

## Rapid Publication

# Missense Mutation of Amylin Gene (S20G) in Japanese NIDDM Patients

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Many studies suggest that amylin, which is cosecreted with insulin from islet  $\beta$ -cells, is a biologically active peptide and modulates plasma glucose levels. We therefore scanned the amylin gene for mutations in 294 Japanese NIDDM patients by single-strand conformational polymorphism, and we found a single heterozygous missense mutation (Ser $\rightarrow$ Gly at position 20: S20G mutation) in 12 NIDDM patients (frequency 4.1%). None of the 187 nondiabetic subjects or 59 IDDM patients had the mutation. Of 12 patients carrying the mutation, 8 were diagnosed as having NIDDM at a relatively early age ( $\leq 35$  years), and they had severe diabetes and strong family histories of late-onset NIDDM. On the other hand, the remaining four patients were diagnosed as having NIDDM after age 51, and they had mild diabetes without family histories of diabetes. In high-performance liquid chromatography analysis, a small amount (16%) of amylin immunoreactivity appeared in the position corresponding to normal amylin and a much larger amount (84%) appeared in the position corresponding to mutant amylin. These findings suggest that the S20G mutation of the amylin gene may play a partial role in the pathogenesis of early-onset NIDDM in the Japanese population and may also provide an important model to investigate the true physiological action of amylin. *Diabetes* 45:1279–1281, 1996

**N**IDDM is thought to be a multifactorial disorder. Although the main susceptibility genes for NIDDM have not been found, some gene mutations have been found in some types of NIDDM, and their significance in the pathogenesis of NIDDM has been discussed. Amylin (islet amyloid polypeptide), a 37-amino acid peptide (1,2) that is cosecreted with insulin from islet  $\beta$ -cells (3), is a major constituent of islet amyloid deposits seen in NIDDM patients (4). Many *in vitro* and *in vivo* studies (5) suggest that amylin is a biologically active

peptide and modulates glucose metabolism. We therefore scanned the amylin gene for mutations in NIDDM patients.

### RESEARCH DESIGN AND METHODS

Genomic DNA was obtained from 294 unrelated Japanese patients with NIDDM, 59 patients with IDDM, and 187 nondiabetic subjects who were  $>60$  years old and had no family history of diabetes. At blood sampling, informed consent was obtained from each individual, according to protocol approved by the Human Studies Committee. Severely obese subjects (BMI  $>30$  kg/m<sup>2</sup>) were excluded. Diabetes was defined according to World Health Organization (WHO) criteria. The HbA<sub>1c</sub> levels of the nondiabetic subjects were  $<6.0\%$ . The clinical data of the subjects are summarized in Table 1. All of the NIDDM patients in the group with relatively early onset had slow onset, no evidence of ketosis, and negative tests for anti-islet cell antibodies and/or anti-glutamic acid decarboxylase antibodies and were treated without insulin for at least 4 years after diagnosis.

For single-strand conformational polymorphism (SSCP) analysis, four DNA fragments were amplified by polymerase chain reaction (PCR) using fluorescence-labeled primers. Forward primer [location (6)], reverse primer [location (6)], and annealing temperature for each DNA fragment are as follows: 5' flanking region: 5'-ACTGCACAAGGACACTGTGT-3' (-202 to -183), 5'-TCCAAGCTTGATCCACTGG-3' (16 to 35), and 60°C; exon 1: 5'-TGCCTGATGTCAGAGCTGAG-3' (-65 to -46), 5'-ACACCAAGTGTGCATTTCTCT-3' (143 to 163), and 55°C; exon 2: 5'-CTCTTGATTTTCAGTGTGGA-3' (401 to 420), 5'-GGCTGTAGTTATTGACAGT-3' (595 to 614), and 51°C; exon 3: 5'-TCACATTTGTTCCATGTTAC-3' (5314 to 5333), 5'-CAATAACTATAGAGTTACATTG-3' (5591 to 5612), and 56°C. The denatured PCR products were separated by 6% polyacrylamide gel at two different exon-specific temperatures (4–22°C) using an ALFred DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

For restriction fragment length polymorphism (RFLP) analysis, the PCR products of exon 3 were ethanol precipitated and then digested by restriction enzyme *Msp* I at 37°C for 3 h. The samples were run on a 2.5% agarose gel and stained by ethidium bromide.

