Original article

“Role of HFE gene in coronary artery disease” – A study from India

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ABSTRACT

Introduction: Hereditary hemochromatosis (HH) is an autosomal recessive genetic disease of iron regulation associated with iron overload and failure of several organs. Whether mutations in the hemochromatosis (HFE) gene increase cardiovascular disease risk is still undetermined. In the present study, we aimed to analyse the influence of HFE C282Y gene mutation on the risk of development of coronary artery disease (CAD) in the north Indian population.

Materials and method: The study included hundred CAD patients who showed presence of greater than 50% luminal stenosis in at least one major coronary artery in angiography. Genomic DNA was extracted from peripheral blood leukocytes of CAD patients using a DNA extraction kit (Gene Aid, India) following the manufacturer's protocol. The HFE C282Y gene mutation was investigated by AS-PCR.

Results: The results showed that out of 100 CAD patients 19 patients were positive for HFE C282Y mutation and 81 patients were negative for mutation. We also evaluated HFE C282Y gene mutation frequency in CAD patients with respect to disease severity and we found there was no significant difference of mutation frequency observed with respect to disease severity in CAD patients cohort i.e., those with single coronary artery or those with 2/3 coronary arteries involvement.

Conclusion: This study concludes that C282Y mutation plays no significant role in coronary artery disease development or its severity. Thus, there is a need to study this mutation in a large cohort of samples so as to elucidate the exact role of C282Y mutation in CAD.

1. Introduction

Coronary artery disease (CAD) or atherosclerotic heart disease is caused by narrowing of lumen of coronary vessels due to atherosclerotic plaque formation.¹ The atheromatous plaques may take years or decades before they finally hinder blood flow in the coronary arteries. When these arteries do become clogged, the supply of oxygen and nutrients to the myocardium is reduced, which leads to angina or myocardial infarction.² Coronary artery disease remains the leading cause of morbimortality and disability in high income countries and there is an alarming increase in countries with lower income economies.³

Hereditary hemochromatosis (HH) is an autosomal recessive genetic disease of iron regulation associated with iron overload and failure of several organs.⁴ The molecular basis of HH was elucidated in 1996 after the positional cloning of the involved gene, currently designated HFE (formerly HLA-H).⁵ HFE gene encodes human hemochromatosis protein (HFE), which is located on surface of primarily liver and intestinal cells

and regulates production of hepcidin protein - considered the master iron regulatory protein. HFE gene is located on short arm of chromosome 6 at location 6p21.3.⁶ Intracellular high levels of iron promote foam cell formation and atherosclerosis by adding to the oxidative burden, instrumental in LDL lipid peroxidation. Counterintuitively HFE gene mutations have not been associated with higher CAD risk in most of the studies.⁷

New frontiers of research in CAD are focussed on identifying these novel risk factors which promote and accelerate the atherosclerotic process and may account for the spiralling high incidence of CAD.² Identification of these risk factors may open new avenues for containment of this life threatening disease.⁸ With this aim in mind this study was planned to investigate the role of aberrations of HFE genes, which may have a bearing in the pathogenesis and progression of CAD.

Iron overload may not only happen only in homozygous carriers of the risk alleles. Multifarious heterozygotes and heterozygotes for the C282Y and H63D mutations also have an indirect elevation in serum

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iron, serum ferritin and transferrin saturation.^{7,9} These minor variations suggest that heterozygotes are more likely to have a slow accumulation of iron, which may lead to pathology later in life. Iron deposition in the arterial wall accelerates low density lipoprotein cholesterol peroxidation and consequently causes the formation of atherosclerotic lesions.¹⁰ An association between coronary artery disease and iron deposition has been suggested for several years. Lee TS et al., demonstrated in an animal model of atherosclerosis that an iron deficient diet reduces the progression of atherosclerosis.¹¹ Epidemiological studies showed a decreased risk of myocardial infarction in blood donors and an association of high body iron with MI.¹² Heterozygosity for the C282Y mutation has been reported to be a risk factor for first MI in men and cerebrovascular death in women.⁷ In this study, we have examined the role of HFE C282Y mutation in pathogenesis and progression of CAD patients and analysed their influence on the risk of development of CAD.

