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Insulin Gene Region Contributes to Genetic Susceptibility to, but May Not to Low Incidence of, Insulin-Dependent Diabetes Mellitus in Japanese

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In the Caucasian population, it has been demonstrated that the insulin gene (*INS*) region contains the insulin-dependent diabetes mellitus locus (*IDDM2*). In the Japanese population, however, there has been no report demonstrating the contribution of *IDDM2* to the pathogenesis of IDDM. We conducted an association study of IDDM in a large number of Japanese subjects with multiple polymorphisms in *INS* region. We found a significant association of the *INS* region with IDDM. Alleles positively associated with IDDM in *INS* region were the same as those positively-associated with IDDM in Caucasian population, although positively-associated alleles are very common (allele frequencies > 0.9) in the Japanese general population. These data suggest that *IDDM2* is involved in the genetic susceptibility to IDDM in Japanese. The high frequencies of disease-associated alleles in the general population suggest that *IDDM2* locus is not responsible for the low incidence of IDDM in Japanese.

Genetic as well as environmental factors are responsible for the pathogenesis of type 1 (insulin-dependent) diabetes mellitus (IDDM) (1). Genes in the major histocompatibility complex (MHC) on chromosome 6p (*IDDM1*) have been repeatedly shown to contribute to genetic susceptibility to IDDM(1). Genes outside MHC gene regions (non-MHC genes) are also reported to contribute to the genetic susceptibility to IDDM both in humans and in animal models. Recent affected sib-pair analyses showed that more than 10 chromosomal regions contribute to the susceptibility to IDDM in humans (2, 3, 4).

Among these, a susceptibility gene in the insulin gene (*INS*) region on chromosome 11p15 (*IDDM2*) has been demonstrated to contribute to genetic susceptibility to IDDM both by linkage studies and association studies. More than 10 years ago, class I alleles at a variable number of tandem repeats (VNTR) situated in the 5′-untranslated region of *INS* were shown to be associated with IDDM in the Caucasian population (5). By analysing other polymorphic loci in and around *INS*, the contribution of *IDDM2* to disease susceptibility was confirmed both by linkage studies and by association studies (6, 7, 8, 9). Recent studies in Caucasians suggested that VNTR at the 5′-untranslated region determines susceptibility to IDDM (10-12) and affects the expression of the insulin gene (11-13).

Previous studies showed that the frequency of class I VNTR alleles was very high in the Japanese population *both in diabetic patients and control subjects, making it difficult to demonstrate the contribution of this locus to IDDM susceptibility,* although no detailed analysis of VNTR or any other polymorphic locus in or around INS region has been published (14, 15). Recent subtyping of VNTR alleles demonstrated that not all class I alleles are associated with IDDM, but some alleles, such as 698mu allele reported by Bennett et al., are protective against IDDM (11). It is, therefore, important to study the VNTR region, together with other polymorphic loci, in the Japanese population, because if the frequency of the "neutral/protective" class I alleles were high in Japanese population, it might explain the low prevalence ot IDDM in the Japanese population. Here, we report extensive analysis of *INS* region polymorphisms, including subtyping of VNTR, in a large number of Japanese subjects.

METHODS

Subjects. Two hundred and ninety subjects (173 control subjects and 117 patients with IDDM) were analyzed. All diabetic patients

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had developed diabetic ketoacidosis at some stage and had required insulin therapy immediately after diagnosis. Their insulin secretion was deficient as assessed by decreased urinary C-peptide (less than 10 μg/day) and/or lack of C-peptide response to intravenous glucagon. Genomic DNA was extracted from peripheral leukocytes and was used for genotyping as described below.

Typings of −2221/MspI, −23/HphI, and +1127/PstI polymorphisms at the insulin gene and microsatellite polymorphism at the tyrosine hydroxylase gene (TH). Genomic DNAs containing polymorphic sites were amplified by the polymerase chain reaction (PCR). For typing of the three polymorphisms in *INS* region, after amplification with primers reported previously (10, 11), PCR products were digested with appropriate restriction endonucleases, electrophoresed in 10% non-denaturing polyacrylamide gels and stained with ethidium bromide. At each locus, an allele what was reported to be associated with IDDM in Caucasians was symbolized by '+'. The microsattellite polymorphism situated in intron 1 of *TH* gene, which is located 9kb apart from the insulin gene and was reported to be associated with IDDM, was typed by PCR amplification with primers reported previously (16) and electrophoresis using 10% non-denaturing polyacrylamide gels.

