Heme Oxigenase 2 Gene Polymorphisms as Genetic Risk Factor in Atherosclerosis in Iranian Patients

M Zamani¹, A Aleyasin¹*, H Fakhrzadeh², M Kiavar³, S Raoufzadeh¹, B Larijani², E Mahmoodi²

¹National Institute of Genetic Engineering and Biotechnology, ²Endocrine and Metabolism Research Center, Tehran University of Medical Sciences and Health Services, ³Shaheed Rajaee Cardiovascular Medical and Research Center, Tehran, Iran

Abstract

Background: Heme oxygenase 2 (HMOX2) is an important antioxidative stress enzyme found in the endothelial cells of blood vessels and adventitial nerves. This enzyme in collaboration with heme oxygenase 1 metabolizes heme molecules into ferrous iron, carbon monoxide (CO) and biliverdin while the later is further converted to bilirubin. Both biliverdin and bilirubin are potent antioxidants, reducing the chance of atherosclerosis. HMOX2 also induces endothelial relaxation by synthesizing CO.

Methods: Heme oxygenase 2 gene mutations were studied in 137 patients with atherosclerosis and in 100 normal controls. Pairs of primers were designed to amplify 2nd, 3rd and 5th exons of HMOX2 gene. These products were analyzed by single strand conformation polymorphism (SSCP) analysis and the shifted fragments were separated from SSCP polyacrylamide gel for further sequencing.

Results: Two sequence variations were observed among 13 patients with atherosclerosis, consisting of C to A substitution in codone A70D (GCC to GAC) which was reported for the first time and A to G substitution in codone K89E (AAG to GAG). A significant association was noticed between A to G mutation in codon K89E of hemoxygenase 2 gene and the risk of atherosclerosis was supported with $p=0.01$ and $\chi^2>6.82$. However, no significant associations were observed among C to A substitution in codon A70D, $p=0.11$ and $\chi^2>2.97$ and the risk of atherosclerosis.

Conclusion: Our findings denoted to the importance of K89E mutation in the development of atherosclerosis in Iranian cases. Further studies are required to show the importance of hemoxygenase 2 gene mutation in other populations.

Keywords: Atherosclerosis; Heme oxigenase 2; Coronary artery disease; Iran

Introduction

Atherosclerosis is a coronary artery disease (CAD) commonly initiated early in life and leading to myocardial infarction and stroke. It is still the leading cause of death in industrial and developed countries with environmental, life style and genetic risk factors. Smoking, high blood pressure, high level of cholesterol and oxidant agents are considered as the classical risk factors that account for 50% of CAD risks factors. Also, diabetes, hyperinsulinemia, low HDL cholesterol level, hypertriglyceridermia, obesity, central obesity and physical inactivity increase the risk for CAD. Genetic factor is one of the major risk factors of atherosclerosis. Heme oxygenase genes are among the genetic factors involved in the anti-atherogenic mechanism and play an important role in the defense against oxidative stress. Heme Oxigenase activities have been associated with vessel endothelial protection by degrading heme, acting as pro-oxidant into bioactive metabolites such as iron, carbon monoxide, and biliverdin while the later is then metabolized into bilirubin.
The antiatherogenic effect of Heme Oxygenase may be attributed to vasodilation properties of carbon monoxide and antioxidant activity of bilirubin.\textsuperscript{5,7} There are two isozymic forms of heme oxygenase in man, the first one is an inducible Heme Oxygenase-1 and the second is a constitutive Heme Oxygenase-2. Heme Oxygenase-2 has been shown by immunohistochemistry to be localized in the endothelial cells and adventitial nerves of the blood vessels where carbon monoxide is synthesized (CO).\textsuperscript{8} CO and nitric oxide (NO) may both have endothelial derived relaxing activity.

This study analyzes the relationship between heme oxygenase 2 gene mutations and premature atherosclerosis. In this way, common Heme Oxygenase-2 gene reported mutations (NCBI, Genbank) have been analyzed in exons 2, 3 and 5 in 137 male under 51 and female under 56 years of age patients affected with atherosclerosis and 100 normal controls after angiographic test.

**Materials and Methods**

This study was carried out on 137 patients selected from 1850 cases who referred to cardiac centers due to symptoms of myocardial infarction and unstable angina. The myocardial infarction or unstable angina was detected in 137 patients due to having 1VD to 3VD atherosclerosis in angiography in males under age 51 and female under age 56 years old. A group of 100 normal individuals with similar age and sexes was obtained with normal angiography reports as control group.

Angiograms were evaluated by an experienced angiographer who was blind to the clinical data. CAD was considered to be present when any stenotic lesion 70% in any of major epicardial coronaries and their branches was observed. Subjects with normal or near-normal arteries (no lesion greater than 30%) considered as a control group.

All angiograms were scored according to the Friesinger index ranging from 0 (completely normal arteries) to 15. Each of the 3 arteries (right coronary, circumflex and anterior descending artery) with 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 without any 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 regarded as 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 were doubled (the lesion was considered in two arteries).

