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MOLECULAR SCANNING OF THE GLYCOGEN SYNTHASE AND INSULIN RECEPTOR SUBSTRATE-1 GENES IN JAPANESE SUBJECTS WITH NON-INSULIN-DEPENDENT DIABETES MELLITUS

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We studied a simple tandem repeat DNA polymorphism in the glycogen synthase gene and polymorphisms at codon 513 (Ala→Pro) and 972 (Gly→Arg) in the insulin receptor substrate-1 (IRS-1) gene in 197 non-insulin-dependent diabetes mellitus (NIDDM) and 178 control subjects in Japan. Eight alleles (-3G, -2G, -1G, 0G, 1G, 2G, 3G, and 4G) were identified in the tandem repeat polymorphism in the glycogen synthase gene. No difference in the frequencies of these alleles was found between diabetics and controls. The codon 972 polymorphism of IRS-1 gene was observed in 7 diabetics (3.6%) and 8 controls (4.5%), whereas the codon 513 polymorphism was not found in either of the two groups. We conclude that the tandem repeat polymorphism in the glycogen synthase gene and the polymorphisms at codons 513 and 972 of the IRS-1 gene are not associated with a higher risk for the development of NIDDM in Japanese subjects.

Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by defects in both insulin secretion and insulin action (1). Impairment of glucose-induced insulin secretion from pancreatic β cells has been shown to be detected in the prediabetic period (2,3). In fact, mutations in the glucokinase gene and the mitochondrial gene can be associated with a certain form of NIDDM with defects in insulin secretion (4,5). On the other hand, several studies in recent years have shown that the clinical onset of NIDDM in different populations is preceded by an undefined time period when insulin resistance may be detected (6-9). For example, mutations in the insulin receptor gene have been shown to cause insulin resistance and diabetes (10). However, subjects with NIDDM are generally associated with post receptor insulin resistance (1). Polymorphisms in the glycogen synthase and insulin receptor substrate-1 (IRS-1) genes, two logical candidate genes causing post receptor insulin resistance, have recently been reported to be associated with a higher risk for the development of NIDDM in Caucasians (11,12).

Glycogen synthase is a key enzyme of the non-oxidative glucose disposal in the skeletal muscle. It has been reported that insulin resistance of the skeletal muscle may be a very early event in

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the development of NIDDM which exists before metabolic derangements occur (13,14). Thus, NIDDM subjects had reduced insulin-stimulated glycogen synthase activities and decreased levels of glycogen synthase mRNA in the skeletal muscle (13-17). This suggests that molecular defects of the glycogen synthase gene may contribute to the pathogenesis of NIDDM. In fact, Groop and coworkers reported that the XbaI polymorphism of the glycogen synthase gene is associated with a higher risk for the development of insulin resistance and NIDDM in the Finnish population (11).

IRS-1 is thought to mediate the signal from the insulin receptor tyrosine-kinase to final biological actions of insulin (18). In fact, when we disrupted the IRS-1 gene in embryonic stem cells by homologous recombination to create mice deficient in IRS-1, these mice showed insulin resistance and growth retardation (19). It has been reported that the 513 and 972 polymorphisms in IRS-1 might be involved in the etiology of a subset of NIDDM (12).

Japanese patients with NIDDM generally have a decreased rather than an increased plasma insulin response to glucose as compared with Caucasian patients with NIDDM (20). Therefore, it is important to determine whether polymorphisms in the glycogen synthase and IRS-1 genes are also associated with the development of NIDDM in Japanese subjects. We studied a simple tandem repeat DNA polymorphism in glycogen synthase and the polymorphism at codon 513 and 972 in Japanese subjects with NIDDM. In contrast to the previous reports (11,12) with the Caucasian NIDDM patients, we failed to confirm the association of the glycogen synthase gene or IRS-1 gene with NIDDM in the Japanese population. These results may further support the notion that there exists a genetic heterogeneity in the pathogenesis of NIDDM.