High-performance liquid chromatography (HPLC) analysis was performed using the reverse-phase column (Beckman, C18, ultrasphere ion-pairing column; 5- $\mu$ m particle size, 25  $\times$  0.46 cm). The amylin-immunoreactive materials extracted from postprandial plasma through Sep-Pak (7) were applied to the column and were eluted (1.0 ml/min) at 50°C with a 25–45% (vol/vol) acetonitrile (ACN) gradient prepared in 0.1% trifluoroacetic acid, and amylin immunoreactivity in the eluates was measured by radioimmunoassay using the Amylin RIA Kit (Peninsula, Belmont, CA). The normal human amylin was eluted at the position corresponding to 41–42 min (ACN 37.5%), and Gly<sup>20</sup>-amylin to 31–32 min (ACN 34.1%), respectively.

Gly<sup>20</sup>-amylin was synthesized by using a solid-phase peptide synthesizer (BOC-strategy, Applied Biosystems model 430A). The homogeneity of the final product was confirmed by analytical HPLC, capillary electrophoresis, and amino acid analysis. The recovery of normal amylin and Gly<sup>20</sup>-amylin through Sep-Pak C18 was identical. The immunoreactivity of these peptides against anti-amylin antibodies was also identical.

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Received for publication 2 April 1996 and accepted in revised form 20 June 1996. ACN, acetonitrile; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformational polymorphism.

TABLE 1  
Clinical data from the subjects and frequencies of the S20G mutation of the amylin gene

	n	Sex (M/F)	Age (years)	Onset age (years)	S20G mutation	
					n	Frequency (%)
NIDDM	294	152/142	60.0 ± 0.7	44.1 ± 0.7	12	4.1
(relatively early onset)	80	40/40	49.8 ± 1.6	28.4 ± 0.7	8	10.0
IDDM	59	26/33	37.7 ± 3.2	21.3 ± 2.0	0	0
Nondiabetic	187	84/103	68.9 ± 0.7		0	0

Data are mean ± SE. Relatively early onset, onset age ≤35 years.

RESULTS AND DISCUSSION

On SSCP analysis, only one pattern with abnormal conformers was observed in the DNA fragment corresponding to exon 3 in 12 NIDDM patients. DNA sequencing of this exon revealed a single heterozygous missense mutation in amino acid 20 of the mature amylin molecule (AGC<sup>Ser</sup> to GGC<sup>Gly</sup>; S20G mutation) (Fig. 1A). This nucleotide change creates a *Msp* I restriction site. Using the *Msp* I RFLP method, all NIDDM patients with abnormally migrating bands were shown to have the same single heterozygous mutation (Fig. 1B). None of the 187 nondiabetic subjects, 59 IDDM patients, or remaining 282 NIDDM patients were shown to have this

mutation by both SSCP and RFLP analysis. The frequency of the mutation in NIDDM was 4.1% (Table 1). It was reported that no mutation of the coding region in the amylin gene was found in 25 unrelated patients with non-Japanese NIDDM by direct sequencing (8). This discrepancy may be due to an ethnic difference or to the small number of patients studied. It thus needs further studies in the different populations.

The clinical data of the affected patients and the results of family studies are summarized in Table 2. Of 12 affected patients, 8 were diagnosed as having NIDDM at relatively early age (≤35 years). The frequency of the affected patients in this relatively early onset NIDDM (onset age ≤35 years) was considerably high (8 of 80, 10.0%). The patients in this group with relatively early onset had severe diabetes (seven out of eight were under insulin treatment and the one remaining patient [patient number 5] also had a history of insulin therapy) with positive family histories of diabetes in two-degree relationship. The onset age in the unaffected family members with diabetes was >40 years. The limited family studies in the relatively early onset group (patients 1, 3, and 4) disclosed that the mutation was transmitted from the parent who was thought to be nondiabetic according to the historical background. Oral glucose tolerance tests performed later revealed that they had very mild glucose intolerance: impaired glucose tolerance (IGT). The family studies also revealed two affected family members: the brother of patient 1 (IGT) and the brother of patient 7 (early-onset NIDDM under insulin treatment). On the other hand, the patients in the late-onset group had mild diabetes (three out of four patients were treated by diet alone, and the remaining patient had mild diabetes but had to be treated with insulin because of renal and hepatic dysfunctions) without family histories of diabetes in two-degree relationship. Family studies (patients 10 and 11) disclosed two family members affected with IGT. These data show that the affected subjects have either relatively early onset and severe NIDDM with a strong family history of late-onset NIDDM or mild glucose intolerance. These findings suggest that the S20G mutation of the amylin gene creates mild glucose intolerance on its own; however, when the mutation is combined with unknown susceptibility genes for late-onset NIDDM, it contributes to the early onset of the NIDDM and makes it more severe.

