Polymorphisms of the Insulin Gene Among Japanese Subjects

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We have sequenced the insulin gene in 72 unrelated Japanese subjects (52 with type 2 diabetes mellitus and 20 with normal glucose tolerance). We identified 6 mutations and all were found at a low frequency (1% to 4%). Three mutations were new. These included a C-to-G substitution in the promoter region, a G-to-A substitution in codon-2 resulting in an Ala-to-Thr replacement in amino acid −2 of the signal peptide, and a G-to-A substitution in intron 2. We have no evidence that any of the mutations that we found are the cause of diabetes. Thus, mutations in the insulin gene do not appear to be an important genetic factor contributing to the development of diabetes in this population.

THE HUMAN INSULIN gene (gene symbol *INS*) (OMIM 176730) consists of 3 exons and 2 introns and spans a region of approximately 1.5 kb in human chromosome band 11p15.5.[1] The type 1 diabetes susceptibility gene IDDM2 maps to the variable number of tandem repeat (VNTR) minisatellite upstream of the *INS*.[2,3] *INS* mutations are a rare cause of diabetes mellitus. To date, 7 missense mutations have been found in the insulin/proinsulin, and all are associated with hyperinsulinemia or hyperproinsulinemia and mild diabetes.[4] Recently, a missense mutation (A7, Cys>Thr) was found in the insulin II protein in the Akita mouse. This mutation appears to function in a dominant negative manner and was associated with hypoinsulinemia and severe diabetes in this mouse model of diabetes.[5] This result suggests that insulin mutations may result in hypo- or hyperinsulinemia depending on the mutation involved. These findings led us to screen the *INS* for diabetes-associated mutations in a group of Japanese subjects with typical type 2 diabetes.

SUBJECTS AND METHODS

The study population consisted of 52 unrelated Japanese subjects with type 2 diabetes and 20 unrelated normal nondiabetic Japanese subjects. The majority of diabetic subjects did not have hyperinsulinemia or hyperproinsulinemia, which characteristics patients with the typical *INS* mutation. Some subjects appeared to be quite insulin-resistant. All of the subjects were recruited from the Fujita Health University Medical School Clinic. A patient was diagnosed with type 2 diabetes based on personal history and medical data, according to the criteria of the Japan Diabetes Society. The clinical and biochemical data of the 52 diabetic patients are summarized in Table 1. Twenty unrelated control subjects who did not have a personal or family history of diabetes and who were confirmed to have a fasting plasma glucose level below 6.0 mmol/L (<110 mg/dL) were selected. The mean age of the control subjects was 44.5 ± 24 years (range, 28 to 70 years) and included 14 men and 6 women.

DNA was prepared from a blood sample. The 72 subjects were screened for mutations in the exons, introns, and promoter region of the *INS* (1,589 bp) by direct sequencing of polymerase chain reaction (PCR) products. The primers used for PCR and sequencing are described in Table 2. PCR was performed using a Perkin Elmer GeneAmp PCR System 9700 (Perkin Elmer, Chiba, Japan), and the conditions of PCR for each primer pair were as follows: initial denaturation at 94°C for 5 minutes and then 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. After the PCR products were purified with an Ultrafree-MC centrifugal filter unit (Milipore, Tokyo, Japan), they were directly sequenced using an ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin Elmer). An ABI PRISM 377 DNA Sequencer was used for sequencing.

Table 1. Clinical and Biochemical Characteristics of the 52 Type 2 Diabetic Patients

Sex (M/F)	35/17		
Age (yr)	54.9 ± 11.3	(20-80)	
Age (yr) at diagnosis of DM	44.8 ± 13.4	(12-70)	
BMI (kg/m^2)	22.9 ± 4.1	(16.5-33.5)	
HbA_{1c} (%)	8.52 ± 3.1	(5.2-12.6)	[4.3-5.8]
Fasting plasma glucose (mmol/L)	8.83 ± 2.79	(5.1-14.3)	[3.3-6.1]
Fasting serum insulin (pmol/L)	62.6 ± 50.7	(8.95-195)	[7.46-62.6]
Fasting serum C-peptide (pmol/L)	728 ± 463	(152-1,886)	[397-662]
Therapeutic method	Diet-5 cases	OHA-25 cases	Insulin-22 cases
Family history of DM	No: 15 cases, Yes: 37 cases		

NOTE. The mean ± SD (range) is shown. The numbers in brackets represent the normal range in our laboratory. Positive family history was defined as a diabetic subject having a grandparent, parent, uncle, aunt, or sibling and/or offspring who had been previously diagnosed with DM.

Abbreviations: DM, diabetes mellitus, OHA, oral hypoglycemic agent.