Subtyping of VNTR. Since almost all Japanese subjects were reported to have at least one class I VNTR allele (14, 15), subtypes of class I alleles were determined by the PCR. PCR amplification was performed with the primers reported by Bennett et al. (11). The reverse primer was labelled with 6-FAM phosphoramidite. After PCR, 1 ml of each PCR product was mixed with 3 ml formamide and 0.5 ml of internal lane size standard (GENESCAN 2500-ROX, Applied Biosystems, USA). After denaturation by heating at 94°C for 3 minutes, each sample was electrophoresed in 4% denaturing polyacrylamide gel using a Model 373 DNA sequencer (Applied Biosystems, USA). PCR fragments were sized with Genescan 672 software (Applied Biosystems, USA), genotyped with GENOTYPER software (Applied Biosystems, USA), and alleles were called using a histogram as described previously (17).

Statistical analysis. Fisher's exact probability test was performed to compare allele genotype frequencies at the three polymorphic loci between the control group and the patient group. For VNTR alleles, frequencies of those carrying a specific allele were compared. For the *+1127/PstI* polymorphism, a meta-analysis was also performed using Mantel-Haenszel procedure using the present data and the data previously published by Undlien et al.(18), after a test for heterogeneity was performed using the Breslow-Day technique.

RESULTS

Polymorphisms at −2221/MspI, −23/HphI, and +1127/PstI and Microsatellite Polymorphism at TH

For the three polymorphisms in *INS* region, all diabetic patients and most control subjects showed "+/+" genotypes at the three loci (Table 1). The frequencies of "+/+" genotypes at these loci was considerably higher than those reported in UK (Bennett et al.)(11) both in IDDM and control subjects (p values $< 2 \times 10^{-10}$). Although the frequencies of "+/+" genotypes were very high at all loci both in IDDM and control subjects, those having "+/−" or "−/−" genotypes were found more frequently in the control group than in the patient group, generating significant differences in allele frequencies between the two groups. Subjects who had the "−" allele at one locus tended to have the "−" allele at other loci, suggesting that "−" alleles at three loci are in linkage disequilibrium with each other. In the typing of TH microsatellite polymorphism, three alleles (Z, Z-8 and Z-16) were identified in the present study. The most common allele (Z) was significantly less frequent in the patient group than in the control group (Table 2).

VNTR

All diabetic patients and control subjects studied showed at least one PCR fragment, indicating that all subjects have at least one class I allele. The histogram showed distinct gaps between bins, making it possible to call alleles unambiguously (data not shown). Frequencies of subjects who carried specific VNTR alleles were calculated and compared between the two groups, but there was no significant difference (Figure 1). We also checked combinations of alleles at VNTR and the four loci described above, but there was no specific combination between them, suggesting that no particular subtype of class I VNTR alleles is in linkage disequilibrium with disease-predisposing alleles at other loci (data not shown).

Meta-Analysis of +1127/PstI Polymorphism

Testing by the Breslow-Day technique showed no significant evidence against homogeneity in the association study (p=0.87). The pooled odds ratio for IDDM of "+/+" versus "+/−" and "−/−" genotypes was 3.71 (95% CI, 1.32-10.5), indicating a significant association of this genotype with IDDM (p value = 0.013) (Table 3).

DISCUSSION

The present study showed that *IDDM2* is involved in genetic susceptibility to IDDM in the Japanese population, although disease-susceptible alleles were much more common both in the control group and the patient group than in Caucasian population. The higher frequencies of disease-susceptible alleles in Japan, where the incidence of IDDM is much lower than in western countries, suggest that *IDDM2* may not be responsible for the low incidence of IDDM in Japanese population.

