ENPP1 K121Q Polymorphism and Ischemic Heart Disease in Diabetic Patients

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Summary

Background: The ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene is a candidate gene for insulin resistance. Insulin resistance is a major component of metabolic syndrome (MetS) and has been implicated in ischemic heart disease (IHD).

Objective: To evaluate the association between the K121Q polymorphism of the ENPP1 gene and IHD in white patients with type 2 diabetes mellitus (DM).

Methods: A cross-sectional study was performed in type 2 DM patients (n = 573, 50.6% males, age 59.5±10.4 years). IHD was defined by the presence of angina or myocardial infarction according to the Worth Health Organization cardiovascular questionnaire and/or compatible electrocardiographic (Minnesota Code), or perfusional abnormalities in myocardial scintigraphy. The K121Q polymorphism of ENPP1 gene was genotyped using PCR-based methods and restriction enzyme digestion.

Results: IHD was present in 209 (36.5%) patients. The distribution of KK, KQ and QQ genotypes among patients with IHD was 60.8%, 34.4% and 4.8%, not different from the genotype distribution in the group without IHD (64%, 32.7% and 3.3%, P=0.574). No difference was found in the clinical and laboratory characteristics between the three genotypes, neither regarding the prevalence of Metabolic Syndrome.

Conclusion: No association was found between polymorphism K121A of ENPP1 gene and the presence of IHD. (Arq Bras Cardiol 2010;94(2): 157-161)

Key words: Polymorphism genetic; Myocardial ischemia; Diabetes mellitus type 2; Metabolic syndrome.

Introduction

Insulin resistance is one of the main mechanisms implicated in the pathogenesis of both type 2 diabetes mellitus (DM) and metabolic syndrome (MetS)[1]. Furthermore, insulin resistance is also associated with the phenotypes involved in MetS definition, such as central obesity, arterial hypertension, dyslipidemia and impaired glucose tolerance. The state of resistance to the action of insulin is more frequent in older, obese, sedentary individuals, and its presence is probably the result of the interaction of environmental and genetic factors.

Genetic influence on insulin action was described in family studies. Reinhard et al[2] evaluated the impact of the diagnosis of MetS on the risk of cardiovascular diseases in German families with a major genetic base for ischemic heart disease (IHD). The authors showed that MetS is an independent predictor of cardiovascular morbidity and mortality, especially in young patients with a family history of early-onset IHD.

The ecto-nucleotide pyrophosphatase / phosphodiesterase 1 (ENPP1) gene is outstanding among the possible candidate genes for the development of MetS, since its K121Q polymorphism appears to be associated with insulin resistance[3-5]. ENPP1 encodes a class 2 membrane glucoprotein which negatively influences sensitivity to insulin action by inhibiting the signal of the insulin tyrosine-kinase receptor. The variant 121Q (risk allele) binds to the insulin receptor with greater affinity compared with its wild allele 121K, resulting in less autophosphorylation of the receptor. In a multicentric study, variant 121Q was associated with a higher risk of early development of type 2 DM and acute myocardial infarction compared to the 121K allele[6]. In a recent meta-analysis of 15,801 patients with type 2 DM and 26,241 control subjects, the 121Q allele was associated with an increased risk for type 2 DM, which was modulated by body mass index (BMI)[7]. However, the precise role of polymorphism K121Q in the development of vascular complications of type 2 DM,

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especially atherosclerotic cardiac disease, has not yet been fully analyzed.

Thus, the purpose of this study was to analyze the association of variant 121Q of the gene ENPP1 in white patients with type 2 DM with the presence of IHD, MetS and related phenotypes.

Methods

Patients

A cross-sectional study was performed with 573 white type 2 DM patients[8] seen at the outpatient clinic of the Endocrinology Division of *Hospital de Clínicas de Porto Alegre*, Brazil. These patients are participants in a study which began in 2002 and is conducted in the state of Rio Grande do Sul for the purpose of studying DM and its chronic complications. Besides the Endocrinology Division of *Hospital de Clínicas de Porto Alegre*, another three hospitals participated in the Project as follows: *Grupo Hospitalar Nossa Senhora da Conceição*, *Hospital São Vicente de Paula*, and *Hospital Universitário de Rio Grande*.