This study was approved by Ethic Committee of the institute and consent forms were obtained from all participants. To investigate the Heme Oxygenase-2 gene polymorphisms and their effect on the incidence of atherosclerosis, five milliliters of the peripheral blood sample on EDTA anticoagulants were obtained from the cases and the controls that had previously undergone selective coronary angiography. Standard salting-out DNA extraction procedure was used to extract DNA from the collected blood samples.\textsuperscript{9} Three pairs of primers named HMOH2E2, HMOH2E3 and HMOH2E5 were designed to amplify fragments related to 2nd exon contained reported SNP T51A, (ID 11542539), for 3rd exon SNP K89N, (ID 11542540), SNP R137Q, (ID 17884623) and SNP P146L, (ID 17880805) and for 5th exon P256 mutation that observed in rats of Heme Oxygenase 2 gene. To amplify these three exons, the PCR thermal cycle was performed in 32 cycles. Each cycle consisted of 95°C denaturation for 30 seconds, 62°C annealing for 1 minute for HMOH2E2, HMOH2E3 primers and 60°C for HMOH2E5 primer and 72°C extension for 30 seconds. The thermal cycles were started with an initial denaturation of 95°C for 5 minutes, being followed by a final extension of 72°C for 10 minutes. The results were visualized, using the 1.5% agarose gel and ethidium bromide staining. At least one PCR products amplified by designed primers for three studied exons were subjected to sequencing to determine their identity.

Single Strand Conformation Polymorphism (SSCP) analysis was used to screen for unknown mutations because of its simplicity and widespread applicability. The technique relies on single-nucleotide variations, modifying the conformation of single-stranded DNA and its mobility in polyacrylamide gels.\textsuperscript{10} All PCR products from cases and controls were analyzed by SSCP Polyacrylamide Gel Electrophoresis (SSCP-PAGE). For the SSCP analysis, five microliters of PCR products were mixed with 25 microliters 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 into the polyacrylamide gels (acrylamide/bisacrylamide, 39:1). The amplified fragments from exon 2 with the length of 175 bp were loaded into a 8% polyacrylamide 5% formimide, those from exon 3 with the length of 297 bp into 8% polyacrylamide, 5% formimide and those from exon 5 with 440 bp into 6% polyacrylamide contained 5% glycerol. The electrophoresis was carried out at 40 V, 11 mA, and 4°C in 1X TBE for 16 hours. Gels were stained, using standard silver staining procedure and then were wrapped in cellophane for preservation.

To identify any mutation, all band shifts observed from normal bands in SSCP analysis were purified from the gels and underwent for sequencing. The SeqMan (DNASTAR) software was used to study their sequences homology to each other and Genbank database.

The Pearson $\chi^2$ test was used to determine the association between Heme oxygenase 2 gene polymorphisms and the genetic risk of atherosclerosis, using SPSS for windows version 12 (Chicago, Illinois) software. A $p$ value less than 0.05 was considered significant.

**Results**

From 1850 patients with myocardial infarction and unstable angina undergone angiography, 137 patients were selected with premature atherosclerosis with genetic family history without other risk factors such as diabetes and hypercholesterolemia. From them, 27% had 3VD, 45% with 2VD and 28% with 1VD. More than 72% of patients had coronary disease at least in two stenotic lesions >70% in a major epicardial coronary. Coronary angiograms showed 100 subjects without any stenotic lesion less than 30%, considered as normal or near-normal arteries. This group was considered as normal control individuals with similar age and sexes as patient group.

The identity of sequencing results obtained from PCR products related to exones 2, 3, and 4 were confirmed by BLAST homology search in NCBI (Figure 1). All sequences showed their homology to Heme Oxygenase-2 related exones (NM 001127206). Sequencing results of SSCP shifted bands presented no polymorphism related to exon 2 and 4 but two mutations were found in exon 3 (Figure 2) among 13/137 patients that had two kinds of substitution consisted of C to A substitution 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 A437G substitution has been reported in other populations. Heme Oxygenase-2 was found in the heterozygous form AC genotype in 3.0% (4/137) and AG genotype of A437G substitution in 6.5% (9/137) in 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 noticed for C381A substitution in codon A70D ($p>0.14$) among Iranian patients.

![Fig.1: PCR products amplified from exons 2-4.](image-url)
Discussion

This is the first study investigating the relationship between Heme Oxygenase-2 polymorphisms and occurrence of premature atherosclerosis in CAD patients. From 137 CAD patients, 13 had two mutations of C381A and A437G which were observed in 3% and 6.5% of patients respectively but without any occurrence in the control group. The first mutation of C381A has been reported for the first time in studied populations and second mutation of A437G has been reported for the first time to be significantly associated with CAD disease ($p<0.01$). This finding is important and shows the relationship of this mutation with artherosclerosis in Iranian cases. The A437G substitution can reduce enzyme activity by structural salt- ing bridge lost in the protein but no structural changes have already been reported for C381A substitution (SNP database).

Heme Oxygenase-2 is part of the calcium-sensitive potassium (BK) complex channel in carotid body and acts as oxygen sensor for respiratory control of BK channels. It enhances channel activity in normoxia which is dependent on Heme Oxygenase-2 expression and was enhanced by Heme Oxygenase-2 stimulation. Knockdown of Heme Oxygenase-2 expression reduced channel activity. Presence of the A437G substitution can cause reduced enzyme activity and inhibition of BK channels during oxygen deprivation. The Heme Oxygenase-2 causes an increase in the level of deducing bilirubin and CO, hence any decrease in protein activity may lead to reduction of bilirubin and CO in carotids cells. In hypoxia, presence of CO also can stimulate the BK channels and decrease CO due to Heme Oxygenase-2 reduced activity that may have a role in atherosclerosis as primary cause in CAD patients.

Our findings denoted to the importance of K89E mutation in the development of atherosclerosis in Iranian cases. Further studies are required to show the importance of Heme Oxygenase-2 gene mutation in other populations.

Acknowledgement

This study was supported by a grant from National Institute for Genetic Engineering and Biotechnology (NIGEB) and Ministry of Science, Research and technology, Tehran, Iran; Endocrine & Metabolism Research Center, Tehran University of Medical Sciences and Health Services, and Shaheed Rajaei Cardiovascular Medical and Research Center Tehran, Iran.

Conflict of interest: None declared.

References


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