2. Methodology

2.1. Study population

The present study was conducted on 100 north Indian consecutive patients aged 40–70 years who underwent elective coronary angiography and angioplasty (PTCA) at Gobind Ballabh Pant Hospital New Delhi, India from January 2016 to May 2017.

2.2. Inclusion criteria

Inclusion criteria for CAD patients were presence of greater than 50% luminal stenosis in at least one major coronary artery.

2.3. Clinical characteristics of CAD patients

Presence of major cardiovascular disease risk factors viz diabetes mellitus, hypertension, positive family history was noted in the cohort of CAD patients. Diabetes mellitus was defined as presence of active treatment with insulin/oral anti diabetic agent or those individuals on dietary control for diabetes for whom documentary proof of diabetic diagnosis as per American Diabetes Association criteria was confirmed. Hypertension was defined as a systolic blood pressure of ≥ 140 mm Hg and/or diastolic blood pressure of ≥ 90 mm Hg on at least 2 separate occasions or presence of antihypertensive treatment. The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India. Written informed consent was obtained from each patient and healthy volunteer.

2.4. Peripheral blood sample collection

A peripheral blood sample (5 ml) was collected in EDTA containing vials from both patients and control subjects. Out of 5 ml of peripheral blood, 3 ml blood sample was taken for the separation of blood plasma. Peripheral blood leucocytes were isolated using RBC lysis buffer. 300 μ L of a peripheral blood sample was diluted with 900 μ L of RBC lysis buffer and incubated for 20 min at room temperature. Centrifuged the RBC lysed blood suspension at 3500 RCF for 5 min, discarded the supernatant and stored at -80°C till analysis.

2.5. HFE gene mutation analysis

2.5.1. AS-PCR procedure

HFE was studied by AS-PCR technique. Briefly, genomic DNA was extracted from peripheral blood leukocytes of CAD patients using a DNA extraction kit (Gene Aid, India) following the manufacturer's protocol. DNA concentration was determined by measuring absorbance at 260 nm. The quality and integrity of DNA was determined by the A260/280 ratio. DNA quality was also checked by ethidium bromide stained 1% agarose gel electrophoresis. The HFE C282Y was analysed

by AS-PCR using HFE gene specific forward primer 1 (5' CTGGGTGCT CCACCTGAC 3') forward primer 2 (5' CTGGGTGCTCCACCTGAT 3') for more frequent and less frequent alleles respectively with a common reverse primer (5'TCGGGCCTTGAAGTAGAC 3') for PCR amplification. PCR was performed in 25 μ L of total reaction volume. The PCR protocol was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 59.2°C for 45 s, extension at 72°C for 90 s and a final extension step at 72°C for 5 min. The PCR amplicons were analysed on 2% agarose gel electrophoresis containing ethidium bromide showed amplified fragments of 324 bp size.

2.6. Statistical analysis

Compliance with Hardy-Weinberg equilibrium was ascertained in the control subjects by the chi square test. The chi-square/Fisher exact test was used for categorical variables. All statistical analyses were performed using Graph Pad Prism 7.03 software package. A p-value of < 0.05 was considered as significant.

3. Results

3.1. Study population

In this study, a total of 100 CAD patients from GB Pant hospital, New Delhi, India, 100 age and sex matched healthy controls subjects were included. The genotypic distributions for HFE C282Y gene mutation in the control subjects were in accordance with Hardy-Weinberg equilibrium. Of the 100 CAD patients 67 were aged ≤ 55 years while 33 were of more than 55 years age. Amongst the CAD patients 81 were male and 19 were female.

3.2. HFE gene mutation

A representative gel electrophoretic image of HFE C282Y by AS-PCR with amplicon 324bp and depicted in Fig. 1.

3.3. HFE C282Y mutation frequency in CAD

Of 100 CAD patients 19 patients were positive for HFE C282Y mutation and 81 patients were negative for mutation.

