Endocrine, *vol. 19, no. 2, 185–189, November 2002* 0969–711X/02/19:185–189/$20.00

Polymorphism in Intron 2 of Islet Amyloid Polypeptide Gene Is Associated with Lower Low-Density Lipoprotein Cholesterol in Nondiabetic Subjects and in Type 2 Diabetic Patients

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The aim of this study was to investigate the presence of mutations in the islet amyloid polypeptide (IAPP) gene in a Spanish population with type 2 diabetes and gestational diabetes mellitus (GDM). Using polymerase chain reaction single-stranded conformation polymorphism, we examined the coding region and the 5′-untranslated region (UTR) of the IAPP gene in 177 unrelated type 2 diabetic patients, 110 healthy control subjects, 38 women with GDM, and 38 gestational control subjects. Mutations were confirmed by DNA sequencing. A heterozygous C-to-A nucleotide substitution at +79 bp in intron 2 of the IAPP gene was detected. The frequencies of the +79-bp polymorphism (A allele) were 6.8% in type 2 diabetic patients, 7.7% in nondiabetic control subjects, 11.8% in women with GDM, and 9.2% in gestational control subjects. No AA genotypes were detected. Nondiabetic subjects and patients with type 2 diabetes bearing the CA genotype had lower low-density lipoprotein (LDL) cholesterol levels than subjects bearing wild genotype. Multivariate logistic regression analysis showed an independent association (*p* < 0.001; odds ratio: 0.33; 95% confidence interval: 0.17–0.63). We did not detect any sequence variant within exons 1 or 2. One diabetic patient was heterozygous for a silent mutation at codon 31 of exon 3 (Asn_{31} AAC→AAT). Our findings indicate that the presence of the +79-bp polymorphism of the IAPP gene in nondiabetic subjects and in patients with type 2 diabetes is associated with lower levels of LDL cholesterol. Furthermore, abnormalities of the coding regions or the 5′-UTR of the IAPP gene are not associated with type 2 diabetes or GDM in the Spanish population.

Received August 1, 2002; Revised September 6, 2002; Accepted September 27, 2002.

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Key Words: Islet amyloid polypeptide; human IAPP gene; type 2 diabetes mellitus; dyslipidemia; low-density lipoprotein cholesterol.

Introduction

Islet amyloid polypeptide (IAPP), also known as amylin, is a normal secretory product of the pancreatic β-cell and is released in response to glucose and nonglucose secretagogues both in vitro and in vivo *(1,2)*. This peptide is the primary constituent of amyloid deposits, which are found in pancreatic islets of individuals with type 2 diabetes *(3,4)*. Other components in islet amyloid are apolipoprotein E (apoE) *(5,6)* and the heparan sulfate proteoglycan, perlecan *(7)*.

In Alzheimer disease, the presence of genetic abnormalities results in aggregation of the constituent protein and cerebral amyloid formation *(8–10)*. By analogy, it has been suggested that abnormalities of the IAPP gene may be involved in the pathogenesis of islet deposits.

The human IAPP gene is located on the short arm of chromosome 12 and contains three exons *(11)*, of which the first is noncoding and the third codes for almost the complete propeptide *(12)*. A serine-to-glycine substitution at position 20 in the IAPP molecule (S20G missense mutation) has been reported in 4.1% of Japanese subjects with type 2 diabetes *(13)*. This association has not been found in other studied populations *(14–17)*, suggesting that ethnic background may be important in the association of the S20G mutation with type 2 diabetes.

There are no studies in Caucasian populations of South European extraction. Therefore, the aim of our study was to investigate the frequency of abnormalities of the IAPP gene in Spanish subjects with type 2 diabetes, and to study the effect of potential polymorphisms on clinical profile. On the other hand, gestational diabetes mellitus (GDM) is considered a prediabetic state and recent studies suggest that IAPP hypersecretion is characteristic for pregnancy *(18,19)*. Subsequently, to evaluate whether abnormalities of the IAPP gene may constitute a genetic risk for GDM, we investigated the

Table 1
Allele and Genotype Frequencies of +79-bp Polymorphism Among Study Groups

	Allele frequency		Genotype frequency	
	C allele	A allele	CC	CA
Control subjects	0.93	0.7	0.86	0.14
Type 2 diabetic patients	0.92	0.8	0.85	0.15
Gestational control subjects	0.88	0.12	0.76	0.24
Patients with GDM	0.91	0.9	0.82	0.18

Table 2
Clinical and Biologic Characteristics of Study Groups[a]

