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Molecular Screening & Influence of Cyp2C9*2 Variant with HHC in CVD Patients

Mehak Maryyam , Khola Kiani, Maryam Bajwa

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ABSTRACT

Cardiovascular diseases are the number one cause of death globally. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. Different risk factors contribute to the development of CVDs, mainly high blood pressure, obesity, diabetes mellitus and physical inactivity. One of the risk factor is the elevated level of homocysteine in blood. Homocysteine accumulates in the body due to the improper working of biochemical transformation process, usually due to deficiency of an enzyme, MTHFR, caused by the mutation. Hyperhomocysteinemia (HHC) is a condition that leads to the development of many cardiovascular diseases due to its clot causing ability. For the metabolism of the drugs taken against cardiovascular diseases, CYP family works. One member of CYP2C family, CYP2C9, is responsible for metabolizing various anticoagulants (such as Warfarin), that are prescribed for thrombophilic condition in HHC patients. In current study it is hypothesized that metabolism by CYP2C9*2 variant of CYP2C9, may have association with elevated homocysteine level in cardiovascular patients. To analyze the association of CYP2C9*2 variant of CYP2C9 family with Hyperhomocysteinemia, DNA was extracted from blood samples. PCR, RFLP, was performed on selected HHC samples with AvaII restriction enzyme and analyzed on gel documentation system. Among the three genotypes, CC, CT and TT, only CC and CT were found in this study. . Calculated P value was 0.924 that was greater than standard P value (P = 0.05), indicating no significant association between CYP2C9*2 and Hyperhomocysteinemia. BMI was found to be a major risk factor associated with CVD's (Mean BMI = 36.39). Allelic frequency of C was 0.86% and frequency of T allele was 0.13% in HHC patients. On the basis of significant results, we may say that in our selected population, no association of CYP2C9*2 variant with elevated homocysteine level in patients with cardiovascular diseases be existent. For further studies, any other variant of CYP2C9 may be analyzed for an association with elevated homocysteine.

Key words: CYP2C9*2 variant, Hyperhomocysteinemia, MTHFR, Cardiovascular disease

Introduction

Any type of disease affecting the cardiovascular system refers to cardiovascular disease Eighty percent of deaths occur due to CVDs and it is bearing 86% of the global burden in the developing countries. Utmost cause of cardiovascular diseases is Hypertension among various causes. There are many other factors that contribute to the development of cardiovascular diseases including high blood pressure, tobacco use, obesity, age, heredity or family history and elevated level of homocysteine. An amino acid, homocysteine, formed as a result of metabolism of methionine. Insufficiency of an enzyme, methylene tetra hydro folate reductase (MTHFR) is a major cause of mildly to moderately high levels of plasma homocysteine (1). Insufficient MTHFR causes variation in MTHFR gene at position 677 where Cytosine is substituted by Thymidine (677C>T). High level of homocysteine is a key risk factor for peripheral arterial and cardiovascular diseases (2, 3, 4). Members of CYP family metabolize the drugs taken against cardiovascular diseases. The cytochrome P450 system (CYP) is involved in the metabolism of various synthetic and naturally occurring compounds. Cytochrome p450 enzymes are monooxygenases, involved in the synthesis and degradation pathways of steroids hormones and in the metabolism of a number of other indigenous compounds, including fatty acids, prostaglandins and leukotriene. (5). The P450 enzymes are encoded by the CYP genes superfamily, which comprises at least ten families in mammals. The largest of these, the CYP2 family, can be further divided into six super families in mammals (6). The CYP2 family is large and complex (7). Location of CYP2C9 gene is 10q24 (8), covering almost 55-kb of nine exons (Gene Bank accession numbers: L16877 toL16883) and encoding a protein of 490 amino acid residues (9). CYP2C9 is an iso-enzyme of the cytochrome P450 super family, involved in many processes including biotransformation and elimination of several common