The plasma glucose concentration was determined using the glucose oxidase method. The hemoglobin A_{1c} (HbA_{1c}) level was measured by high performance liquid chromatography (normal range, 4.3% to 5.8%). The immunoreactive insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (DAINABOT, Tokyo, Japan). The immunoreactive C-peptide concentration was measured using a radioimmunoassay (RIA) kit with the antibody raised against amino acids 5-31 of the C-peptide protein (Shionogi, Osaka,

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Table 2. Sequences of Primers for PCR Amplification and Sequencing of the *INS*

Region	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Product Size (bp)
Promoter, exon 1, and intron 1	InE1F-agcaccagggaaatggtcc	InE2R-caggggcagcaatgggcggtt	604
Exon 2, exon 3, and intron 2	InE2F-caaggcagggcacctggcctt	InE3R-acagcagggctggttcaag	1,268
	InE2P1-ggcttcttctacacacccaag*	InE3P1-ggccaaggggctggcaggctgc*	
	InE3F-gctgttccggaacctgctct*		

*These primers were used only for sequencing.

Japan). The immunoreactive proinsulin level was measured with a sandwich ELISA kit using an insulin antibody and a C-peptide antibody. The normal range of insulin, C-peptide, and proinsulin concentrations is 37.3 to 111.9, 397.2 to 662, and 1.7 to 8.6 pmol/L, respectively.[6] The hyperglycemic-euglycemic glucose clamp study, the 75-g oral glucose tolerance test (OGTT), and the intravenous glucagon test were performed according to standard methods.[7]

RESULTS

We found 6 mutations in the *INS* in the 72 subjects (Table 3). They were all found in the heterozygous state and included 3 new mutations (c. −153C>G, c.67G>A, and IVS2nt+88G>A) and 3 that had been previously described[1] and are not thought to contribute to the development of type 2 diabetes (IVS1nt-6T/A, c.342T/C, and c.355A/C). The later three mutations were found in the same subjects suggesting that they may be in complete linkage disequilibrium. The c.67G>A mutation results in an alanine (A)-to-threonine (T) substitution in amino acid −2 of the signal peptide. The 4 subjects with new mutations do not have more than 1 mutation. We examined the 4 subjects with the new mutations.

Case 1 (mutation: c.67G>A) is a 60-year-old Japanese man who was found to have type 2 diabetes in 1979 when he was 41 years of age (Fig 1, Table 4). His physical examination was normal. After he was diagnosed with diabetes, he started taking oral hypoglycemic agents. For the last 3 years, he has received insulin therapy. In 1998, he suffered from multiple myeloma. The insulin secretory response in the glucagon test and glucose levels in the hyperglycemic clamp study were low. The level of urinary C-peptide was 13 μg/d.

We performed the OGTT on 7 other family members of case 1 (5 sisters, 1 son, and spouse). Three of the 5 sisters inherited the mutant allele, and 1 of them has been diagnosed with diabetes. The 2 sisters with the mutation and 1 without it had a low insulin secretory response to glucose (Table 5). There were no significant differences in the insulin, C-peptide, and proinsulin levels between family members who did or did not have the mutation.

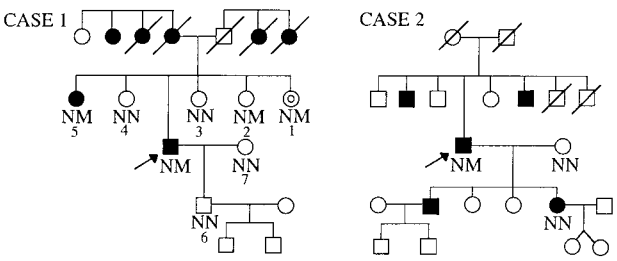

Fig 1. Pedigrees of 2 diabetic patients with missense mutation A(−2)T in the signal peptide (cases 1 and 2). The proband is indicated by the arrow. Males are indicated by squares and females are indicated by circles. Individuals with type 2 diabetes are noted by black, filled symbols, and nondiabetic subjects are noted by open symbols. The individual with impaired glucose tolerance is indicated by a double circle. The *INS* genotype, if known, is indicated below the symbol: N, normal allele; M, mutant allele.

Case 2 (mutation: c.67G>A) is an 80-year-old Japanese man who was diagnosed with type 2 diabetes in 1989 when he was 70 years of age. His physical examination was normal. After being diagnosed with diabetes, he started to take oral hypoglycemic agents. In 1998, he suffered from polymyalgia rheumatica (PMR) and needed to be treated with prednisolone. At the same time, he received insulin therapy. His family history is shown in Fig 1. His daughter has type 2 diabetes, and her treatment consists of only dietary therapy with good diabetic control (HbA_{1c}, 6.1%). However, she does not have the mutant allele. The insulin secretory responses in the glucagon test and the glucose levels in the hyperglycemic clamp study were relatively low (Table 4). The level of urinary C-peptide was 28 μg/d.

