Heme oxygenase-2 gene mutations and blood bilirubin level in Iranian patients with premature atherosclerosis

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Abstract
Heme oxygenase-2 (HO-2) is a critical antioxidative stress enzyme found in endothelial cells and adventitial nerves. This enzyme in conjunction with other HOs (1 and 3) metabolize heme molecule into ferrous iron, carbon monoxide (CO), and biliverdin which is further converted to bilirubin. Both biliverdin and bilirubin are potent antioxidants, reducing the risk of atherosclerosis. HO-2 also induces endothelial relaxation by synthesizing CO. This is the first study to evaluate the association of HO-2 gene mutation in patients affected with atherosclerosis. Blood samples from patients (n=137) and normal controls (n=100) were collected. Three pairs of primers were designed to amplify exons 2 to 4 related to human HO-2 gene. The PCR products were analyzed by SSCP and sequencing to find out mutations. Iron and bilirubins (Total, Direct and Indirect) levels were determined in patients and controls. Two nucleotide substitutions were found among 10% of patients, consisted of a newly reported transversion mutation, C to A substitution in codon A70D (GCC to GAC) (Ala to Asp) and a previously reported transition mutation, A to G substitution in codon K89E (AAG to GAG) (Leu to Glu). Significant associations were obtained between risk of atherosclerosis and A437G substitution in codon K89E of HO-2 gene ($P< 0.006$ and $\chi^2 >6.82$) and reduced level of total ($P< 0.016$ and $\chi^2 >6.01$), and indirect ($P< 0.016$ and $\chi^2 >5.99$) bilirubins with no significant association with serum iron and direct bilirubin. No significant associations were observed among C381A substitution in codon (A70D, $P< 0.11$ and $\chi^2 >2.97$), level of serum iron, bilirubin and risk of atherosclerosis. These findings indicate the importance of A437G substitution in the development of atherosclerosis. Further studies are required to study the association of HO-2 gene mutations with atherosclerosis in other populations.
Keywords: Atherosclerosis; Heme oxygenase-2; Bilirubin; CAD

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INTRODUCTION

Atherosclerosis is the first leading cause of global morbidity and mortality, with more than 17.5 million worldwide deaths attributable to the cardiovascular complications of this disease (Gaziano *et al*., 1995; WHO, 2007). The common risk factors for developing atherosclerosis such as smoking, type 2 diabetes, hypertension, and dyslipidemia, increase the production of reactive oxygen species (ROS) and damage the vascular wall (Ross 1999; Kim *et al*., 2006). The disturbed vasculature walls become sensitive to lipid deposition, thrombus induction, immune cell penetration, inflammation, and hemodynamic pressure. Hence, oxidant stress can disturb vascular homeostasis and leading to the development of atherogenesis. One reported marker of oxidantive stress is F_2-isoprostanes that formed by the free-radical and catalyzed peroxidation of phospholipid-bound arachidonic acid and released it into the circulation (Morrow *et al*., 1992; Awad *et al,*. 1993; Davi *et al*., 1999). Elevated levels of F_2-isoprostanes have been detected in patients with risk factors of coronary heart disease, including hyperlipi-

demia, diabetes mellitus, hypertension, tobacco use, and obesity (Stocker *et al*., 1987; Davi *et al*., 1997; Minuz *et al*., 2002).

To reduce oxidative stress, the expression of endogenous antioxidative stress proteins including heme oxygenase (HO) is increased. HO is a rate-limiting enzyme in the degradation of heme to biliverdin producing equimolar amounts of free iron and carbon monoxide, a potent anti-apoptotic and proliferation- -promoting compound (Kim *et al*., 2006). The reduction of biliverdin by biliverdin reductase leads to the generation of bilirubin, a potent endogenous antioxidant (Baranano *et al*., 2002; Ollinger *et al*., 2005). In fact, bilirubin has recently been shown to have an antiproliferative effect on vascular smooth muscle cells (Ollinger *et al*., 2005).

