A Variant of Human Paraoxonase/Arylesterase (*HUMPONA*) Gene Is a Risk Factor for Coronary Artery Disease

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Abstract

Coronary artery disease (CAD) is a complex trait caused by a number of genetic and environmental factors. Recently, paraoxonase/arylesterase (PONA) enzyme has been implicated in the pathogenesis of atherosclerosis. There is a 10–40-fold variability in the activity of this enzyme among individuals. This variability is due to the presence of an A/G polymorphism in the coding region of the gene (*HUMPONA*). The A and G alleles code for glutamine (A genotype) and arginine (B genotype), respectively. Individuals with A genotype have a lower enzymatic activity than those with B genotype. We determined the HUMPONA genotypes and alleles in 223 patients with angiographically documented CAD and in 247 individuals in the general population. The distribution of genotypes were in Hardy-Weinberg equilibrium in patients and in controls. Genotypes A and B were present in 120 (49%) and 28 (11%) individuals in controls and in 68 (30%) and 40 (18%) patients with CAD, respectively (χ^2 = 16.5, P = 0.0003). The frequency of the A allele was 0.69 in controls and 0.56 in patients (OR = 1.7, P = 0.0001). There were no differences in the distribution of HUMPONA genotypes in the subgroups of patients with restenosis, myocardial infarction, or any of the conventional risk factors for CAD as compared with corresponding subgroups.

In summary, variants of the HUMPONA gene are involved in predisposition to coronary atherosclerosis. (*J. Clin. Invest.* 1995. 96:3005–3008.) Key words: atherosclerosis • genetics

Introduction

Atherosclerotic cardiovascular disease is the leading cause of death in the western hemisphere and a major public health concern. Genetic factors play an important role in predisposition to atherosclerotic coronary artery disease (CAD)[1] and its thrombotic complications (1, 2). The evidence that implicated genetic factors in predisposition to atherosclerosis in the past was derived from epidemiological studies, studies of monozygotic and dizygotic twins, and studies of first degree relatives of patients with CAD (1, 2). However, identification and characterization of the genes involved in atherosclerosis and its thrombotic complications awaited the development of modern techniques of molecular genetics. These advances have provided the opportunity to identify and characterize the genes involved in polygenic diseases such as CAD. A commonly used method for identification of genetic risk factors for complex traits is allelic association studies (3). These case-control studies compare the distribution of alleles or genotypes of a particular gene in unrelated affected and unaffected individuals. Such studies are performed to analyze the gene variants that are thought to be candidates in the pathogenesis of a disease or play a role in modifying its phenotypic expression (4, 5).

A large number of genes are likely to be involved in the pathogenesis of coronary artery disease. Genes involved in lipoprotein synthesis, modification, and metabolism are excellent candidates for CAD. Human paraoxonase/arylesterase enzyme (EC 3.1.8.1, aryldialkylphosphatase) which hydrolyzes paraoxon, the toxic metabolite of organophosphate anticholinesterases, also has been implicated in the pathogenesis of atherosclerosis (6). Paraoxonase (PONA) is a Ca^{2+}-dependent glycoprotein that is associated with HDL and has been shown to prevent LDL oxidation in vitro (6, 7). A decreased paraoxonase (PONA) activity has been documented in patients with myocardial infarction (8). While the serum level of PONA in a given individual is relatively stable over time, the enzymatic activity of PONA varies among individuals by 10–40-fold (9, 10). The PONA enzymatic activity polymorphism is substrate dependent and varies among population with different ethnic background (11, 12, 13). The genetic basis of the inter-individual variability of PONA activity has recently been attributed to the presence of an A ⇒ G polymorphism in the coding region of the gene (*HUMPONA*) coding for this enzyme (10, 11, 12). The A/G polymorphism corresponds to glutamine (Gln)/arginine (Arg) polymorphism at amino acid position 192 (10–12). Individuals homozygous for Arg at position 192 (B genotype) show a significantly higher serum PONA activity than those homozygous for Gln (A genotype) (11). Thus, given the potential role of PONA in atherosclerosis and the presence a functionally

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1. *Abbreviations used in this paper:* Arg, arginine; CAD, coronary artery disease; Gln, glutamine; HUMPONA, human paraoxonase/arylesterase gene; OR, odds ratio; PONA, paraoxonase/arylesterase; PTCA, percutaneous transluminal coronary angioplasty.

significant genetic polymorphism, we determined the *HUMPONA* genotypes and alleles in 223 patients with angiographically documented CAD and compared them with those in 247 healthy individuals in US population.

