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Hind III Site Causing Proinsulin Kyoto and Pst I Site Polymorphism of the Insulin Gene in Japanese: Its Lack of Association with Either IDDM or NIDDM

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Abstract. The gene encoding Proinsulin Kyoto has been isolated and characterized by DNA sequencing, indicating that the molecular basis of the disorder is a G-T point mutation in the insulin gene which creates a Hind III site. In addition, in the 3'-untranslated region of the mutant insulin gene, a Pst I site negative, α type allele was found, and in the normal gene, a Pst I site positive, β type allele was found. In order to clarify the frequency of the mutation and to determine whether this mutation is associated with diabetes mellitus or not, we have investigated Hind III polymorphism in 91 normal Japanese subjects and patients with IDDM and NIDDM. No cases with the Proinsulin Kyoto gene were found among the subjects examined. Secondly, to determine whether this α type allele is associated with DM in Japanese, we investigated Pst I polymorphism in the same subjects. The frequencies of the α type and β type alleles were 92% and 8%, respectively. No significant difference in genotypic frequency was found among normal, NIDDM, and IDDM. We conclude that the Proinsulin Kyoto gene is not a common cause of DM and the occurrence of the α type insulin gene in Japanese diabetes is more frequent than in other races, so this Pst I polymorphism is not a marker for diabetes mellitus in Japanese.

Key words: Proinsulin Kyoto, NIDDM, Insulin gene, RFLP, Pst I site.

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A NEW TYPE OF hyperproinsulinemia has recently been reported by our laboratory in a 65-yr-old non-obese Japanese man who developed NIDDM [1]. A family study revealed that his son and daughter also showed signs of fasting hyperinsulinemia. Analysis of their sera by reverse phase high performance liquid chromatography demonstrated a minor peak of human insulin (5%) and a major peak of Proinsulin-like material (95%). Amplification and sequencing of the products of the insulin gene by polymerase chain reaction (PCR) disclosed that the molecular basis of the disorder is a G-T point mutation in the insulin gene, which corresponds to the second codon position of amino-acid residue 65 of Arg at the junction of the C-peptide and A chain. By isolating and characterizing the insulin gene of Proinsulin Kyoto, we have further demonstrated that the nucleotide substitution creates a Hind III site in the coding regions of the insulin gene and accounts for its association with diabetes. We also found that a Pst I site negative, α type allele [2, 3] is found in the 3'-untranslated region of the mutant insulin gene. It is important to know whether or not this polymorphism is associated with Proinsulin Kyoto. RFLP analysis of the insulin gene may also provide a genetic marker of diabetes mellitus [4, 5]. Since the frequency of Pst I polymorphism in the

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3'-untranslated region of the insulin gene is different in several races [6, 7], in the present study we investigated the association of Pst I polymorphism in normal Japanese subjects and patients with IDDM and NIDDM.

Materials and Methods

A genomic DNA preparation from peripheral leukocytes of the proband was prepared as previously described [1]. A size-fractionated human genomic library was constructed. A library of approximately 2×10^5 independently derived bacteriophage EMBL4 clones consisting of large (15–20 kb) fragments of human DNA covalently joined to the bacteriophage EMBL4 was prepared, as previously described [8]. In brief, the patient's leukocyte DNA was subjected to nonlimit digestion with the restriction endonuclease EcoR I, and the products were sizefractionated by gel electrophoresis. The human library was then screened for clones containing insulin sequences by means of the *in situ* plaque hybridization. ^{32}P-labeled nick translated cDNA containing the human insulin gene phins 96 was used as a hybridization probe, and isolation and characterization of genomic DNA clones bearing the Proinsulin Kyoto sequences was done. Four independently derived clones bearing insulin sequences were identified and plaque-purified. The location of various restriction endonuclease sites in the recombinant DNA was determined as a means of characterizing the DNA sequence organization of the human insulin gene. The agarose gel electrophoresis pattern of DNA digested with enzymes showed three similar clones and one different. We have designated these clones NHI and PIK, respectively.

Sequence and general organization

The complete nucleotide sequences of the cloned human insulin genes, NHI and PIK were determined by the chain-termination method.

Polymorphism study

Subjects: Ninety-one Japanese subjects, normal controls (n=23), NIDDM (n=39, including the Proinsulin Kyoto patient), and IDDM (n=29), were analyzed.

PCR primers: The following oligonucleotide primers were used to amplify the C peptide A chain junctional region including the ^{65}Arg coding region and the 3'-untranslated region of the insulin gene:

5'GCTGTTCCGGAACCTGCTCT 3'
5'ACAGCAGGGCTGGTTCAAG 3'

To amplify the 221 base pair fragment including nucleotide 1,628, the following oligonucleotide primers to the 3'-untranslated region of the insulin gene were used:

5'AAGCGTGGCATTGTGGAACAA3'
5'CTGGGAGGGGCTCACAACAACAGT3'

PCR procedure: 1 μg of genomic DNA was amplified with 0.5 μg of each primer in a total volume of 100 μl containing 1×Taq polymerase buffer and 200 μM dNTPs. Samples were heated to 97°C for 5 min, after which 2 units of Taq polymerase was added. Reactions were cycled 30 times at 94°C for 1 min at 60°C for 1 min, and at 73°C for 2 min. Pst I restricted PCR products (10 μl aliquots digested in a total volume of 50 μl) were analyzed by electrophoresis through 3% agarose gels. When digested with Hind III, DNA fragments of various sizes were detected; the NHI allele had a 266 base pair fragment, whereas the PIK allele had two fragments: 152 and 114 base pairs. Digestion with Pst I showed the α allele had a 221 base pair fragment and the β allele had a 137 and a 84 base pair fragments.