There are two isoforms of heme oxygenase, HO-1 and HO-2, have been identified in humans. HO-1 is the inducible isoform, which is up-regulated by oxidative stress, hypoxia, or its own substrate heme (Maines 1997; Otterbein *et al*., 2000), and is observed in atherosclerotic lesions, vascular smooth muscle cells, endothelial cells, and several other tissues (Wang *et al*., 1998). HO-2 has been shown by immunohistochemistry to be located in the endothelial cells and adventitial nerves of the blood vessels where carbon monoxide (CO) is synthesized (Zakhary *et al*., 1996). CO and nitric oxide (NO), may both have endothelial derived relaxing activity. There are several reports, which indicate that HO-2 has protective activity in the cardiovascular system. The antiatherogenic effect of HO-2 may be attributed to vasodilation properties of CO and antioxidant activity of bilirubin (Maines 1997; Siow *et al*., 1999; Namiki *et al*., 2002). This is the first study to analyze the relationship between heme oxygenase 2 gene mutations and premature arthrosclerosis. The aim of this study was to investigate the HO-2 gene polymorphisms and their effects on the incidence of atherosclerosis, and correlating them with the levels of blood iron and bilirubin.

MATERIALS AND METHODS

This study was carried out on 137 patients selected from 1850 cases referred to the cardiac center, at the Shaheed Rajaei Cardiovascular Medical and Research Center (Tehran, Iran), showing symptoms of myocardial infarction and unstable angina. These symptoms were confirmed by the presence of 1VD (Blockage of one main epicardial coronary artery) to 3VD atherosclerosis following angiography of males below the age of 51 and females under 56 years old. A normal control group consisting of 100 normal individuals (all of the same sex, and with similar ages) having normal angiography reports was also used in the study.

Coronary angiography: Angiograms were evaluated by an experienced angiographer unaware of the clinical data. Coronary arterial disease (CAD) was considered to be present when up to 70% blockage induced by stenotic lesions were observed in the major epicardial coronaries and their branches. Subjects with normal or near-normal arteries (no lesions greater than 30%) formed a control group.

All angiograms were scored according to the Friesinger index. This index ranges from 0 (completely normal arteries) to 15. Each of the 3 arteries (right coronary, circumflex and the anterior descending artery), including their major branches, was analyzed independently and scored from 0 to 5. The final score was the sum of the results for each artery. An artery with no wall irregularity was scored as 0. Score 1 was determined by parietal irregularities, less than 30%. If the artery had a single stenotic lesion causing a narrowing of less than 70%, the score was 2. The same degree of obstruction, but at more than one specific site of the artery, was scored as 3. An artery with any lesion greater than 70% was scored as 4. A score of 5 was assigned when complete occlusion of the proximal right coronary, circumflex or anterior descending artery was found. Lesions in the left main coronary were assessed using the same scoring system, but doubled (the lesion was considered in two arteries).

Sampling and DNA extraction: This study was approved by Ethics Committee at the Institute and consent forms were obtained from all participants. Five milliliters of the peripheral blood samples were taken and sera were then sent to the laboratory at the Shariati hospital, University of tehran, to determine the levels of serum iron and bilirubin (total, direct and indirect). Five milliliters of the peripheral blood samples were taken from the cases and the controls that had previously undergone selective coronary angiography. The standard salting-out DNA extraction procedure was used to extract DNA from the blood samples (Sambrook and Frisch, 1989).

Genetic analysis: Three pairs of primers were designed to amplify fragments related to exon2 SNP T51A, (ID 11542539), exon 3 SNPs K89N, (ID

11542540), R137Q, (ID 17884623) and P146L, (ID 17880805) and exon 4 P256 mutations that were observed in rats carrying the HO-2 gene (Table 1). The PCR thermal cycle was performed in 32 cycles. Each cycle consisted of denaturation at 95°C for 30 s, annealing at 62°C for 1 min followed by extension at 72°C for 30 ss, except for amplification of exon 4 annealing temperature that was carried out at 60°C. The thermal cycles were started with an initial denaturation at 95°C for 5 min, followed by a final extension at 72°C for 10 min. The amplified products were visualized following electrophoresis on 1.5% (w/v) agarose gel and ethidium bromide staining. Determination of the PCR products idendity from the three studied exons were approved by sequencing.