3.4. HFE C282Y gene mutation frequency in CAD patients with respect to disease severity

There was no significant difference of mutation frequency observed with respect to disease severity in CAD patients cohort i e, those with

L3: 100bp Ladder

L1: Mutation Positive

L2: Mutation Negative

Fig. 1. Gel electrophoretic image of HFE C282Y.

Table 1
HFE C282Y gene mutation in CAD patients with respect to disease severity.

HFE C282Y Mutation	Disease severity		p value
	1 vessel n = (62) n (%)	> 1 vessel n = (38) n (%)	
No Mutation	49(79)	32(84.21)	0.70
Mutation present	13(20.96)	6(15.78)

Fig. 2. HFE C282Y gene mutation in CAD patients with respect to disease severity.

single coronary artery or those with 2/3 coronary arteries involvement. Table (1, Fig. 2).

4. Discussion

Hereditary haemochromatosis is a common disease in Caucasians with an autosomal recessive inheritance pattern, with the prevalence of the C282Y mutation approximately ten times that of cystic fibrosis causing allele.⁴ In Irish population, the frequency of the C282Y allele has been calculated as 0.099 and H63D allele is 0.141.¹³ The prevalence of these mutations in non-white populations is much lower. A large population study in the US provided estimates for C282Y homozygosity in native Americans, Hispanics, African Americans, Pacific Islanders and Asians, that were at least one quarter of the frequency in the non-Hispanic whites.¹⁴ The allele frequencies for the H63D mutation are similar in haemochromatosis patients and the general population, however when extremely rare occurrence of the C282Y/H63D haplotype are taken into account, H63D is more predominant in haemochromatosis than in the general population.¹⁰ The presence of either C282Y or H63D alleles even in the heterozygous state is able to influence iron overload.¹⁵ Population studies have elucidated that compound heterozygosity for C282Y and H63D presents a higher risk for the development of HH than either mutation alone, and the extent of iron loading generally follows the trend C282Yhomozygotes > C282Y/H63D compound heterozygotes > H63D homozygotes > C282Y heterozygotes > H63D heterozygotes > wildtype.^{16,17}

The C282Y mutation in HFE gene has been shown to be the major reason behind hereditary haemochromatosis in the western population.⁵ In this study, we analysed the frequency of C282Y mutation in the HFE gene in 100 north Indian CAD patients whose coronary anatomy was defined by means of coronary angiography. We observed that 19% of CAD patients in our cohort were positive for C282Y mutation. It has been reported that heterozygotes for C282Y mutation have a slightly increased serum iron concentration, transferrin saturation and serum ferritin concentration.¹⁸ Some studies have suggested that there is an association between the C282Y mutation and development of CAD or MI.^{6,7} Further, heterozygous men for C282Y mutation have been found to have an increased risk for first MI.¹⁹ Besides, heterozygous women have been seen to have an increased risk for cardiovascular death.²⁰ In contrast, Battiloro E et al. found no relation of C282Y mutation and CAD in a case control study which compared 174 patients with angiographically confirmed CAD and healthy subjects.²¹ Moreover, there are

reports which suggest that C282Y mutation has no influence on the extent of CAD, represented by angiographic scores and stratification into single, double, or triple vessel involvement.⁷ The results of this study also go with support to reports which state that C282Y mutation does not play a significant role either in the development of CAD or its severity. These contradictions regarding the involvement of C282Y mutation in the development of CAD necessitate a study of C282Y mutation in a large cohort to establish the facts.

5. Conclusions

This study concludes that C282Y mutation plays no significant role in coronary artery disease development or its severity. Thus, there is a need to study this mutation in a large cohort of samples so as to elucidate the exact role of C282Y mutation in CAD.

Compliance with ethical standards

The study was conducted in accordance to GCP and ICMR guideline and approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India.

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Ethical approval

The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India.

Informed consent

Written informed consent was obtained from each patient and healthy volunteer.

Declaration of competing interest

There is no conflict of interest to declare in this study.

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