Amylin is a main constituent of the islet amyloid that may disturb β-cell function (9). Recent studies have indicated that a limited segment or an amino acid residue of the human amylin has an intrinsic capacity to form amyloid fibrils (10). Altered amyloidogenic properties of Gly<sup>20</sup>-amylin caused by the substitution of an amino acid may thus influence the age at onset and the clinical course of NIDDM in individuals who have inherited other unknown susceptibility genes for late-

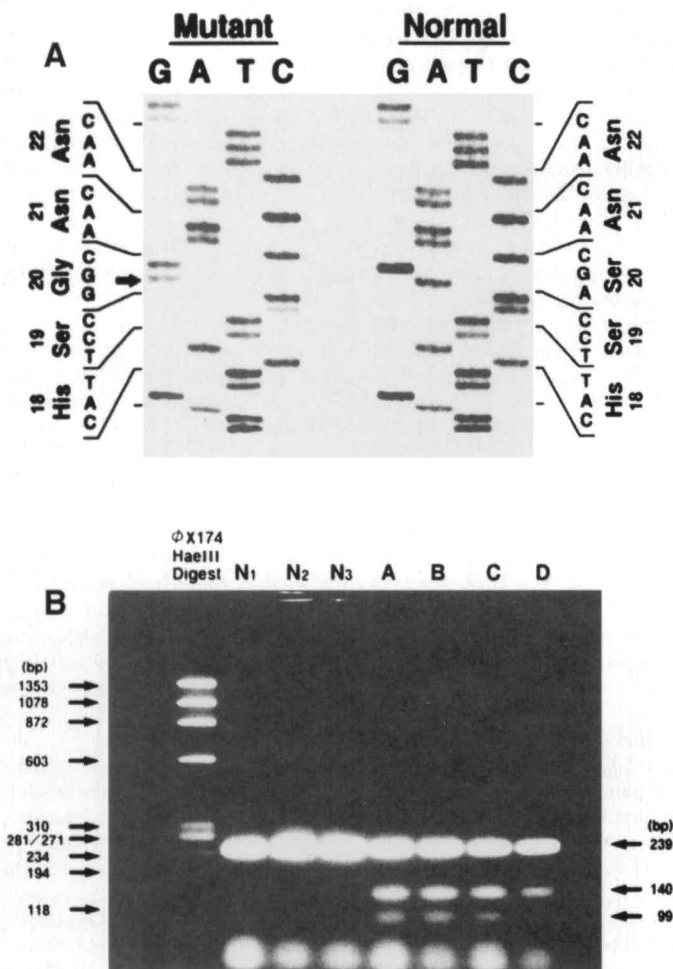


FIG. 1. A: Nucleotide sequences of exon 3 in the amylin gene of patient 2 are shown. Arrow indicates an A→G one-point missense mutation at position 20 (Ser to Gly) of the amylin molecule. B: *Msp* I RFLP of the PCR product (239 bp) of exon 3 in the amylin gene is shown. The normal pattern is represented in lanes N1 to N3. The heterozygous S20G mutation creates an *Msp* I site, resulting in the presence of the additional 140-bp and 99-bp bands represented in lanes A, B, C, and D.