Single strand conformation polymorphism (SSCP) analysis was used to screen for unknown mutations because of its simplicity and widespread applicability (Hayashi, 1991). All PCR products from cases and controls were analyzed by SSCP polyacrylamide gel electrophoresis (SSCP-PAGE). For the SSCP analysis, 5 µl of the PCR products were mixed with 25 µl of SSCP loading dye (95% formamide, 100 mM NaOH, 0.025% bromphenol blue, 0.025% xylene cyanol). The samples were denatured at 94°C for 2 min and cooled on ice. A 20 µl aliquot of this mixture was loaded onto the polyacrylamide gels (acrylamide/bisacrylamide, 39:1). The amplified fragments from exon 2 with a size of 175 bp and exon 3 with a size of 297 bp were loaded onto 8% polyacrylamide, containing 5% (v/v) formimide. The PCR products relating to exon 4 with a size of 440 bp were loaded onto 6% (w/v) polyacrylamide gel containing 5% (v/v) glycerol. Electrophoresis was carried out at 40 V, 11 mA, and 4°C in 1X TBE buffer for 16 h. The gels were stained, using the standard silver staining procedure and then wrapped in cellophane for preservation. To identify mutations, all band shifts observed from normal bands during SSCP analysis were gel purified and subsequently sequenced. The SeqMan (DNASTAR) software was used to study their sequences homologies with each other and reference sequences from the Genbank database.

Table 1. Primer sequences used for amplification of exons 2, 3 and 4 of human heme oxygenase-2 gene.

primers	Forward	Reverse
HO-2	agggctaattgacacacaaa	gctgtttaaggtttgtgccc
HO-3	ccaaagatggctcagtcgat	cagccatagctgttcggaag
HO-4	atgtggtgtgctcaggcata	cttgaactgctgggcattgt

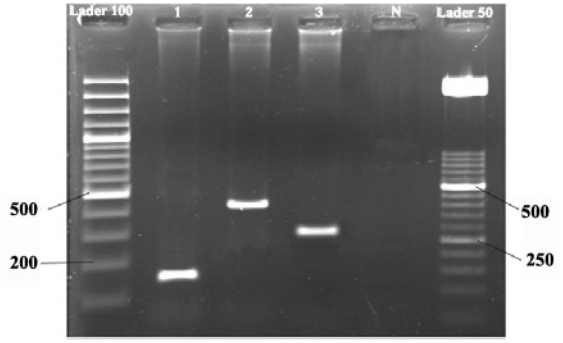

Figure 1. Amplified PCR products derived from exons 2-4. From left side, first lane is 100 bp size DNA marker ladder and last line is 50 bp ladder (Fermentas, Okrain), lane number 1 is 175 bp amplified product from exon 2, lane number 2 is 440 bp amplified product from exon 3 and lane number 3 represents the 297 bp product derived from exon 3.

Statistical analysis: Allele frequencies were calculated for each genotype by the allele counting method. Descriptive values were expressed as the mean ± standard deviation (SD). Comparisons of the case allele frequencies with those of the control groups were determined by the Pearson χ^2 test using the SPSS for windows version 11.2 (Chicago, Illinois) software. Differences between patients carrying a mutation in HO-2 and the control group were assessed by the student T test for continuous variables (iron and bilirubin). The Fisher exact test was used when the number of observations in any group was less than or equal to 5. All tests were two-tailed and $p<0.05$ was considered as a significant value.

RESULTS

From a total of 1850 patients with myocardial infarction and unstable angina who had undergone angiography, 137 patients with premature atherosclerosis and a genetic family history without other risk factors such as diabeties and hypercholesterolemia were selected. In this group, 27% were diagnosed with 3VD, 45% with 2VD and 28% with 1VD stenosis. More than 72% of the patients had coronary disease at least in two stenotic lesions >70% in a major epicardial coronary. Control individuals were chosen acording to their coronary angiograms results that were consisted of 100 subjects were devoid of stenotic lesions less than 30%, which indicated normal or near-normal arteries. The control group individuals were at the same sex and age with case group.

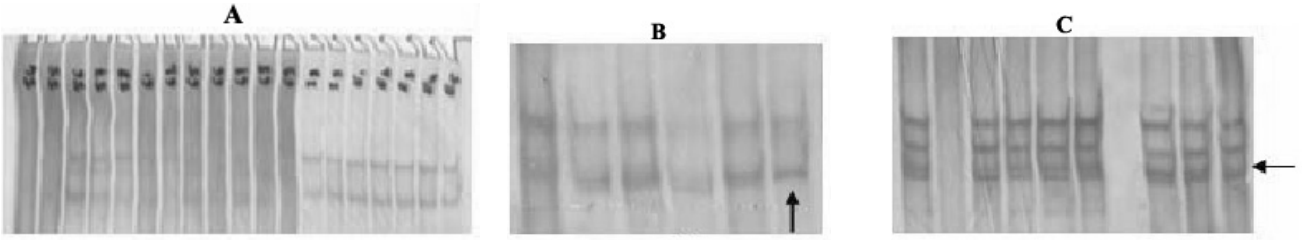

Figure 2. SSCP gel results for amplified fragments from exon 3. A: normal controls without shifted bands, B and C: are patients with shifted band polymorphisms (indicated by arrows).

