Haptoglobin genotypic distribution
in Iranian patients with coronary heart disease

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ABSTRACT

Haptoglobin is a plasma protein with hemoglobin binding capacity. Haptoglobin has important biological functions such as binding to free hemoglobin and removes it from the circulation, thus preventing iron loss and kidney damage during intravascular hemolysis, superior antioxidant capacity, protection against free radicals. Studies on the distribution of Hp show that the gene frequencies of Hp dependent on geographical and genetic family. In the other, several authors have showed the correlation between HP types and different diseases, such as inflammation, infection, cardiovascular diseases and malignant tumors.Smoking, hypertention,diabetes mellitus and serum lipid concentrations are risk factors for developing cardiovascular diseases. In addition, Hp polymorphism has been proposed as a risk factor for developing atherosclerotic vasculare disease. In this study, the association between Haptoglobin genotypic distribution and the incidence of coronary heart disease investigated. 50 Iranian patients with coronary heart disease were randomly selected. Genomic DNA extracted from peripheral blood leukocytes. In PCRs with primers A and B, amplification products of 1757 and 3481 bp were amplified from genomic DNA containing alleles Hp^1 and Hp^2, respectively. In the population studied, the distribution of haptoglobin polymorphism was 36% (n = 18) for the Hp1-1 type, 62% (n =31) for the HP2-1 type, and 2% (n = 1) for the HP2-2 type. The trend in this study showing a lower frequency of 3-vessel disease and less severe coronary artery stenosis in patients with the HP1-1 phenotype than in patients with the HP2-1 phenotypes may be indicative of a protective effect of the HP1-1 phenotype against the development of atherosclerotic coronary artery disease.

Keywords: Haptoglobin; Cardiovascular; coronary heart disease

INTRODUCTION

The importance of oxidative stress and reactive oxygen intermediates in the atherosclerotic lesions in humans and animals has been well-described [1-9]. Accordingly, differences between persons in their level of antioxidant protection may influence their risk of developing restenosis. Hemoglobin is an important mediator of oxidative tissue damage that is released from red blood cells at sites of vascular injury. Haptoglobin (Hp) is a serum protein that serves as an antioxidant by virtue of its ability to bind to hemoglobin and prevent hemoglobin-mediated oxidative tissue damage [10,11]. The immunological and antioxidant capacities of haptoglobin point towards a possible role in the pathogenesis of atherosclerosis [12]. In humans, HP is characterized by a genetic polymorphism of the α - chain, with three major phenotypes: HP1-1, HP2-2 and HP2-1, which are the expression of two alleles (HP1 and HP2) on chromosome 16q22.1 [9]. Haptoglobin contains β- (40 kDa) and α- (α_1 = 8.9 kDa and α_2 = 16KDa) chains. The β- chains are identical in all, with variations dependent on different α- chains. Hp1-1 expresses only the α_1-chain and is the smallest form (86 KDa). Hp2-1 and Hp2-2 express α_2- chains, which can form polymers of 86-300 KDa (Hp2-1) and up to 900 KDa (Hp2-2) [14]. The strength of several Hp functional properties differs depending on the

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specific genotype. Hp1-1 has a strong capacity for hemoglobin binding and antioxidative function. Hp2-2 functions weakly in hemoglobin binding and antioxidative capacity. The Hp2-1 heterozygot is a functional intermediate [15].

This functional allelic polymorphism in the haptoglobin gene, may determine susceptibility to a wide variety of vascular disorders associated with an increase in oxidative stress [15-19]. In this study we characterized the frequency of Haptoglobin phenotype in Iranian patients with coronary heart disease.