Methods

Study population

Patients with CAD. The study population was comprised of 223 adult Caucasian patients who had angiographically documented coronary artery narrowing exceeding 75% luminal diameter who underwent percutaneous transluminal coronary angioplasty (PTCA) at The Methodist Hospital, Baylor College of Medicine, Houston, Texas. Conventional risk factors for CAD such as family history of CAD, history of hypertension, diabetes mellitus, hypercholesterolemia, and smoking were obtained from the patients or their medical records.

In 98 patients a follow-up coronary angiogram was performed at least three months after angioplasty due to recurring symptoms, or the presence of an abnormal stress test. The availability of follow-up angiograms provided the opportunity to identify those patients who had developed restenosis and to determine their *HUMPONA* genotypes. Restenosis was defined by the presence of 50% or greater luminal diameter narrowing at the site of the previous angioplasty.

General population. HUMPONA genotypes were determined in 247 (104 male and 143 female) healthy Caucasian individuals in the general population of US. These individuals had no history of CAD, and had a normal electrocardiogram and echocardiogram.

Collection of blood and DNA extraction

The techniques used for extraction of DNA are conventional and have been previously published (14).

HUMPONA genotyping. Genotyping was performed by laboratory personnel who had no knowledge of the angiographic data. *HUMPONA* genotypes were determined using PCR and restriction mapping with Alw1 as per previously published protocols (10). In brief, a set of primers was designed to encompass the polymorphic region in the *HUMPONA* gene (sense primer 5'TATTGTTGCTGTGGGACCTGAG3' and antisense primer 5'CACGCTAAACCCAAATACATCTC3'). The PCR reaction contained 100 ηg of DNA template, 0.100 μM of each primer, 200 μM of 4dNTPs, 1 unit of Taq DNA polymerase, and 1.5 mM $MgCl_2$. DNA was amplified for 40 cycles, each cycle comprised of denaturation at 94°C for 1 min, annealing at 61°C for 45 s, extension at 72°C for 45 s with a final extension time of 5 min. The PCR products were digested with 8 units of AlwI restriction endonuclease per manufacturer's recommendation. The digested products were separated by electrophoresis on 3.5% agarose gel and identified by ethidium bromide staining.

Statistical analysis

Chi-square test for independence, and Fisher's exact test were performed to compare the distribution of *HUMPONA* genotypes and alleles frequency, respectively. ANOVA was used to compare the mean values of variables among groups. A two-tailed P value of < 0.05 was considered to indicate an association between variables.

Results

Study patients. A total of 223 patients with CAD and 247 healthy individuals in US general population were enrolled in the study. One hundred fifty eight patients with CAD (71%) and one hundred four individuals in the general population (47%) were male. 60% of all patients with CAD (134/223) had history of myocardial infarction. The mean luminal stenosis pre-PTCA was 89.7±12. 85 patients (38%) had a family history of CAD, 88 patients (39%) had history of hypercholesterolemia, 101 patients (45%) had hypertension, and 47 (21%) had diabetes mellitus.