	Control subjects	Type 2 diabetic patients	Gestational control subjects	Patients with GDM
n	110	177	38	38
Age (yr)	50.8 ± 13.2	63.06 ± 10.9[b]	30.9 ± 4.5	31.7 ± 4.6
Sex (M/F)	57/53	94/83	F	F
BMI (kg/m^2)	27.6 ± 4.08	28.5 ± 5.3	24.7 ± 4.5	26.6 ± 4.9
Fasting glucose (mmol/L)	5.09 ± 0.46	9.6 ± 3.4[b]	4.30 ± 0.45	4.76 ± 0.86[b]
Cholesterol (mmol/L)	5.46 ± 1.06	5.39 ± 1.22	6.23 ± 1.37	6.42 ± 1.25
HDL cholesterol (mmol/L)	1.40 ± 0.38	1.17 ± 0.38[b]	2.0 ± 0.53	1.87 ± 0.46
LDL cholesterol (mmol/L)	3.81 ± 0.78	3.52 ± 1.09	3.43 ± 1.25	3.57 ± 1.08
Triglycerides (mmol/L)	1.20 ± 0.66	1.73 ± 0.86[b]	1.95 ± 0.61	2.17 ± 0.80

[a]Data are *n*, means ± SD, or %.
[b]$p < 0.001$ between type 2 diabetic subjects and control subjects. $p < 0.01$ between patients with GDM and gestational control subjects.

presence of mutations in the IAPP gene in a cohort of patients with GDM.

Results

Identification of Polymorphisms in IAPP Gene

One electrophoretic variant pattern was detected in the region of the exon 2 that was explored by polymerase chain reaction (PCR) and single-stranded conformational polymorphism (SSCP). DNA sequencing revealed a C-to-A nucleotide substitution at +79-bp in intron 2 of the IAPP gene. The allele frequencies of the +79-bp polymorphism (A allele) among groups were 6.8% in type 2 diabetic patients, 7.7% in nondiabetic control subjects, 11.8% in women with GDM, and 9.2% in gestational control subjects. No differences in frequencies were observed among the study groups. No homozygous carrier of this variant was identified. The allele distribution and genotype frequencies of the +79-bp polymorphism according to glucose tolerance status are summarized in Table 1. None of the allelic frequencies were statistically significantly different from Hardy-Weinberg equilibrium.

On SSCP analysis of exon 3, which encodes the mature IAPP, only one pattern with abnormal conformers was detected in a patient with type 2 diabetes mellitus. DNA sequencing of this sample revealed a silent polymorphism at codon 31: Asn_{31} (AAC→AAT), which does not involve any change in the amino acid sequence of IAPP. No sequence variant was observed within exon 1, which encodes most of the 5'-untranslated region (5' UTR) of the mRNA, within exon 2, which encodes the signal peptide, or in other exon-intron boundaries.

Clinical Profile of Subjects with +79-bp Polymorphism of IAPP Gene

The clinical characteristics of the study groups are given in Table 2. Of the 177 type 2 diabetic patients, 12% were treated with diet, 56% with insulin, 19.5% with oral hypoglycemic drugs, and 12.5% with combination therapy. Fifteen percent of diabetic patients were treated with lipid-lowering drugs. Of the patients, 65% developed microvascular complications (35% retinopathy and 47% nephropathy with 27% microalbuminuria, 16% proteinuria, and 7% renal failure), and 38% suffered from clinical macrovascular complications (8% cerebrovascular disease, 13% peripheral vascular disease, and 26.5% coronary disease).

Study of the clinical and biologic features of the subjects revealed that this polymorphism of the IAPP gene was associated with a specific lipid profile. Patients with type 2 diabetes and nondiabetic control subjects harboring the +79-bp polymorphism had lower total cholesterol and low-density lipoprotein (LDL) cholesterol levels than noncar-

Table 3
Univariate Study of Nondiabetic Subjects and Patients with Type 2 Diabetes According to Presence (CA Genotype) or Absence (CC Genotype) of +79-bp Polymorphism [a]