TABLE 2  
Clinical data from the 12 patients with NIDDM carrying the S20G mutation of the amylin gene

Patient no.	Sex	Age (years)	Mode of treatment	Onset age	Glucose tolerance status in family members		
					Father	Mother	Other family members
Relatively early onset group							
1	F	31	Insulin	11	[IGT]**	[DM]*#	B[IGT]**, m-U[DM]**# m-A[DM]**#, +m-GF(DM)
2	F	26	Insulin	13	+(N)	+(DM)	m-GM(DM)
3	F	62	Insulin	25	[IGT]**	+(DM)	B(DM)#
4	M	57	Insulin	28	+(DM)	[IGT]**	B(DM)
5	M	63	SU	30	+(?)	+(?)	S(DM), B(DM)
6	M	50	Insulin	34	+(DM)	+(N)	
7	M	60	Insulin	35	+(?)	+(?)	B[DM]**#
8	M	75	Insulin	35	+(N)	+(DM)	
Late onset group							
9	F	61	Insulin	50	+(N)	+(N)	
10	F	61	Diet	57	+(N)	+(N)	m-A(DM), B[IGT]**
11	F	77	Diet	59	+(N)	+(N)	S[IGT]**
12	M	66	Diet	61	+(N)	+(N)	

B, brother; S, sister; U, uncle; A, aunt; GF, grandfather; GM, grandmother; m, maternal; SU, sulfonylureas; #, under insulin treatment; \*\*, affected; \*, unaffected; +, deceased. State of glucose tolerance is shown in parentheses (from historical background) or square brackets [diagnosed by oral glucose tolerance test and/or plasma glucose]. N, normal; ?, unclear; IGT, impaired glucose tolerance; DM, diabetes mellitus.

onset NIDDM. From such a point of view, further examinations, including examinations of the amyloidogenic properties of the mutant amylin, are required.

The serine residue at the position 20 of the amylin molecule is conserved among at least eight mammals (11). The possibility thus arises that the mutant amylin has reduced biological activity. In HPLC analysis, amylin immunoreactivity extracted from postprandial plasma of the affected patient (number 11) appeared at the position corresponding not only to normal human amylin (16%) but also to synthesized Gly<sup>20</sup>-amylin (84%). Although amylin immunoreactive peaks did not migrate with synthetic amylin, the identity of these peaks was not confirmed. Microsequencing of these HPLC peaks would be required to identify them. More than 20 ml of plasma is required for the HPLC study even in the case of the subject whose plasma amylin level ( $\beta$ -cell secreting ability) is well preserved. Thus, it is very difficult to perform the HPLC study in diabetic patients who have low  $\beta$ -cell secretion (patients 1–8). In the remaining four patients of the late-onset group, whose  $\beta$ -cell secreting ability is relatively preserved, only one patient (number 11) accepted our proposal to take enough blood for HPLC analysis. Although the data for the HPLC study was obtained from a single subject and the identity of the peaks was not confirmed, they suggest that the mutant amylin is expressed *in vivo* and that the slow metabolic clearance rate of the Gly<sup>20</sup>-amylin caused by reduced biological activity (receptor binding affinity) would lead to the accumulation of this peptide in the circulation. This finding controversially supports the notion that amylin is a biologically active peptide, although its true action is still unknown. Studies are in progress to characterize the action(s) of the synthesized Gly<sup>20</sup>-amylin. Fasting plasma amylin and insulin concentrations in the three affected patients (patients 2, 7, and 11) were measured in 1991 (7). At that time, the patients 2 and 7 were treated without insulin. Concentrations of amylin and insulin (both in picomoles per liter) were as follows: patient 2, 7.9 and 37.5; patient 7, 8.3 and 40.3; and patient 11, 11.4 and 51.4, respectively. The amylin/insulin molar ratio of these three affected patients ( $2.12 \pm 0.04 \times 10^{-1}$ , mean  $\pm$  SE) was significantly ( $P < 0.001$  by Student's *t* test) higher than that of unaffected NIDDM patients treated without insulin

( $1.23 \pm 0.09 \times 10^{-1}$ ,  $n = 32$ ). This indicates relatively high concentrations of amylin to insulin in the affected patients and supports the results of the HPLC analysis.

We have presented the first report of a missense mutation of the human amylin gene. Although we found a small number of affected patients, we suggest that the S20G mutation may play an important partial role in the pathogenesis of the early-onset NIDDM seen in the Japanese population and may provide a model to investigate and interpret the true physiological action of amylin.

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