MATERIALS AND METHODS

Blood samples obtained from 50 adult patients with coronary heart disease (male, age: 45-70). Specimens were collected in tubes containing EDTA. Data on smoking habits, medical history and level of physical activity collected by self-administered questionnaires. The Hp 1- and Hp 2-specific sequences are contained in the EMBL/GenBank Data Libraries under accession numbers AC004682 and M69197, respectively (19,20). According to the sequence present in AC004682, the Hp 1-specific DNA region has a length of 1711 bp; it extends, in an inverse orientation, from nucleotide position 188616 to nucleotide position 186906. In the sequence present in M69197, the 3435-bp Hp 2-specific DNA segment starts at position 2804 and ends at position 6238; it contains two units of similar sequences, consisting of 1724 and 1711 bp. The sequences of the 1711-bp segments present in AC004682 and M69197 are complementary with the exception of one divergence located at position 188141 in AC004682, which corresponds to position 5003 in M69197. DNA was extracted from peripheral bloods by sodium per chlorate method have set up in my laboratory. Briefly, 500 μl of blood sample was suspended with 1 ml of lysis buffer (10mM Tris-HCl pH 8, 0.1mM EDTA, 0.15M NaCl, 0.5% Triton X-100,) and centrifuge for 5 min at 8000 rpm at 4 °C and removed supernatant. 100 μl sodium per chlorate (4M), 10 μl SDS (10%) and 400 μl TE buffer were added to Pellete. 100 μl of 5N NaCl was added to the reaction, vortexed and centrifuged at 12000 RPM for 10 min. supernatant was transferred to a new micotube and DNA was precipitated by alcohol

and dissolved in 100 μl dH2O. Oligonucleotide primers A (5-GAGGGGAGCTTGCCTTTCCATTG-3) and B (5-GAGATTTTTGAGCCCTGGCTGGT-3) were used for amplification of a 1757-bp Hp 1 allele-specific sequence and a 3481-bp Hp 2 allele-specific sequence. Primers were synthesized by Applied Biosystems. In Hp 1 and Hp 2, the annealing sites for primer A are located [immediately] upstream of the 1711-bp unit and the 1724-bp unit, respectively.

The nucleotide at the 5' end of primer A corresponds to position 188639 in AC004682 (Hp 1) and position 2781 in M69197 (Hp 2). Primer B has binding sites just downstream of the 1711-bp elements of Hp 1 and Hp 2. The nucleotide at the 5' end of primer B corresponds to position 186883 in AC004682 (Hp 1) and position 6261 in M69197 (Hp 2). Depending on the genotype represented by the template DNA, a Hp 1-specific product of 1757- bp and/or a Hp 2-specific product of 3481-bp is generated in PCRs with primers A and B. The 50-μL reactions contained 1 U of *Taq* polymerase (SinnGen), 100–1000 ng of DNA, and 200 μM each of dATP, dCTP, dGTP, and dTTP (Invitrogen); PCR buffer was used as suggested by the supplier (SinnGen) with no supplements added. After initial denaturation at 94 °C for 5 min, the two-step thermocycling procedure consisted of denaturation at 94 °C for 1 min and annealing and extension at 66 °C for 1 min (in the presence of primers A and B), repeated for 30 cycles, and followed by a final extension at 72 °C for 5 min. The thermocyclers used were Corbet PCR systems (Applied Biosystems). For genotype assignments, the PCR products were separated in 0.8% agarose gels. Genotype determinations were done without knowledge of the phenotyping results.

RESULTS

In PCRs with primers A and B, amplification products of 1757 and 3481 bp were amplified from genomic DNA containing alleles Hp1 and Hp2, respectively. After electrophoresis of the reaction products in 0.8% agarose gels, Hp genotype-specific banding patterns were obtained: genotypes Hp 1-1 and Hp 2-2 were characterized by single bands representing the 1757- and 3481-

bp products, respectively, and genotype Hp 2-1 was characterized by the presence of both the 1757- and 3481-bp products (fig. 1).With the heterozygous genotype Hp2-1, the HP1-specific 1757-bp band was considerably more intense than the Hp2-specific 3481-bp band (fig. 1, lane 4). The haptoglobin genotypes of 50 nondiabetic cardiovascular patients were determined with genomic DNA prepared from blood samples. In the population studied, the distribution of haptoglobin polymorphism was 36% (n = 18) for the Hp1-1 type, 62% (n =31) for the Hp2-1 type, and 2% (n = 1) for the Hp2-2 type.