SUBJECTS AND METHODS

Subjects -The study subjects with NIDDM (n=197) were recruited from the Institute for Diabetes Care and Research, Asahi Life Foundation (Tokyo, Japan). All NIDDM diagnosed according to World Health Organization (WHO) criteria was treated with diet and/or oral hypoglycemic agents without insulin therapy. The 178 control subjects recruited from the Institute for Diabetes Care and Research, Asahi Life Foundation and Toranomon Hospital (Tokyo, Japan) for their general health examination, showed normal glucose patterns in a 75 g or a 100g oral glucose tolerance test (OGTT) according to WHO criteria and had a negative family history of diabetes mellitus.

Genotyping of subjects using a simple tandem repeat DNA polymorphism in the glycogen synthase gene -Genomic DNA was obtained from peripheral white blood cells. Two oligonucleotide primers (GS-CA1: 5'-AGCTAATTTTTGTATCTGTG-3' and GS-CA2: 5'-CCTGGGCATCAGAGCAAGAC-3') flanking the TG repeat in the glycogen synthase gene were used to amplify an 86 bp fragment as described by Vionnet and Bell (21). Genomic DNA was digested with a restriction endonuclease (Hind III) before use in the polymerase chain reaction (PCR). The PCR was conducted in a volume of 25 μl and included 100ng of DNA, 10 pmol of ^{32}P-labeled GS-CA1 (1 pmol ^{32}P-labeled and 9 pmol unlabeled), and 1 pmol of unlabeled GS-CA2 (21). PCR conditions were initial denaturation at 94°C for 5min followed by 35 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min and extension at 72°C for 1min, and a final extension step of 5 min. The PCR products were separated on an 8% polyacrylamide sequencing gel and visualized by autoradiography.

Polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP) analysis of IRS-1 gene and sequencing of amplified DNA -To amplify two regions of codon 513 and 972 polymorphisms of IRS-1, two oligonucleotide primer pairs (513 forward primer: 5'-GCGGTGAGGAGGAGCTAAGC-3' and reverse primer: 5'-GCCACT GAGGACTGGGACGGG-3', 972 forward primer: 5'-CTTCTGTCAGGTGTCCATCC-3' and reverse primer: 5'-TGGCGAGGTGTCCACGTAGC-3') were used as described by Almind et al.

(12). PCR conditions were initial denaturation at 94°C for 5min followed by 30 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min and extension at 72°C for 1min, and a final extension step of 5 min. The labeled PCR products were loaded onto 5% polyacrylamide gel with 1% glycerol at 35W constant power for 2.5-4h at 4°C and with 5% glycerol at 50W constant power for 1.5-2.5h at room temperature (22,23). When shifted bands were observed, the amplified DNAs were subcloned into plasmid and the sequences were determined by the dideoxy chain termination method (24).

Statistical analysis-All results were expressed as mean±standard error of mean (SEM). Statistical analysis was performed using paired or unpaired two tailed Student's *t* tests. Chi-square analysis with Yates' correction was used to determine the significance of differences in frequency.

RESULTS

Characterization of a simple tandem repeat DNA polymorphism in the glycogen synthase gene-The simple tandem repeat DNA polymorphism in the glycogen synthase gene was highly polymorphic, and 8 alleles (-3G, -2G, -1G, 0G, 1G, 2G, 3G, and 4G) were identified in Japanese subjects (Fig. 1 and Table 1). An 0G allele had the 18 TG repeats, and each allele (-3G, -2G, -1G, 1G, 2G, 3G, and 4G) had 15, 16, 17, 19, 20, 21, and 22 TG repeats, respectively. The allele frequency in the Japanese population was different from that reported in the Caucasian (21). However, no difference in allele frequency was found between 197 NIDDM and 178 control subjects in the Japanese population (Table 1).