Case 3 (mutation: c. −153C>G) is a 54-year-old Japanese woman who was found to have type 2 diabetes in 1984 when she was 39 years of age. Her physical examination was normal. After she was diagnosed with diabetes, she started taking oral hypoglycemic agents. She started insulin therapy 2 years ago. Her parents do not have diabetes. Hyper- and euglycemic glucose studies were performed on case 3. The results are summarized in Table 4. The level of urinary C-peptide was 20 μg/d.

Case 4 (mutation: IVS2nt+88G>A) is a 38-year-old Japanese man who was found to have diabetes in 1998 when he was 37 years of age. His physical examination was normal. After he was diagnosed with diabetes, he has undergone only dietary therapy. His parents and 2 sisters do not have diabetes. His OGTT showed marked impaired glucose tolerance (IGT) with mild hypoinsulinemia (Table 4). The level of urinary C-peptide was 30 μg/d.

DISCUSSION

We have screened the insulin gene for mutations in a group of 52 Japanese subjects with type 2 diabetes. We found 6 mutations, 3 of which had been described previously. One of the new mutations that we found (c. −153C>G) was located in the promoter region and another (c.67G>A) resulted in an Ala-to-Thr substitution of amino acid −2 of the signal peptide. However, we have no evidence that either mutation is the cause of diabetes in these subjects. The promoter mutation (c. −153C>G) is not located in a region of the promoter implicated in the control of insulin gene transcription,[8] nor is it

Table 3. Polymorphisms and Mutations in the *INS* Among Japanese Subjects

Location	Nucleotide	Nucleotide Change	Designation	Amino Acid Change	Designation	Frequency	
						Type 2 DM	Nondiabetic
Promoter	−153(−94)	C>G	c.−153C>G			1/52	0/20
Intron 1	−6	T/A	IVS1nt-6T/A	IVS1nt-6T/A		2/52	1/20
Exon 2 codon(−2)	67	G>A	c.67G>A	Ala(GCA)>Thr(ACA)	A(−2)T	2/52	0/20
Intron 2	+88	G>A	IVS2nt+88G>A	IVS2nt+88G/A		1/52	0/20
Exon 3 3′-UTR	342	T/C	c.342T/C			2/52	1/20
	355	A/C	c.355A/C			2/52	1/20

NOTE. Nucleotide numbering: The A of the ATG of the initiator Met codon of the cDNA sequence is denoted nucleotide +1, and the lowercase c for cDNA in front of the nucleotide number indicates that the reference sequence is the cDNA sequence. The polymorphism in the promoter region is located −94 bp upstream of the start of transcription. Codon numbering: The first amino acid of proinsulin is denoted codon +1.

Abbreviations: UTR, untranslated region, IVS, intervening sequence.

Table 4. Results of the Glucagon and Glucose Loading Tests and Hyperglycemic and Euglycemic Clamp Studies in Four Diabetic Patients Who Had Mutations in the *INS*

Subject (mutation)	Glucagon Test (min)		Hyperglycemic Clamp (min)					OGTT (min)			
	0	6	0	15	30	60		0	30	60	120
Plasma glucose (mmol/L)											
Case 1	6.00	6.67	4.44	10.0	12.5	12.7					
Case 2	8.28	9.28	8.33	14.0	14.6	14.0					
Case 3			5.67	13.6	13.0	13.3					
Case 4								6.28	10.5	12.1	10.0
Plasma insulin (pmol/L)											
Case 1	54.4	102	23.1	54.4	41.7	47.4					
Case 2	74.6	160	96.9	114	102	89.5					
Case 3			73.1	105	114	134					
Case 4								52.5	238	240	219
Plasma C-peptide (pmol/L)											
Case 1	231	397	152	264	258	364					
Case 2	430	827	628	695	662	662					
Case 3			562	761	827	1,059					
Case 4								827	1,555	1,787	2,052
Plasma proinsulin (pmol/L)											
Case 1	4.83	6.04									
Case 2	16.8	23.8									
M value (mg/min/kg)											
Case 1							3.02 [5.51]				
Case 2							2.61 [3.76]				
Case 3							3.11 [4.45]				
C-peptide/insulin ratio (mol)											
Case 1	4.25	3.86	6.58	4.85	6.18	7.80					
Case 2	5.76	5.16	6.48	6.10	6.49	7.40					
Case 3			7.69	7.24	7.25	7.88					
Case 4								15.8	6.53	7.45	9.36
Proinsulin/insulin ratio (mol)											
Case 1	0.088	0.059									
Case 2	0.225	0.149									