Clinical evaluation

Patients were submitted to a previously described standardized evaluation[9] that includes: age, DM duration, and current medications in use. Physical examination and laboratory tests were performed in order to evaluate diabetic chronic complications and cardiovascular risk factors. Weight and height were measured with patients wearing light clothes and without shoes on. BMI was calculated with the weight (kg)/height^2 (m) ratio. Waist was measured at the level of the umbilical scar[10]. Arterial hypertension was defined as blood pressure $\geq$140/90 mm Hg or current use of antihypertensive medications[11]. Patients who had smoked regularly in the previous year were considered active smokers.

IHD was diagnosed in the presence of angina or possible myocardial infarction according to the Word Health Organization (WHO) cardiovascular questionnaire and/or compatible electrocardiographic changes (Minnesota Code: Q and QS [1.1-2, 1.3] patterns; S-T [J] junction and segment depression [4.1-4]; items of the T [5.1-3] wave, and complete left bundle block [7.1])[12] and/or perfusional abnormalities (fixed or variable) in myocardial scintigraphy at rest and after dipyridamol. These diagnostic tools for myocardial ischemia in type 2 DM patients were previously evaluated in a prospective study[13].

The diagnosis of peripheral vasculopathy was based on the presence of intermitted claudication (WHO cardiovascular questionnaire) and/or absence of lower limb pulses at physical examination.

Diabetic retinopathy (DR) was evaluated by an experienced ophthalmologist by direct fundoscopy on mydriasis. DR was classified as absent, non-proliferative DR (NPDR) (microaneurisms, hard exudates and hemorrhage) or proliferative DR (PDR) (presence of neovessels and/or fibrous tissue in the vitreous cavity). Diabetic nephropathy (DN) was defined by an increased urinary albumin excretion (UAE) in the absence of urinary infection or other renal abnormalities in at least two isolated measurements, and it was classified as microalbuminuria (UAE 20-199 μg/min) or macroalbuminuria (UAE $\geq$200 μg/min)[14].

MetS was defined according to the criteria of the National Cholesterol Education Program - Adult Treatment Panel III as the presence of two or more of the following besides DM: abdominal obesity (waist circumference >102 cm in men and >88 cm in women); high serum triglycerides (≥150 mg/dl); low HDL cholesterol (<40 mg/dl in men or <50 mg/dl in women), and high blood pressure (≥130/85 mmHg or use of anti-hypertensive medication)[15].

Laboratory tests

UAE was measured using the immunoturbidimetry technique in sterile timed 24-h urine samples, without the use of converting enzyme inhibitors or angiotensin receptor blockers. Fasting plasma glucose levels were determined by the glucose-oxidase method; creatinine by the Jaffe reaction, and HbA1c, by high performance liquid chromatography (HPLC; Merck-Hitachi 9100; reference values: 4-6%); triglycerides and cholesterol levels were measured using the enzymatic method, and LDL was calculated by the Friedewald equation. Serum insulin was measured by radioimmunoassay (Elecsys R Systems 1010/2010/ modular analysis E170 - ROCHE) in a sample of patients who were not using insulin and who had serum creatinine <1.3 mg/dl. Insulin resistance was estimated by Homeostasis Model Assessment {HOMA-IR = [fasting insulin (mUI/ml) X fasting plasma glucose (mmol/l)] / 22.5)}, as recently validated[16].