Table I. Humpona Genotypes and the Frequency of Alleles

	Control	CAD
Genotypes	(n = 247)	(n = 223)
A	120 (49%)	68 (30%)
A/B	99 (40%)	115 (52%)
B	28 (11%)	40 (18%)
	X^2 = 16.5	
	P = 0.0003	
Alleles		
A (Gln)	0.69	0.56
G (Arg)	0.31	0.44
	OR = 1.7 (95% CI: 1.3–2.2)	
	P = 0.0001	

HUMPONA genotypes and CAD. The genotype of each individual was established with gel electrophoresis of the PCR product following digestion with Alw1 restriction endonuclease per previously published protocol (10). Each genotype was read by two individuals independently. The amplified PCR product was 99 bp. The A ⇒ G (Gln ⇒ Arg) transition creates a unique AlwI site in the amplified fragment. Individuals homozygous for Gln/Gln showed only the presence of 99-bp product and those homozygous for Arg/Arg showed 69- and 30-bp products. Heterozygous individuals showed the 90-, 69-, and 30-bp products.

The distribution of *HUMPONA* genotypes and the frequency of alleles in patients with CAD and in the control group are shown in Table I. The A/B (Gln/Arg) genotype was the most common in patients with CAD (115/223). In contrast, the A genotype was present in the majority of the controls (120/247). Sixty-eight patients with CAD and 120 individuals in the control group had A genotype ($P < 0.0001$, odds ratio = 2.2, 95% confidence interval 1.5–3.2). The frequency of G allele was also significantly higher in patients with CAD as compared with that in the general population (0.44 vs. 0.31, respectively, $P = 0.0001$).

There were no gender-dependent differences in the distribution of *HUMPONA* genotypes neither in patients with CAD or in the control group. Analysis of *HUMPONA* genotypes in patients with CAD showed no association between a particular genotype and the severity of pre-PTCA mean luminal diameter stenosis, history of myocardial infarction, a family history of CAD, or presence of any of the conventional risk factors for CAD (Table II). The distribution of genotypes in patients with myocardial infarction were significantly different as compared with controls ($x^2 = 14.7$, $P = 0.0006$).

Paraoxonase genotype A has been shown to be a risk factor for CAD in patients with diabetes mellitus (15). Therefore, in order to determine whether paraoxonase genotypes were risk factor for CAD in patients without diabetes mellitus, the data was reanalyzed following exclusion of 47 patients with diabetes mellitus. In the remaining 176 the distribution of A, A/B, and B genotypes were 54 (31%), 94 (53%), and 28 (16%), respectively ($\chi^2 = 13.6$, $P = 0.0011$ as compared with controls). The frequency of A and B were 0.57 and 0.43 in nondiabetic pa-

Table II. Humpona Genotypes in Patients with CAD

n = 223	A n = 68 (30%)	A/B n = 115 (52%)	B n = 40 (18%)
Male (n = 158)	46	81	31
Female (n = 65)	22	34	9
Age (mean±SD)	65.1±10	62.6±11	64±10
Pre-PTCA Stenosis (mean±SD)	90.4±8	88.5±15	91.6±7
Restenosis (n = 68)	19 (28)	37 (54)	12 (18)
No Restenosis (n = 30)	7 (23)	15 (50)	8 (27)
Family History (n = 85)	25 (29)	43 (50)	18 (21)
Myocardial Infarction (n = 134)	38 (28)	76 (57)	20 (15)*
Hypercholesterolemia (n = 88)	20 (23)	54 (61)	14 (16)
Hypertension (n = 101)	27 (27)	50 (49)	24 (24)
Diabetes (n = 47)	14 (30)	21 (45)	12 (25)
Smoking (n = 97)	29 (30)	52 (54)	16 (16)

None of subgroups showed any significant difference when compared with the matching subgroup without the variable. * x^2 = 14.7, P = 0.0006 compared with controls.

tients with CAD, respectively (P = 0.0005, OR = 1.6, 95% CI 1.1–2.2).

68 patients had developed restenosis, 30 patients had no significant restenosis on follow-up angiography. The distribution of *HUMPONA* genotypes and the frequency of alleles in patients with restenosis compared to those with no restenosis or in the control group was not significantly different (Table II).

