilibrium of linkage with another gene located near GYS1 should also be kept in mind. Further examinations including expression studies are necessary to clarify the mutant GYS1 activity.

Recently, a possible association between GYS1 gene locus and hyperglycaemia induced by high calorie intake has been reported using B/6J mouse [28]. The B/6J mouse had a normal insulin level on a normal diet, whereas a diabetogenic diet (high fat and high carbohydrate) increased insulin levels 3 to 4 times. In this mouse, hyperglycaemia was shown to be associated with the mouse GYS1 locus in chromosome 7 which is shown to correspond to the region of human muscle GYS1 gene in chromosome 19. After the second world war, the number of NIDDM patients in Japan has gradually but steadily increased [29]. The increasing number of patients has been definitely correlated with the increased production of cars and increased fat intake, both of which are thought to cause insulin resistance through lack of exercise and obesity, respectively [29]. Taking such a social background into consideration with the finding obtained from the B/6J mouse, the M416 mutation, which is widely found in the Japanese, is thought to be an important factor in amplifying insulin resistance and leading to the onset of NIDDM in the Japanese population, although its diabetogenic power is weak.

Recently, two major susceptibility loci for late-onset NIDDM, NIDDM1 on chromosome 2 in Mexican Americans [30] and NIDDM2 on chromosome 12 in Finnish families [31], were reported by using genome-wide scanning. However, it is possible that NIDDM1 differs between ethnic groups [30], and that NIDDM2 is linked to limited homogeneous families characterized by low insulin secretion [31], which indicates the genetic complexity of NIDDM. Given the present situation, it would be informative to clarify the genetic factors such as the M416V mutation of GYS1 gene which exists widely and contributes to the insulin resistance which is thought to be one of the pathophysiological characteristics of NIDDM.

In conclusion: we found a novel GYS1 gene mutation (M416V) which correlates to insulin resistance in vivo in the Japanese population.

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