Molecular analysis

Individuals were genotyped for polymorphism ENPP1 K121Q as previously described[17]. DNA was isolated from lymphocytes using standard procedures[18]. All the PCRs were run in a final 25 μl volume containing 50 ng of genomic DNA, 20 mmol/l Tris-Cl, (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1 unit of *Taq*DNA polymerase, and 1 μmol/l of specific primes to obtain exon 4 (forward: 5'-CTG TGT TCA CTT TGG ACA TGT TG-3' and reverse: 5'-GAC GTT GGA AGA TAC CAG GTT G-3'). The PCR products were digested by enzyme restriction *Ava*II and separated in agarose gel. The allele encoding variant K appeared as a single fragment of 238 base-pairs and the allele encoding variant Q as two fragments of 148 and 90 base-pairs each.

Statistical analysis

Student's t test for independent samples or Mann-Whitney U test were used to compare the characteristics of patients with and without IHD as appropriate. The chi-square test and One-Way ANOVA were used to compare the clinical and laboratory characteristics of patients divided according to genotypes. The Tukey test was used for multiple post hoc comparisons. Hardy-Weinberg Equilibrium was calculated using allele frequencies and the chi-square test. Variables with a non-normal distribution were submitted to logarithmic transformation. The continuous variables were described as mean ± standard deviation, or median and range, and the categorical variables were expressed as number of cases and

percentage. A value of P <0.05 (two-tailed) was considered significant. The analyses were performed using the Statistical Package for the Social Science program (SPSS, version 14.0 for Windows).

The study protocol was approved by the Ethical Committee of the *Hospital de Clínicas de Porto Alegre*, and all patients signed a written informed consent.

Results

Patients

The mean age of the 573 patients included was 59.5 ± 10.4 years with a known duration of DM of 11.7 ± 9.2 years and BMI of 28.9 ± 5.2 kg/m^2. Women were 49.4% (n = 283) of the sample. IHD was present in 209 (36.5%) of the patients. The clinical and laboratory characteristics of the patients, divided according to the presence of IHD, are shown in Table 1.

Patients with IHD had longer DM duration and the frequency of smokers was higher (P = 0.010) (Table 1). The other clinical characteristics, such as age, sex distribution, blood pressure, BMI and abdominal waist were not different. Regarding the laboratory data, the group with IHD had higher serum creatinine levels (1.61 ± 1.8 *vs* 1.30 ± 1.37 mg/dl, P = 0.024) and higher rates of UAE [15 (0.8-3162) *vs* 5.3 (0.1-7680) μg/min, P <0.001] than patients without IHD. MetS occurred in 96.1% (n = 323) of the patients without IHD and in 91.8% (n = 178) of the group with IHD (P = 0.118).

The presence of IHD was more frequent among those with any degree of DR (NPDR/PDR) (44.2% *vs* 33.7%, P=0.003), DN (micro/macroalbuminuria) (47.4% *vs* 32.1%, P <0.001) or peripheral vascular disease (52.5% *vs* 33.0, P <0.001) than among those without these complications.

K121Q polymorphism and ischemic heart disease and metabolic syndrome

The genotypes evaluated were in Hardy-Weinberg Equilibrium (P >0.05), and the frequency of the risk allele (allele Q) was 20.5%. The clinical and laboratory characteristics of patients divided according to the different genotypes were not different among the three groups (Table 2).

When patients were divided according to the presence of IHD, the distribution of KK, KQ and QQ genotypes was 60.8%, 34.4% and 4.8%, not different from the genotype distribution observed in the group without IHD (64%, 32.7% and 3.3%, P = 0.574) (Table 1). Allele Q occurred in 22% (n = 81) of the patients with IHD and in 19.6% (n = 131) of the patients without IHD (P = 0.328). The prevalence of MetS according to the KK, KQ and QQ genotypes was 96.3%, 95% and 88.2% (P = 0.279). These results did not change when patients were divided assuming a dominant model (KQ/QQ *vs* KK).