There was a statistically significant difference in the distribution of the 3 Hp types between nondiabetic cardiovascular patients (P<0.001).There was 49 of the 50 patients with the Hp-1 or 1-1 (98 %) but only one patients with the Hp2-2, for this reason we eliminated Hp2-2 type from analysis. As given in Table 1, there were no significant differences in age, lifestyle characteristics, body mass index and smoking between these two groups. The distribution of the Hp phenotypes tested against Hardy-Weinberg equilibrium according to $\chi 2$ test [27]. Among the two groups Hp1-1 and Hp2-1, the equality of distributions of continuous variables was statistically evaluated according to the Kruskal–Wallis test, while proportions were compared using Fisher's exact test. In this matched case-control study, the association between the Hp polymorphism and mortality from CHD, independently of classical coronary risk factors, was modeled using conditional logistic regression for matched sets [29].

Multivariately adjusted odds ratios were calculated together with their 95% confidence intervals. Statistical significance of the estimated regression coefficients judged according to the Wald $\chi 2$ statistic. The global level for statistical significance was taken as $\alpha = 0.05$.
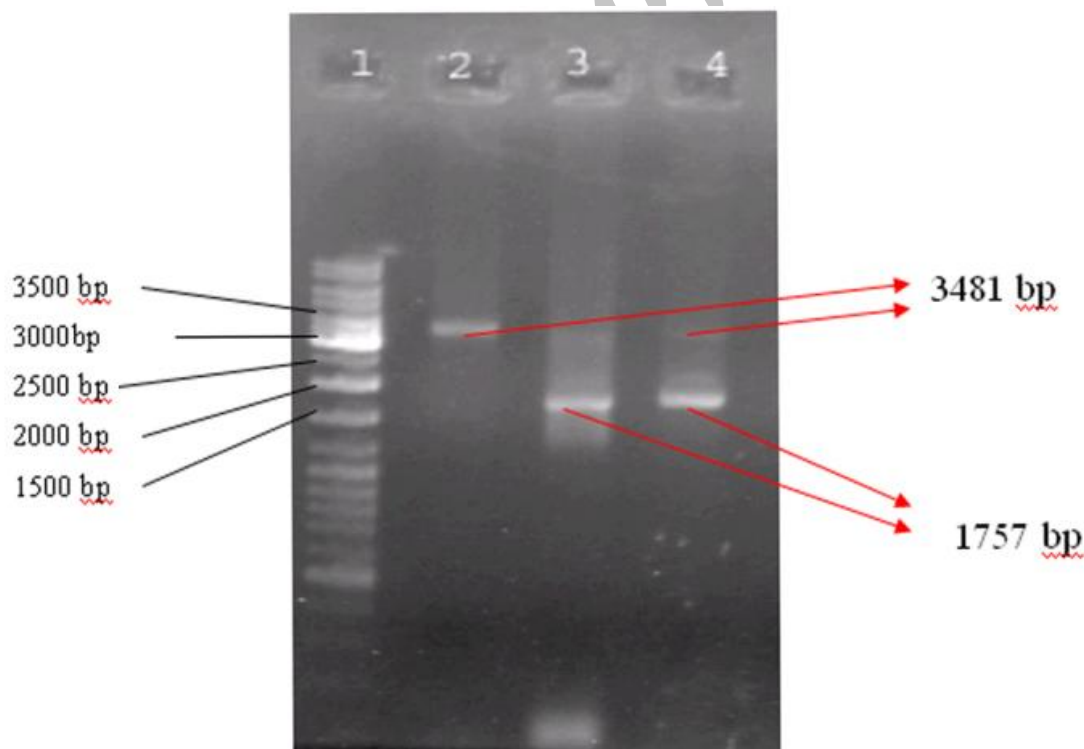