Discussion

We performed an allelic association study, and determined the *HUMPONA* genotypes in 223 patients with angiographically documented CAD and 247 healthy Caucasian individuals in the US population. Our results showed an increased frequency of B and a decreased frequency of A genotypes in patients with CAD as compared to those frequencies in controls. This association was independent of gender and conventional risk factors for CAD. This is the first study to implicate *HUMPONA* gene variants as genetic risk factors for atherosclerotic CAD and the largest to determine the *HUMPONA* genotypes in white population of US.

The exact pathogenesis of atherosclerosis remains unknown. A number of risk factors such as smoking, high levels of LDL, low levels of HDL with hypertriglyceridemia, diabetes, hypertension and genetic factors are known to predispose to coronary atherosclerosis. Identification of the genetic risk factors is expected to enhance our understanding of the molecular basis for atherosclerosis, the leading cause of death in the industrialized world. Allelic association studies are commonly used to identify the susceptibility genes for complex traits such as atherosclerosis (3–5, 16–19). Using such approach, a number of gene variants as genetic risk factors for CAD have been identified including T235M variant of angiotensinogen, 4G variant (in the promoter) of plasminogen-activator inhibitor, and DD variant of angiotensin-1 converting enzyme genes, have been identified as genetic risk factors for CAD (4, 5, 18–20). We report, for the first time, that a variant of *HUMPONA* gene is also a genetic risk factor for CAD.

Association studies are subject to spurious results. Several factors such as the small number of study subjects, admixture of genetically and ethnically non-homogenous population, lack of an appropriate control group, and poorly defined phenotypes are likely to results in false associations. We took such potential flaws into consideration. The study was performed in a large number of genetically homogenous (white) patients with a well defined phenotype. Although no coronary angiography was performed in the control group, these individuals were asymptomatic and had a normal electrocardiogram and echocardiogram. It is possible that a certain number of the individuals in the control group had silent CAD. However, this could pose a potential problem in a negative association study and is unlikely to deter from our results showing a positive association. The observed frequency of *HUMPONA* alleles [A (Gln) = 0.69 and G (Arg) = 0.31] in the general population in this study is similar to the previously reported frequencies of PONA allozymes in unrelated Caucasians from US population pools (21). Furthermore, the distributions of *HUMPONA* genotypes in patients with CAD as well as in control groups were in Hardy-Weinberg equilibrium.

This study does not provide a mechanism by which variants of *HUMPONA* gene predispose to CAD. The *HUMPONA* gene is located on to chromosome 7q21–22 and codes for a protein of 355 amino acids (11). The protein contains two polymorphic sites; one at amino acid position 55 (Met/Leu) and the second at amino acid position 192 (Gln/Arg) (10–12). The latter polymorphism determines the activity of the enzyme (10–12). Arginine at position 192 (B genotype) confers high activity and glutamine (A genotype) low activity to PONA (10–12). PONA serum levels and enzymatic activity exhibits a weak correlation with conventional lipid risk factors such as total cholesterol, triglycerides and apo A-1 levels (22). PONA also has been implicated in the prevention of LDL oxidation in in vitro studies (6, 7). Oxidized LDL is cytotoxic and mitogenic agent that activates expression of acetyl-LDL or scavenger receptors by monocyte-derived macrophages. However, the observed increase in the frequency of the more active enzyme (B genotype) and the decrease in the frequency of the less active enzyme (A genotype) in patients with CAD indicate that PONA is involved in atherosclerosis through a mechanism(s) other than lipid oxidation. The oxidized LDL has also been implicated in the development of restenosis following PTCA (23). Therefore, we determined the frequency of *HUMPONA* genotypes in 68 patients with restenosis. We found no significant differences in the distribution of genotypes and frequency of alleles in patients with restenosis, those without restenosis and in patients with CAD.

In summary, we have identified a new genetic risk factor for CAD. Our results show that variants of *HUMPONA* gene are involved in predisposition to coronary atherosclerosis. The G allele (Arg) is more common and A allele (Gln) is less common in patients with CAD. Further studies are needed to characterize the molecular mechanism(s) by which paraoxonase/arylesterase enzyme is involved in atherosclerosis.

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