The contribution of *IDDM2* to disease susceptibility in the Japanese population *has been difficult to study due to the high frequency of disease-associated alleles in the general population and*, as a consequence, lack of useful genetic markers. It was previously reported that *INS* region was not associated with IDDM in Japanese (14, 18, 19), but, these studies should be interpreted as being unable to demonstrate significant association due to lack of informative markers in Japanese rather than lack of contribution of *INS* region to IDDM susceptibility in Japanese. In the present study, we studied polymorphisms in *INS* region including *−23HphI*, which is more closely associated with

TABLE 1

Genotypes at Three Polymorphic Loci in *INS* Region

Locus:	−2221/*MspI*		−23/*HphI*		+1127/*PstI*	
Genotype:	+/+	+/− or −/−	+/+	+/− or −/−	+/+	+/− or −/−
UK						
Control	112	78	94	96	112	78
(n=190)	(59%)	(41%)	(49%)	(51%)	(59%)	(41%)
IDDM	163	65	167	61	173	55
(n=228)	(71%)	(29%)	(73%)	(27%)	(76%)	(24%)
Odds ratio	1.8		2.8		2.2	
p value	0.01		0.001		0.001	
Japanese						
Control	167	6	161	12	162	11
(n=173)	(97%)	(3%)	(93%)	(7%)	(94%)	(6%)
IDDM	117	0	116	1	116	1
(n=117)	(100%)	(0%)	(99%)	(1%)	(99%)	(1%)
Odds ratio	9.1		8.6		7.9	
p value	0.04		0.01		0.02	

Note. The disease-associated allele at each locus is symbolized by '+'.

IDDM2 than other markers, in a large number of subjects, and demonstrated for the first time that *INS* region does contribute to *IDDM* susceptibility in Japanese. The contribution of *IDDM2* to disease susceptibility in Japanese is further supported by a previous study in Japanese where, although not statistically significant, the frequency of a disease-associated allele was more frequent in IDDM patients than in control subjects (18). In fact, the evidence of association of *INS* with IDDM is strengthened when the data of the previous study are combined with those of the present study (Table 3).

In this study we analyzed three polymorphic sites in the insulin gene and, although the allele distributions were skewed greatly, we found significant differences between diabetic patients and control subjects. The alleles positively associated with IDDM at the three loci were the same as those in Caucasian population, suggesting that the linkage disequilibrium of IDDM and the disease-causing haplotype may be global.

It may be difficult, however, to narrow down the region of *IDDM2* using Japanese population. The "Crossmatch" haplotype analysis method used by Bennett et al.(11) to map *IDDM2* to within VNTR would be difficult to apply, because there were too few people having "+/−" or "−/−" genotype. A recent report by Doria et al.(20) suggested that *IDDM2* spans *INS* and *TH* loci. Our findings of the association of the *TH* locus with IDDM support their view. The association of *TH* microsatellite with IDDM shown in this study makes this marker very informative in family-based studies in Japanese because the frequencies of disease-associated alleles in general population are much lower than those at *INS* region polymorphisms.

TABLE 2

Allele Distributions of Microsatellite Polymorphism at Tyrosine Hydroxylase Gene (*TH*)

Allele	Control (n = 173)	IDDM (n = 117)	Odds ratio	p value
Z-16	122 (35%)	109 (47%)	1.6	0.006
Z-8	97 (28%)	67 (29%)		
Z	127 (37%)	58 (25%)	0.6	0.003

Matsumoto et al. previously reported that there was no association between IDDM and *INS* region polymorphisms using 3′ PstI and four class I subtypes divided according to their lengths (19). The association of class I subtypes with diabetes, however, may not be straightforward; Bennett et al. reported that, while many class I subtypes were associated positively with diabetes, the "814" subtype, the most common subtype, did not show positive association, and the "698" allele was suggested to show a negative association (11). These findings demonstrate that it is not valid to combine class I subtypes according to their length and that precise subtyping of class I alleles is essential for the analysis of *IDDM2*. The fluorescence-based VNTR-subtyping method in this study made it possible to call alleles unambiguously between lanes and between gels due to the size standard in each lane. Our data indicate that subtypes of class I alleles contribute little, if at all, to IDDM susceptibility in Japanese and that class I alleles as a whole appear to contribute to disease susceptibility.