NOTE. A serum sample was collected to determine the levels of plasma glucose, insulin, C-peptide, and proinsulin during glucagon (1 mg IV injection) and OGTT (75 g orally) and for hyperglycemic and euglycemic clamp studies according to the methods reported previously.[7] In the hyper- and euglycemic glucose clamp studies, case 2 was treated with 15 mg of prednisolone because the patient had PMR as mentioned in the text. The normal range of C-peptide concentration at 0 and 6 minutes of the glucagon test are 529 ± 132 and 1,986 ± 311, respectively. The normal range of insulin concentration at 0, 15, 30, and 60 minutes of the hyperglycemic glucose clamp was 74.6 ± 37, 350 ± 55.2, 447 ± 22 and 447 ± 22, respectively. The normal range of insulin concentration at 0, 30, 60, and 120 minutes on the OGTT was 74.6 ± 37, 462 ± 224, 376 ± 187, and 301 ± 137, respectively. The normal range of C-peptide concentration at 0, 30, 60, 90, and 120 minutes during the OGTT was 52.9 ± 132, 2,184 ± 463, 2,681 ± 761, 2,250 ± 529, and 1,655 ± 695, respectively. The normal range of the C-peptide/insulin ratio was 5 to 15. The normal range of the proinsulin/insulin ratio was 0.1 to 0.2. The M values are for the hyperglycemic clamp. The values in brackets are for the euglycemic clamp. The normal range of M values in the hyperglycemic and euglycemic glucose clamp studies was 8.03 ± 0.68 and 7.00 ± 0.52, respectively.

Table 5. Results of OGTT in Family Members of Case 1

		Minutes					Age (yr)	BMI (kg/m^2)	HbA_{1c}	HOMA Index	Insulinogenic Index
		0	30	60	90	120					
1	a	5.7	9.8	11.2	10.2	8.1	71	23.4	5.9	2.02	0.50
	b	58.9	335.7	384.1	537.8	377.4					
	c	400.5	1,340.5	1,942.9	2,648.0	2,383.2					
	d	1.2	1.5	2.5	3.7	7.1					
2	a	5.1	8.4	9.3	8.1	6.9	68	21.5	5.9	0.38	0.16
	b	12.6	84.6	123.0	151.4	141.7					
	c	185.3	519.6	873.8	1,115.4	1,138.6					
	d	1.1	1.4	1.9	3.2	3.6					
3	a	5.6	10.6	9.2	4.1	6.1	66	23.6	6.0	1.00	0.33
	b	29.8	254.3	813.1	203.6	165.6					
	c	251.5	1,019.4	2,373.2	1,691.4	1,244.5					
	d	1.3	2.4	10.7	9.4	5.3					
4	a	5.7	7.9	8.8	7.3	5.2	57	21.6	5.9	1.02	0.20
	b	29.8	88.0	155.1	176.8	111.1					
	c	235.0	503.1	817.5	1,022.7	860.6					
	d	1.6	2.2	3.3	4.9	4.4					
5	a	7.1	12.5	14.1	14.0	13.2	53	22.3	6.5	1.49	0.11
	b	35.0	117.1	148.4	183.5	205.1					
	c	390.5	708.3	993.0	1,324.0	1,416.6					
	d	1.5	2.0	4.0	4.1	8.5					
6	a	6.0	13.7	12.5	9.0	5.9	32	20.2	5.5	0.94	0.30
	b	26.1	337.1	359.5	262.5	270.0					
	c	74.0	1,529.2	2,264.0	1,790.7	1,946.2					
	d	1.2	1.8	3.2	2.8	3.2					
7	a	5.8	9.4	8.8	5.7	5.6	54	19.2	5.8	0.88	0.56
	b	25.3	296.9	456.5	270.0	129.0					
	c	271.4	1,224.7	2,227.6	2,025.7	1,320.6					
	d	1.6	2.2	3.2	3.8	3.2					

NOTE. Family numbering is the same as the numbering for case 1 in Fig 1. a, plasma glucose (mmol/L); b, plasma insulin (pmol/L); c, plasma C-peptide (pmol/L); d, plasma proinsulin (pmol/L). Insulin resistance was estimated by the HOMA index. The level of HOMA index = FPG (mg/dL) × IRI (μU/L)/405. Insulin secretion was estimated by the insulinogenic index. Insulinogenic index = IRI (30 minutes to 0 minute) (μU/L)/PG (30 minutes to 0 min) (mg/dL). If the insulinogenic index was below 0.4, it indicated impaired insulin secretion.

Abbreviations: HOMA, homeostasis model assessment, FPG, fasting plasma glucose; IRI, immunoreactive insulin; PG, plasma glucose.

conserved among human, monkey, dog, or rat/mouse insulin genes. Similarly, the mutation in the signal pepetide (A-2T) is an amino acid residue that is not conserved in the insulin signal peptide (Ala in human, Gln in rat, mouse, hamster, and pig, Pro in monkey, and Arg in dog and cow).

In conclusion, there is variation in the sequence of the insulin gene in Japanese people. However, this variation does not appear to be a contributing factor in the development of diabetes.

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