Figure 1 : Determination of Hp^1 and Hp^2 alleles with primers A and B, respectively. In a polymerase chain reaction with primers A and B, alleles Hp^1 and Hp^2 were characterised by amplification bands of 1757 and 3481 bp, respectively. lane1, DNA size marker; lane 2, DNA from an individual with the Hp^1Hp^1 genotype; Lanes 3, DNA from an individual with the Hp^2Hp^2 genotype;Lanes 4 DNA from an individual with the Hp^2Hp^1genotype

DISCUSSION

Haptoglobin has been demonstrated to have activities related to modulation of oxidative stress [10,11] and inflammation [21], phenomena that have been shown to be important in the restenotic process [1 – 9]. Haptoglobin is an antioxidant by virtue of its ability to bind to hemoglobin and prevent hemoglobin-mediated oxidative injury [10,11]. The different haptoglobin phenotypes appear to differ in their antioxidant capacity, with the Hp1-1 protein being the most superior antioxidant [21]. Moreover, the ability of the different haptoglobin types to enter the extravascular space, where they are needed to neutralize hemoglobin deposited at sites of vascular injury, are different [21,22].Hp1-1 protein, a dimer of 86 kd is better able to sieve into the extravascular space than Hp2-1 protein, which is a liner polymer with an effective molecular weight of 86 to 300 kd. Haptoglobin has also been demonstrated to play a role as an immunomodulator that may be related to its role in hemoglobin metabolism. A specific receptor for the haptoglobin-hemoglobin complex has been identified on monocyte/macrophages as CD163, a member of the group B scavenger receptor cysteinerich superfamily. CD163 binds the complexes of hemoglobin and multimeric Hp2-1 or 2-2 stronger as compared to dimeric Hp1-1 [23]. Ligand binding to CD163 has been shown to induce a tyrosine kinase-dependent signal cascade, resulting in secretion of a number of inflammatory cytokines [24].

According to our results, because there are no significant differences between coronary risk factors and haptoglobin phenotypes, it seems that the mechanism by which haptoglobin phenotype is related to coronary heart disease, is different from the mechanism by which the risk factors cause cardiovascular disease. The trend in this study showing a lower frequency of 3-vessel disease and less severe coronary artery stenosis in patients with the Hp1-1 phenotype than in patients with the Hp2-1 phenotypes may be indicative of a protective effect of the Hp1-1 phenotype against the development of atherosclerotic coronary artery disease [25]. Indeed, it has been demonstrated in a prospective longitudinal population study using the Strong Heart Study [26] cohort that patients with the Hp2-2 and 2-1 phenotypes are at significantly greater risk of developing coronary heart disease than patients with the Hp1-1 phenotype [15].

Table 1: Characteristics of the study sample by Hp phenotype.

	Hp1-1 (n=18)	Hp2-1 (n=31)	Significance
Age (years, mean, S.D.)	58.27 (11.8)	58.22 (10.58)	0.98
BMI (kg/m^2, mean, S.D.)	29.10 (2.27)	30.52 (2.97)	0.066
Obesity (% yes, n)	27.8 (5)	51.6 (16)	0.1
Smoking (% current, n)	55.6 (10)	48.4 (15)	0.62
Clostrol (mg/dl ,mean, S.D.)	232.06 (58.55)	231.48 (36.95)	0.97
LDL (mg/dl, mean, S.D.)	145.61 (44.11)	140.55 (34.18)	0.65
HDL (mg/dl, mean, S.D.)	35.94 (10.26)	37.74 (10.26)	0.55
Cystolic pressure (mmHg, mean, S.D.)	142.94 (31.06)	126.71 (25.88)	0.055
Diastolic pressure (mmHg, mean, S.D.)	86.56 (12.99)	80.87 (13.08)	0.14

CONCLUSION

We performed haptoglobin genotyping by polymerase chain reaction (PCR) using allele-specific primer-pairs. The haptoglobin genotypes of 50 subjects were as follows: Hp1-1: 34%; Hp2-1: 62 %; Hp2-2: 4%; The results indicate that Hp polymorphism, in Iranian population, does not predispose to the occurrence of cardiovascular diseases. It seems that Hp1-1 and Hp2-1 are independent of risk factor.

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