Table 1 - Clinical and laboratory characteristics of type 2 diabetes mellitus patients with and without ischemic heart disease

	Ischemic Heart Disease		P
	No N = 364	Yes N = 209	
Age (years)	59.5 ± 10	60.7 ± 10	0.157
Diabetes duration (years)	10.9 ± 8.5	14.4 ± 9.4	<0.001
Male - n (%)	181 (49.7%)	109 (52.2%)	0.576
Current Smokers - n (%)	53 (14.9%)	48 (23.6%)	0.010
Hypertension - n (%)	244 (63.5%)	140 (67%)	0.991
Systolic blood pressure (mmHg)	143.1 ± 23.7	142.3 ± 24.3	0.710
Diastolic blood pressure (mmHg)	86.1 ± 13.3	84.8 ± 12.3	0.261
Fasting plasma glucose (mg/dl)	173.5 ± 66.8	172.6 ± 72.6	0.892
HbA1c (%)	6.36 ± 1.83	6.40 ± 1.81	0.833
Abdominal circumference (cm)			
Male	100.2 ± 11.9	98.9 ± 10.1	0.343
Female	96.6 ± 12.7	97.5 ± 10.9	0.465
Body mass index (kg/m^2)	28.4 ± 4.8	28.5 ± 4.9	0.943
Creatinine (mg/dl)	1.30 ± 1.37	1.61 ± 1.8	0.024
Total cholesterol (mg/dl)	209.1 ± 45.9	212.6 ± 47.6	0.489
HDL cholesterol (mg/dl)	44.1 ± 11.3	42.7 ± 12	0.169
LDL cholesterol (mg/dl)	130.5 ± 40.6	134.7 ± 48.5	0.420
Triglycerides (mg/dl)*	155 (27-900)	167 (47-1265)	0.552
HOMA - IR*	5.18 (0.46-30.2)	4.6 (0.3-28.2)	0.301
Metabolic Syndrome - n(%)	323 (96.1%)	178 (91.8%)	0.118
Albuminuria (μg/min)*	5.3 (0.1-7680)	15 (0.8-3162)	<0.001
K121Q polymorphisms			
KK genotype	60.8%	64.0%	0.574
KQ genotype	34.4%	32.7%	
QQ genotypes	4.8%	3.3%	

*Data showed as number (%), mean ± standard deviation or *median (range). HOMA-IR - homeostasis model assessment.*

Discussion

In this study of white Brazilian patients with type 2 DM, allele 121Q of the gene ENPP1 was not associated with the presence of IHD, neither with characteristics associated with insulin resistance, including BMI, abdominal obesity, high blood pressure, worse lipid profile, glycemic control or presence of MetS. The absence of an association of this polymorphism with insulin resistance characteristics deserves some comments. First, this association may not exist, and relations described in other studies are in fact spurious due to a type 2 error. If this hypothesis is rejected, the relevance of a positive association should be discussed. In order to detect a significant difference in the frequency of Q allele in patients with (22%) and without IHD (19.6%) ($\alpha = 0.05$ and $\beta = 80\%$) it would be necessary to evaluate about 4500 patients. Even if this would be significantly different, its applicability would be questionable because of its small effect and might depend on other polymorphisms

Table 2 - Clinical and laboratory characteristics of patients according to genotype