Identification of codon 513 and 972 polymorphisms in IRS-1 gene-When we analyzed the 197 NIDDM and 178 control subjects, we identified an SSCP variant in the PCR fragment encompassing codon 972 (Fig. 2A). By determining the nucleotide sequence of this PCR variant, we identified a single base pair substitution which substitutes Arg (GGG) for Gly (AGG) at codon 972 (Fig. 2B). This missense mutation was identical to the 972 polymorphism reported in the Caucasian population (12). The 972 polymorphism was observed in 7 diabetic (3.6%) and 8 controls (4.5%) in the Japanese population. No significant differences in frequencies were noted between these two groups. However, we did not detect the codon 513 polymorphism either in diabetics or control subjects in the Japanese population.

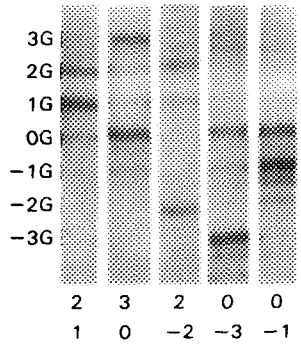

Fig.1. Genotypes using a simple tandem repeat DNA polymorphism in the glycogen synthase gene. The different alleles are defined at the left side of the figure and genotypes of individuals are shown at the bottom of the figure.

TABLE 1. Characterization of a simple tandem repeat DNA polymorphism in the glycogen synthase gene

Genotype	NIDDM (n=394)		Control (n=356)	
	n	(%)	n	(%)
4G	1	(0.3)	1	(0.3)
3G	9	(2.3)	8	(2.2)
2G	68	(17.3)	61	(17.1)
1G	105	(26.6)	101	(28.3)
0G	159	(40.4)	149	(41.9)
-1G	16	(4.1)	16	(4.5)
-2G	8	(2.0)	6	(1.7)
-3G	28	(7.1)	14	(3.9)

Clinical characteristics of individuals with 972 polymorphism in IRS-1 gene-Clinical characteristics of NIDDM and control subjects with or without 972 polymorphism in the IRS-1 are shown in Table 2. Age, body mass index, known duration of diabetes, HbA_{1c}, fasting plasma glucose, and plasma insulin levels were not significantly different between those with and without this polymorphism in both NIDDM and control subjects.

DISCUSSION

Several studies have shown that impaired activation of glycogen synthase in the skeletal muscle was observed in patients with NIDDM and occurs in patients at increased risk for this condition (13-

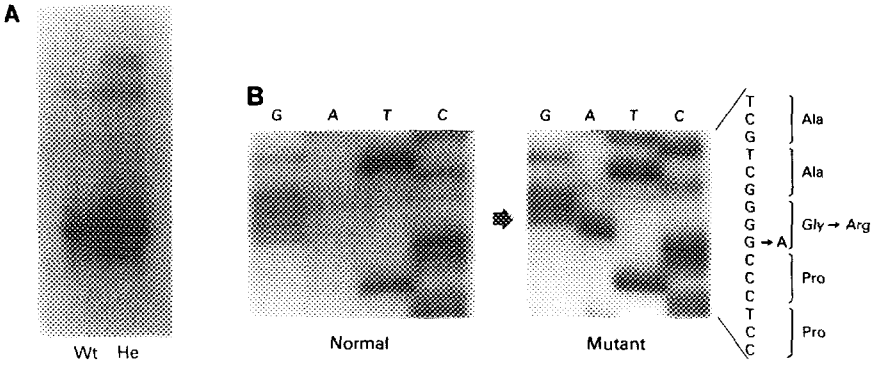

Fig.2. SSCP analysis and nucleotide sequence of codon 972 polymorphism in IRS-1.
A. SSCP patterns with wild type (Wt) and heterozygous type (He) sequences at codon 972 polymorphism are shown.
B. The sequence of codon 972 polymorphism (Gly→Arg) is shown.