	No diabetes		Type 2 diabetes	
	CA	CC	CA	CC
n	17	93	24	153
Sex (M/F)	5/12	52/41	14/10	80/73
Age (yr)	47.3 ± 11.7	51.4 ± 13.4	64.4 ± 9.9	62.8 ± 11.1
Diabetes duration (yr)	—	—	10.5 ± 8.7	12.9 ± 9.3
BMI (kg/m^2)	29.8 ± 6.9	27.2 ± 3.2	29.7 ± 4.8	28.3 ± 5.4
Insulin treatment (%)	—	—	62.5	55.5
Known hypertension (%)	—	—	58.3	55.3
Smoking (%)	29.4	30.1	33.3	32.6
Menopause (%)	41.6	68.2	100	89
Lipid-lowering drugs (%)	—	—	10.7	14.1
Fasting glucose (mmol/L)	5.07 ± 0.51	5.10 ± 0.46	9.3 ± 3.7	9.7 ± 3.4
HbA1c (%)	4.18 ± 0.45	4.59 ± 0.64	7.3 ± 1.8	7.8 ± 1.9
Triglycerides (mmol/L)	1.08 ± 0.53	1.22 ± 0.70	1.62 ± 0.75	1.75 ± 0.88
Cholesterol (mmol/L)	4.87 ± 0.79	5.58 ± 1.07 [b]	4.87 ± 0.77	5.48 ± 1.27
LDL cholesterol (mmol/L)	3.15 ± 0.56	3.88 ± 0.78 [b]	2.69 ± 0.60	3.65 ± 1.09
HDL cholesterol (mmol/L)	1.29 ± 0.45	1.42 ± 0.38	1.01 ± 0.26	1.19 ± 0.38
Apolipoprotein E (g/L)	48.1 ± 2.7	36.5 ± 13.3	39.7 ± 7.4	46.3 ± 19.6

[a] Data are *n*, means ± SD, or %.
[b] $p < 0.05$ (CA vs CC genotype) within the nondiabetic group. $p < 0.005$ and $p < 0.001$ (CA vs CC genotype) within type 2 diabetic subjects.

riers (Table 3). By contrast, no differences between the two groups of genotypes could be detected for other variables such as sex, body mass index (BMI), fasting plasma glucose, microalbuminuria, or treatment with hypolipidemic agents. The multivariate logistic regression model showed that LDL cholesterol plasma level was the only variable independently associated with the +79-bp polymorphism ($p < 0.001$; odds ratio: 0.33; 95% confidence interval: 0.17–0.63). In the cohort of women with GDM, univariate analysis showed that LDL cholesterol levels were significantly lower in carriers of the +79-bp polymorphism than in noncarriers (2.93 ± 1.1 vs 3.79 ± 0.99 mmol/L; $p = 0.05$).

Discussion

There is increasing evidence suggesting that IAPP plays an important role in the pathogenesis of type 2 diabetes, by its ability to form amyloid fibrils *(20–22)*. The possible role of the IAPP gene mutations in the development of type 2 diabetes remains controversial; both positive association and negative results have been reported *(13–17)*. However, only a few studies are available, and these studies have been performed on a limited number of subjects.

In the present study, we examined the coding region, the 5'UTR region, and exon-intron junctions of the IAPP gene for genetic variability in a cohort of Spanish subjects with type 2 diabetes and GDM. The S20G missense mutation reported in the Japanese population was not detected in any subject, a result that is in agreement with the study of Birch et al. *(17)*.

However, we detected a nonpreviously reported C/A polymorphism at +79 bp in intron 2 of the IAPP gene. The allele distribution of diabetic patients was not significantly different from that of control subjects with normal glucose tolerance, suggesting that this polymorphism is not associated with genetic predisposition to type 2 diabetes mellitus or GDM. It is noteworthy that the +79-bp IAPP gene polymorphism was associated with a specific phenotype. Both nondiabetic subjects and type 2 diabetic patients bearing the CA genotype had statistically significant lower levels of LDL cholesterol than subjects bearing the wild genotype. Moreover, this association was also present in the cohort of women with GDM.

The +79-bp polymorphism of the IAPP gene does not involve any known transcription factor or any structural change in IAPP. One possible explanation for the detected association is the linkage of the polymorphism to other gene polymorphisms on chromosome 12. Two possible candidate genes related to lipid metabolism and atherosclerosis have recently been mapped close to the IAPP locus: the LOX-1 gene, assigned to the p12.3-p13.2 region on chromosome 12, which encodes the human lectin-like oxidized

LDL receptor-1 *(23)*; and the LRP6 gene, mapped to chromosome 12p11-p13.3, which encodes the LDL receptor–related protein 6 *(24)*. Further investigation of this is now in progress.

It has been hypothesized that apo E may have a role in islet amyloidogenesis given its presence in amyloid fibrils, and that an enhanced production of apo E could be associated with type 2 diabetes *(6)*. We measured plasma apo E levels and did not find any significant difference between diabetic patients and the nondiabetic control group, neither between diabetic subject carriers nor noncarriers of the +79-bp polymorphism. This result is in agreement with recent reports suggesting that apo E does not play a critical role in islet amyloid formation in type 2 diabetes *(25,26)*.