Polymorphism in HO-2: The identity of the sequenced PCR products related to exons 2, 3, and 4 were confirmed by BLAST homology search (NCBI) (Fig. 1). All the sequences were shown to have homology with HO-2 relating to exon (NM 001127206). Sequencing results of the SSCP shifted bands presented no polymorphism relating to exon 2 and 4, but two mutations were found in exon 3 (Fig. 2) among 13 out of the 137 which had two kinds of substitution, C to A in codon A70D (C381A), and A to G substitution in codon K89E (A437G). The C381A substitution was reported for the first time in this study but the A437G substitution has already been reported in other populations (SNP databases in NCBI). The heterozygots, C381A mutation was found in 3.0% (4/137) and A437G substitution was observed in 6.5% (9/137) in studied Iranian cases. There was a significant association between A437G substitution ($P<0.01$) and presence of premature atherosclerosis among Iranian cases. No significant association was found for C381A substitution in codon A70D ($P>0.14$) among the Iranian patients.

HO-2 polymorphism and serum iron and bilirubin: The levels of serum iron and bilirubin (total, direct and indirect) were compared among CAD patients carrying the C381A and A437G substitutions and a subgroup of CAD patients without these substitutions. The proportions of iron, total bilirubin and indirect bilirubin were substantially lower among CAD patients carrying the C381A and A437G substitutions than those observed in other patients. The mean levels of iron 79.16. ± 30.12 < 80.97 ± 55.71, TBil 0.65 ± 0.05 < 0.72 ± 0.47, DBil 0.21 ± 0.06 < 0.22 ± 0.11 and IBil 0.43 ± 0.62 < 0.50 ± 0.39 were lower among patients with A437G than those of the control patients (Table 2). Statistical analysis of A437G, showed that the differences in the blood TBil (P= 0.016) and IBil (P= 0.016) levels between the two groups were significant but with no significant associations with the levels of serum iron (P> 0.10) and DBil (P> 0.24) (Table 2). The AG genotype was associated with lower levels of serum bilirubin when compared to the AA genotype. No significant associations were observed between C381A substitution in codon A70D (P< 0.11 and χ^2 >2.97), levels of serum iron (P> 0.57) and TBil (P> 0.50), DBil (P> 0.34) and IBil (P> 0.45), and the risk of atherosclerosis (Table 3).

Table 2. Human heme oxygenase 2 A437G polymorphism.

A437G	Alleles	Number of Patients	Mean±SD	*P*-value
Iron	G	6	79.16±30.12	0.10
	A	81	80.97±55.71	
TBil	G	6	0.65±0.05	0.016
	A	81	0.72±0.47	
DBil	G	6	0.21±0.06	0.249
	A	81	0.22±0.11	
IBil	G	6	0.43±0.62	0.016
	A	81	0.50±0.39	

Number of patients carrier for altered allele G and number of patients with wild type allele A and their levels of serum iron and bilirubins (total, direct and indirect).

Table 3. Human heme oxygenase 2 C381A polymorphism in exon 3.

C381A	Alleles	Number of Patient	Mean± SD	*P*-value
Iron	A	4	65± 69.75	0.57
	C	83	81.61±53.78	
TBil	A	4	0.52± 0.32	0.5
	C	83	0.73± 0.46	
DBil	A	4	0.16± 0.06	0.34
	C	83	0.22±0.11	
IBil	A	4	0.36± 0.25	0.45
	C	83	0.5±0.38	

Number of patients carries the altered allele A and number of patients with wild type allele C and their levels of serum iron and bilirubins (total, direct and indirect).