TABLE 2. Clinical characterization of NIDDM and control subjects with or without 972 polymorphism in IRS-1

	NIDDM (n=197)		Control (n=178)	
	P972	Wild type	P972	Wild type
Number (F/M)	3/4	65/125	4/4	73/97
Age (yr)	51.3±8.7	56.7±9.1	52.3±13.6	55.6±8.8
BMI (kg/m2)	22.9±5.3	23.6±8.1	21.2±3.6	22.2±2.9
Known duration of diabetes (yr)	8.3±4.5	8.3±6.3	-	-
HbA1c (%)	8.6±1.5	8.3±1.7	6.0±0.2	6.0±3.5
Plasma glucose (mmol/L)	8.8±2.0	8.0±2.1	4.5±0.57	4.5±0.42
Plasma insulin (pmol/L)	47.4±20.8	48.1±25.8	50.2±47.4	44.5±25.1

17). This suggests that glycogen synthase may be a candidate gene for NIDDM. In fact, Groop and coworkers reported that the A_2 allele of the XbaI polymorphism of the glycogen synthase gene is associated with a higher risk for the development of insulin resistance and NIDDM in the Finnish population (11). The A_2 allele was observed in 30 percent of patients with NIDDM, whereas it was observed in only 8 percent of normal subjects. However, in the French population, this polymorphism of the glycogen synthase gene was not associated with NIDDM (25). Kuroyama and colleagues in a Japanese group (Wakayama) (26) have reported that they could not find the A_2 allele in 98 Japanese patients with NIDDM and 86 non-diabetic subjects by Southern blot analysis (11) or by PCR (27). Recently, we also confirmed that there is no XbaI polymorphism in the glycogen synthase gene in Japanese NIDDM (unpublished observation). Therefore, in order to assess the role of this gene in the pathogenesis of NIDDM, we studied another polymorphism (a simple tandem repeat DNA polymorphism (21)) in glycogen synthase gene in 197 NIDDM and 178 control subjects. We found that the simple tandem repeat polymorphism also occured in the Japanese population, although we found no association between this polymorphism and NIDDM (Table 1). Kuroyama and colleagues also studied this polymorphism in 164 patients with NIDDM and in 115 non-diabetic subjects and showed that the 2G allele is positively associated with non-obese NIDDM in Japanese subjects (26). The 2G allele was found more frequently in patients with NIDDM than in non-diabetic subjects (17.7% vs. 8.7%). In our findings, the 2G allele was found in 17.3 percent of subjects with NIDDM and in 17.1 percent of control subjects (Tokyo, Japan). This apparent difference in Japanese subjects may be due to a regional difference (Wakayama vs. Tokyo) in the genetic background for NIDDM. We think that a simple tandem repeat DNA polymorphism in glycogen synthase gene may not be a major candidate gene in Japanese subjects with NIDDM, although this

study cannot exclude the possibility that mutations in this gene may be the cause of NIDDM in some cases.

IRS-1, a major endogenous substrate for the insulin receptor tyrosine-kinase, is thought to play an important role in mediating insulin actions (18). Almind and coworkers reported that two amino acid polymorphisms of IRS-1 (codons 513 and 972) are associated with NIDDM in Danish whites (12). Taylor and coworkers also reported that mutations in the IRS-1 gene may have a causal role in Caucasian NIDDM (28). On the other hand, Froguel and coworkers have reported that the codon 513 and 972 polymorphisms do not cosegregate with NIDDM in the French population (29). We also found no association between these polymorphisms in the IRS-1 gene and Japanese NIDDM. We next studied whether polymorphisms of IRS-1 may affect clinical features in diabetic and non-diabetic individuals. In this respect, it has been reported that carriers of the codon 972 variant had lower plasma levels of fasting insulin (12). However, fasting plasma glucose and plasma insulin levels were not significantly different between those with or without this polymorphism in either NIDDM or control subjects (Table 2). These results may be due to ethnic differences in the genetic background for NIDDM. In light of the findings that mice deficient in IRS-1 show insulin resistance (19), other deletions and mutations than the 513 and 972 polymorphisms in the IRS-1 gene may contribute to the pathogenesis of insulin resistance and NIDDM.

We conclude that the simple tandem repeat DNA polymorphism in the glycogen synthase gene and the polymorphisms at codons 513 and 972 in the IRS-1 gene are not associated with a higher risk for the development of NIDDM in Japanese subjects and suggested that there may be ethnic differences in the genetic background for this disease.

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