Statistical analysis: Statistic analysis was performed by Chi-squares test.

Results

Analysis of the 5' flanking region and 3' untranslated region of the Proinsulin Kyoto gene clone

According to the adopted classification of restriction fragments [9], the Bgl I fragments of PIK and NHI correspond to class 1 and class 3 alleles, respectively. The complete sequence of PIK and NHI reveals that the former is an α-type gene with a point mutation and the latter is a β-type gene.

The Proinsulin Kyoto gene-related zamplification

Using Hind III (to detect Proinsulin Kyoto) three fragments resolved were 266, 152, and 114 size

base pairs. In none of the 91 Japanese subjects was polymorphism detected, thus ruling out mutation at this restriction site as a common cause of NIDDM in Japanese (data not shown).

3′ UT related amplification

Using Pst I, two patterns were detected, as shown in Fig. 1. The subjects homozygous for the α-type showed the pattern of fragment sized 221 base pairs, those homozygous for the β-type showed two fragments, 137 and 84 base pairs, and those heterozygous for both showed three fragments, 221, 137 and 84 base pairs. β-type homozygotes were found in only 2 individuals with NIDDM. Heterozygotes were found in 2 normal, in 5 NIDDM, and in 3 IDDM. The remaining ones were all α-type homozygotes.

Genotypic analysis shows the frequency of β-type alleles to be 7.7% overall, 4.3% in normal, 11.5% in NIDDM and 5.2% in IDDM, respectively (Table 1). There was no statistical difference among the three groups.

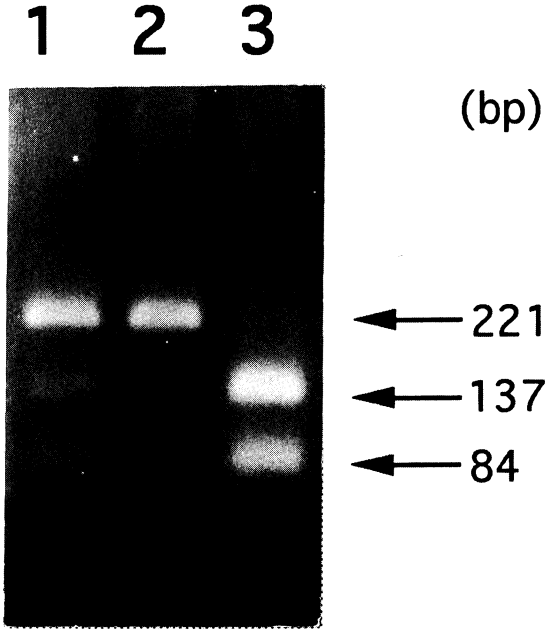

Fig. 1. The Pst I polymorphisms within the 3-untranslated region of the insulin gene are shown. The 84 bp, 137 bp and 221 bp bands are polymorphic. Lane 1, α β; lane 2, αα; lane 3, ββ.

Discussion

In a previous study, we demonstrated that nucleotide substitution produces a Hind III site in the coding region of the insulin gene of Proinsulin Kyoto [1]. However, there was no case found in normal subjects or diabetic patients in the present study. Therefore, one mutant proinsulin, Proinsulin Kyoto, is not a factor common in NIDDM in Japanese. In the present study, we also analyzed RFLP in the Pst I site at 1,628 in the 3′-untranslated region of the insulin gene [2] in the same individuals by using PCR and have obtained results quite different from those in some previous reports [6, 7]. We found a lower frequency of the Pst I present allele (8%) and a higher frequency of the Pst I absent allele (92%) in Japanese. In South Indians and Punjabi Sikhs, the corresponding frequencies of present/absent are 0.22/0.78 and 0.24/0.76, respectively [6]. Furthermore, 92% of the Japanese subjects examined had a Pst I site negative allele, compared to 76% in South Indians, 81% in Punjabi Sikhs [6], and 71% reported by the Royal Manchester Childrens Hospital (RCMH) [7]. In addition, we have found a lower heterozygosity (0.11), as RCMH showed 0.30 [7]. Although some reports suggest that polymorphism of this site of the insulin gene, which was first described as an isolated mutation, can be used as a genetic marker for diabetes mellitus [2, 3], there was no significant difference in the frequency of the Pst I positive allele among normal subjects, NIDDM, and IDDM in Japanese, as shown also by our previous study of RFLP of the 5′ portion of the human insulin gene [4]. It is known that there is disequilibrium in the linkage between this Pst I polymorphism and hypervariable region 5′ to the insulin gene [6], but

Table 1. Genotype distribution and allele frequency of Pst I site RFLP in Japanese

		Genotype distribution			Allele frequency
	n	+ +	+ −	− −	+ / −
Normal	23	0	2	21	0.04 /0.96
NIDDM	39	2	5	32	0.12 /0.88
IDDM	29	0	3	26	0.05 /0.95
Total	91	2	10	79	0.08 /0.92

+(site present) −(site absent)

we have been unable to confirm the relationship. 92% and 97% of Japanese show signs of Pst I-absent and class 1 polymorphism [4], respectively. It seems likely that the basis for the dominance of the α type allele is its linkage to the class 1 in Japanese, because the reported α type frequency in class 1 homozygotes is 0.94 [6].

In conclusion, one mutant proinsulin, Proinsulin Kyoto, is not a factor common in NIDDM in Japanese and the Pst I site RFLP linked to the human insulin gene cannot presently be used as a marker of diabetes mellitus in persons of Japanese ancestry.

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