DISCUSSION

This is the first study which has investigated the relationship of the HO-2 gene polymorphisms as a genetic risk factor with the occurrence of premature atherosclerosis in CAD patients. From 137 CAD patients, 4 (3%) had transversion substitution C381A and 9 (6.5%) had transition substitution A437G, with 0.0% occurrence in the control group. The C381A mutation has been observed for the first time in this study, but the second mutation, A437G that had been reported previously was found to be associated with CAD ($P< 0.01$). This finding is important and shows the relationship of A437G substitution with arthrosclerosis in Iranian cases. The A437G substitution changes Lucine to the glutamine amino acid in the HO-2 protein and can reduce enzyme activity by salting bridge lost in the protein structure (Williams *et al*., 2004).

The data of this study are in line with several previous studies that have found reduced serum bilirubin concentrations in CAD patients (Ghem *et al*., 2010). Different circulating forms of bilirubin are powerful antioxidants and elevating physiological concentration of serum bilirubin may reduce the atherogenic risk (Wu *et al*., 1996). All forms of bilirubin have been noted to be effective in protecting LDL against peroxidation (Stocker *et al*., 1987; Neuzil and Stocker 1994). Bilirubin also appears to be cytoprotective to erythrocytes and ventricular myocytes when these cells are exposed to oxyradicals (Neuzil and Stocker, 1994).

When Bilirubin oxidized, it converts to biliverdin and in turn bilirubin is created by the activity of biliverdin reductase on biliverdin. This cycle not only demonstrates the potent antioxidant activity of bilirubin, also has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant **(**Baranano *et al*., 2002). Bilirubin, the metabolic end product of heme degradation by HO, has found as the most abundant endogenous antioxidant in mammalian tissues (Gopinathan *et al*., 1994) known as important endogenous anti-inflammatory and antioxidant molecule (Perlstein *et al*., 2008). The antioxidant and anti-inflammatory effects of bilirubin limiting the development of atherosclerotic vascular disease by limiting LDL oxidation, monocyte migration, vascular smooth muscle cell proliferation, vascular inflammation, vascular reactive oxygen species generation and endothelial dysfunction (Erdogan *et al*., 2006; Kawamura *et al*., 2005; Durante *et al*., 2002). These properties induce strong inverse association of bilirubin level with carotid atherosclerotic plaque and thrombus formation (True *et al*., 2007; Ishizaka *et al*., 2001).

HO-2 is part of the BK channel complex in the carotid body and acts as an oxygen sensor for the respiratory control of calcium-sensitive potassium (BK) channels. It enhances channel activity in normoxia that is dependent on HO-2 expression and is enhanced by HO-2 stimulation. The knockdown of HO-2 expression reduces channel activity. The presence of the A437G substitution can cause reduced enzyme activity and inhibition of BK channels during oxygen deprivation (Williams *et al*., 2004). The HO-2 enzyme causes an increase in the levels of deducing bilirubin and CO, hence, any decrease in protein activity may lead to reduction of bilirubin and CO in the carotid cells. In hypoxia, the presence of CO can also stimulate the BK channels and decreased CO due to heme oxygenase reduced activity may have a role in athrosclerosis as a primery cause in CAD patient (Williams *et al*., 2004).

It is also important to mention that CO, one of the end products of HO-2, also has an effective role in cardiovascular homeostasis (Anderson *et al*., 1989) and its imbalance due to HO-2 dysfunction may be important in the pathogenesis of vascular diseases (Lüscher 2000; Dijkhorst-Oei *et al*., 1999). An imbalance characterized by reduced nitric oxide production or increased production of reactive oxygen species may promote endothelial dysfunction (Lüscher 2000; Dijkhorst-Oei *et al*., 1999; Drexler and Hornig 1999).

The pathogenesis of atherosclerosis as well as the mechanisms underlying the ability of bilirubin concentrations to protect against atherosclerosis and CAD has not been well understood. This study showed the relationship between HO-2 mutations and reduction of bilirubin for the first time, underlying their importance risk factors for atherosclerosis. This study determined one of the potent genetic risk factors and its relationship with bilirubin concentrations and showed the significance of its synthetic pathway, which could be involved in the bilirubin protective action. The results of this study are in accordance with previous studies that suggest HO enzyme abnormalities appear to play a role in the pathogenesis of CAD patients. This research highlights the potential cardiovascular prognostic significance of the A437G substitution in the HO-2 gene of patients with coronary artery diseases in the Iranian population. It could be suggested that the HO-2 mutation may also play a role in other reported bilirubin related diseases, such as strokes (Todd *et al*., 2008).

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