In conclusion, we have identified a +79-bp polymorphism in the IAPP gene that is associated with a less atherogenic lipid profile in nondiabetic subjects and in type 2 diabetic patients. Further studies, including linkage analysis of the probands' pedigrees, will provide important insights into the mechanism of such association.

Materials and Methods

Patients

The total study population included 363 unrelated Spanish subjects. One hundred seventy-seven patients with type 2 diabetes (94 men and 83 women, ages 63.1 ± 10.9 yr) were consecutively selected from our outpatient clinic. These patients fulfilled the World Health Organization criteria for type 2 diabetes *(27)*. The control group consisted of a sample of 110 healthy subjects (57 men, 53 women) without family history of diabetes recruited from patients' spouses and hospital staff. They did not have any chronic diseases, nor did they have any drug treatment that could influence glucose or lipid metabolism.

The second study group comprised a cohort of 76 unrelated women referred to our diabetes unit for the assessment of glucose tolerance at 22–29 wk of gestation (median: wk 25). Thirty-eight women with GDM and 38 women with normal glucose tolerance matched for age and BMI were also selected and included.

The study was approved by the local medical ethics committee. It was carried out in accordance with the Declaration of Helsinki, and all participants gave their written, informed consent.

Analytical Methods

Blood samples were obtained after an overnight fast. Serum glucose was determined by the glucose oxidase method. Serum total cholesterol and triglyceride levels were measured using enzymatic reagents (Trinder, Bayer, New York) adapted to a Cobas Mira autoanalyzer (Hoffman-La Roche, Switzerland). High-density lipoprotein (HDL) cholesterol was determined by a commercial direct method, using an anti human β-lipoprotein antibody (Sigma, St. Louis, MO). LDL cholesterol was estimated using the Friedewald formula *(28)* when triglyceride did not exceed 3.45 mmol/L and by ultracentrifugation when triglyceride levels were ≥3.45 mmol/L. Serum apo E was measured by a nephelometric method coefficients of variation for apo E measurement were 2.9 and 4.1%, respectively.

DNA Analysis

Genomic DNA was extracted from peripheral leukocytes using standard methods and amplified by PCR. The reaction conditions were as follows: an initial 5-min denaturation step at 95ºC, 30 cycles of denaturation at 95ºC for 1 min, annealing at 51ºC (exon 1)/49ºC (exon 2)/50ºC (exon 3) for 1 min, and an extension at 72ºC for 1 min. The reaction was concluded with a 10-min extension at 72ºC. Three different sets of oligonucleotide primers were designed to individually amplify each exon of the IAPP gene including the exon/intron boundaries. The specific primer sequences and their respective PCR fragment length were as follows: exon 1 forward primer: 5'-ATGACAGAGGCTCTCTGA GCT-3' and reverse primer: 5'-ACACCAAGTGTGCATT TCTCT-3' (248 bp); exon 2 forward primer: 5'-GAACTGT AAGAAATCTCTTG-3' and reverse primer: 5'-GATATA GTCAGAAATCTAAGGCTG-3' (247 bp); exon 3 forward primer: 5'-GGCTGGATCCAGCTAAAATTC-3' and reverse primer: 5'-GCAAGTAATTCAGTGGCTCTC-3' (241 bp).

Amplification products were examined by SSCP analysis, and the samples showing an electrophoretic variant pattern were sequenced on an ABI Prism™ 377 DNA sequencer (Perkin Elmer, Norwalk, CT).

Statistical Analyses

Statistical analyses were done with the STATA statistical software package, version 6.0 (Stata 1999, College Station, TX). Means ± SD were determined for quantitative data, and frequencies were determined for categorical variables. In univariate comparisons, differences in continuous variables between groups of subjects were tested with student's *t*-test or the Wilcoxon rank sum test. Logarithmic transformations were used for variables with skewed distributions (fasting glucose, total cholesterol, triglycerides, HbA1c, and apo E), and data are presented as geometric mean. The Pearson χ^2 test and Fisher's exact test were used to analyze group differences for categorical variables. A multivariate stepwise forward logistic regression analysis was applied to identify variables independently associated with the +79-bp polymorphism of the IAPP gene. A p value <0.05 was considered significant.

Acknowledgments

We are grateful to Drs. J. Vidal, A. Costa, C. dell'Anna, and P. Cristobal for their collaboration. We also wish to thank Dr. A. Clark for scientific advice. This work was supported by grants FIS 99/0378 (Health Ministry, Spain) and Marató TV3 1998 991730/31/32.

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