	Polymorphism ENPP1 K121Q			
	KK (N = 361)	KQ (N = 192)	QQ (N = 20)	P
Age (years)	61.4 ± 8.9	61.7 ± 8.3	61.1 ± 12.6	0.973
Diabetes duration (years)	11.7 ± 8.8	12.4 ± 8.8	10.7 ± 6.6	0.372
Male - n (%)	14 (49.5)	36 (50.8)	3 (33.3)	0.615
Current smoking - n (%)	5 (18.9)	17 (24.6)	0 (0)	0.240
Hypertension - n (%)	19 (69.7)	18 (73.8)	6 (66.7)	0.822
Systolic blood pressure (mm Hg)	143.3 ± 23.8	141.3 ± 22.4	145.3 ± 26.0	0.377
Diastolic blood pressure (mm Hg)	85.9 ± 13.8	86.0 ± 12.4	86.4 ± 13.0	0.956
Fasting plasma glucose (mg/dl)	177 ± 70.1	168.2 ± 70.7	189.7 ± 88.4	0.114
HbA1c (%)	6.4 ± 1.8	6.4 ± 2.1	6.6 ± 1.9	0.740
Waist circumference (cm)				
Male	95.92 ± 8.52	100.63 ± 9.07	85.50 ± 4.95	0.032
Female	95.92 ± 13.53	96.52 ± 12.96	99.50 ± 1.91	0.799
Body Mass Index (kg/m^2)	28.5 ± 4.9	28.7 ± 5.2	26.4 ± 4.06	0.451
Serum creatinine (mg/dl)	1.5 ± 1.9	1.6 ± 16	2.1 ± 2.8	0.131
Total cholesterol (mg/dl)	212 ± 48.7	209 ± 46.0	207 ± 54.8	0.665
HDL cholesterol (mg/dl)	44 ± 12.3	45 ± 12.0	46 ± 13.7	0.301
LDL cholesterol (mg/dl)	133 ± 46.2	130 ± 41.8	137 ± 45.0	0.739
Triglycerides (mg/dl)*	155 (27–1470)	144 (26–2236)	155 (56–659)	0.216
HOMA-IR*	5.69 (0.27-61.98)	4.3 (0.37-31.91)	11.1 (1.14-82.73)	0.497
Albuminuria (µg/min)*	19.25 (0.8-5104)	18.9 (1-3055)	5.0 (1-232)	0.145

*Data showed as number (%), mean ± standard deviation or *median (interval). HOMA-IR - homeostasis model assessment.*

or gene interactions. A more plausible explanation is that the expression of this gene variant depends on the ethnic group that is being studied. Actually, even among European Caucasians, the phenotypic expression of this polymorphism varies. Indeed, the relationship between polymorphism K121Q and sensitivity to insulin action was shown in Caucasian individuals[19-21], but not in all of them[20,22,23]. The absence of the risk allele effect shown in some groups may be the result of differences at the genetic base of these populations, or it may be due to the fact that this polymorphism is in linked disequilibrium with other unidentified gene variants.

The association of allele 121Q with risk and severity of IHD was evaluated recently in a case-control study, which included individuals from Italy and the United States[6]. In that study, the authors did not show a significant association between polymorphism K121Q and the presence of IHD in patients with type 2 DM. However, the ischemic events occurred at an earlier age in the group of patients with allele Q, even after analysis adjusted for sex, BMI, smoking, hypertension and site of recruitment (P=0.04). The cross-sectional design of the present study precludes any analysis of the influence of this polymorphism on the course of IHD.

A possible limitation of this study is that only one polymorphism of the gene was evaluated. This allows the exclusion of the idea that this polymorphism is connected to IHD, but not the gene ENPP1 per se, since polymorphism K121Q is only one among the most studied. Another aspect could be related the adopted IHD criteria. Patient was diagnosed with IHD when one of the evaluated tests (WHO cardiovascular questionnaire, rest ECG, myocardial scintigraphy) was positive. We did not have detail information about witch test was positive in all 573 patients in this multicentre cross-sectional study. However, since all studied patients underwent the WHO cardiovascular questionnaire and rest ECG, we can state that in the group of patients without IHD 100% of these tests were negative. Furthermore, we had previously demonstrated[13] that the presence of IHD according the WHO cardiovascular questionnaire had a RR for cardiac events of 2.13 (95%CI 1.11-4.07; P = 0.022) even better than the risk conferred by resting ECG or myocardial scintigraphy. Furthermore, when WHO cardiovascular questionnaire was associated with a normal ECG the negative predictive value for cardiac events was 82.1%. In this situation, further additional tests are probably not necessary.

In conclusion, in this sample of white Brazilian patients with

type 2 DM, polymorphism K121Q of the gene ENPP1 was not associated with the presence of IHD neither with phenotypes related to insulin resistance or MetS.

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Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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