The incidence and prevalence of IDDM in Japanese population is considerably lower than those in Caucasian populations. The low prevalence may be attributed to environmental and/or genetic factors. Since familial

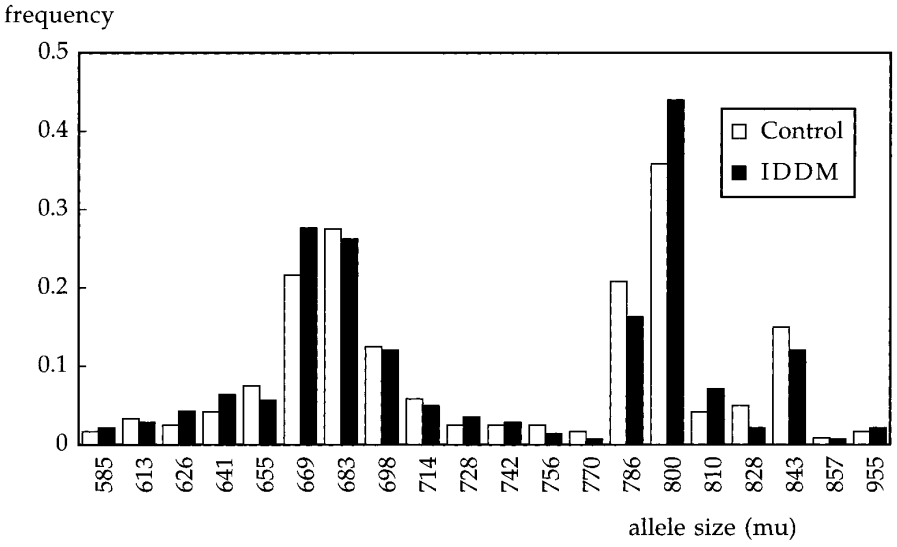

FIG. 1. Distribution of subtypes of class I VNTR alleles.

clustering of type 1 diabetes in the Japanese population is very strong (λs =271 in Japanese population vs. λs = 15 in UK population)(21), genetic factor appears to play a major role in the low prevalence. The frequencies of the susceptibity alleles at the three loci in *INS* region, however, were higher in the Japanese population with low incidence and prevalence of IDDM than those in Caucasian populations. There may be several explanations. Mutation(s) around *INS* at loci other than the three loci analyzed may predispose to IDDM. Although there was no difference in frequencies of those carrying specific VNTR allele between IDDM patients and control subjects, VNTR is still worth studying because it is suggested that sequence differences of repeat elements of VNTR are important in IDDM susceptibility. We are currently studying the sequence of repeat elements in Japanese in comparison with those in Caucasians. It must be noted, however, that to conduct an association study at loci composed of polymorphisms with many alleles, many more samples should be analyzed due to multiple comparisons. This is particularly true if we are to consider not only repeat numbers at VNTR but also sequence differences of repeat elements. Another explanation is that disease-predisposing alleles at loci other than *IDDM2* are rare in the Japanese population. In this sense it may be quite attractive to conduct a genome-wide linkage study using the Japanese population to determine to what extent each diabetes susceptibility gene contributes to IDDM. If λs for a disease-linked locus is higher in the Japanese population than that in Caucasian populations, such a locus may explain the low-prevalence of IDDM in Japanese.

In conclusion, this study demonstrated that *IDDM2* plays a role in genetic predisposition to IDDM in the Japanese population, further emphasizing the importance of a large number of samples for genetic analyses. The high frequencies of disease-associated alleles in the general population suggest that *IDDM2* is not responsible for the low incidence of IDDM in Japanese.

TABLE 3

Meta-Analysis of *+1127/Pst*I Polymorphism in the Japanese Population

	Previous study (Undlien *et al.*) (18)		Present study	
Genotype	Control (n=141)	IDDM (n=114)	Control (n=173)	IDDM (n=117)
+/+	133 (94%)	111 (97%)	162 (94%)	116 (99%)
+/− or −/−	8 (6%)	3 (3%)	11 (6%)	1 (1%)

Note. Pooled odds ratio of "+/+" was 3.71 (95% CI, 1.32-10.5, p value = 0.013).

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