drugs like anti-convulsants, anti-depressants, anticoagulants, hypoglycemic, antibacterial, the alkylating anticancer prodrugs cyclophosphamide and some nonsteroidal anti-inflammatory drugs. (10, 11, 12). Several single nucleotide polymorphisms of CYP2C9 has been recognized (13, 14). Three CYP2C9 alleles, CYP2C9*1, CYP2C9*2, and CYP2C9*3, are found in most ethnic populations. CYP2C9*1 is most common among these alleles and it is considered to be a wild type allele. The CYP2C9*2 is a result of single based substitution (430C>T) located in exon 3. The base changes at exon 7 causes following variations, CYP2C9*3 (1075 A>C), CYP2C9*4 (1076 T>C) and CYP2C9*5 (1080 C>G). The CYP2C9*2 and CYP2C9*3 encodes a protein with reduced enzymatic activity in vitro (15, 16). The genotype distributions vary with ethnic background among different individuals. The allelic frequencies of CYP2C9 differ among different ethnic groups (17, 18, 19). The CYP2C9*2 is more common in Caucasian (10-20%) than Asian (1-3%) or African (0-6%) populations (21). The activity and substrate specificity of CYP2C9 is affected by the change in amino acid sequence of CYP2C9. Previously, CYP2C9*1, CYP2C9*2 and CYP2C9*3 were identified in Caucasian population. The wild-type protein is encoded by CYP2C9*1 and C to T transition occur in CYP2C9*2, causes the replacement of cysteine by arginine at amino acid position 144. The CYP2C9*3 is defined by A>C nucleotide substitution that causes change of leucine by isoleucine at amino acid position 359.

1. Material and Methods

Subjects:

To analyze the association of CYP2C9*2 with hyperhomocysteinemia, blood samples were collected from selected Pakistani population. Total 100 hyperhomocysteinemic blood samples were collected from KRL General Hospital and forty blood samples were collected to be used as control in our study.

Methodology:

Genomic DNA from all the samples was extracted. CYP2C9*2 genotyping was done by polymerase chain reaction (PCR) and RFLP. For the identification of CYP2C9*2 variant, one forward primer (5' GGAGGATGGAAAACAGACTTA3') and one reverse primer (5' TGAGCTAACAACCAGGACTCAT3') was used.

The PCR amplification was carried out in a total reaction volume of 20ul that consisted 0f 2.5ul of 10X TBE buffer, 1ul Mgcl2, 1ul DNTP's, 0.2ul Taq DNA Polymerase enzyme 0.5ul of forward and 0.5ul of reverse primers, 3ul DNA template and 11.3ul of dH2O. The amplification steps for CYP2C9*2 was performed with an initial denaturation step at 95 _C for 4 min, followed by 35 cycles of 94 _C for 45sec, 61 _C for 45sec and 72 _C for 45sec, with a final extension step of 72 _C for 10 min.

PCR product was then digested with AvaII enzyme. Digestion mixture contained 0.5ul of AvaII enzyme, 10ulof PCR product; 3ul of 10X PCR buffer and 16.5ul d.H2O.AvaII enzyme cleaved the restriction site of amplified variants. RFLP was performed for 6 hours at 30°C. 3% agarose gel was prepared for the analysis of digested product.

Statistical analysis:

For statistical analysis, the significance of clinical items related to homocysteine level, level of folic acid, level of uric acid, systolic & diastolic blood pressures, age, waist, height, weight, BMI, hips and WHR were determined by Chi square tests. The Hardy-Weinberg test of genetic equilibrium was applied using the Chi square test to ensure that there was no significant difference between observed and expected genotype frequencies. A minimum level of statistical significance was considered at a p level of <0.05.

2. Results

In this study a total number of 100 HHC patients along with 40 controls were participated for analyzing the role of CYP2C9*2. CVD patients were evaluated for homocysteine level, level of folic acid, level of uric acid, systolic & diastolic blood pressures, age, waist, height, weight, BMI, hips and WHR. Averages of these parameters were calculated by using SPSS version 16.0.

PCR products were digested with Ava II enzyme. Enzyme cleaved on rs1799853 of CYP2C9*2 gene. Enzyme produced bands of three different sizes; 171bp, 224bp and 396bp. In current study, SNP of CYP2C9*2 was C>T in which C is wild type and T is polymorphic. CC genotype (homozygous major) produced two bands of 171bp and 224bp. CT genotype (heterozygous carrier) produced three bands of 171bp, 224bp and 396bp. TT genotype (homozygous minor) produced bands of 171bp. Gel picture showed that two genotypes CC and CT were present in our study group of HHC patients and controls. Therefore to analyze these genotypes SPSS software Version 16.0 was used. This tool was used to predict the significance of clinical parameters of HHC patients and measure the association of rs1799853 with HHC by finding genotypic and allelic frequencies. In this study, out of 100 samples 73 patients were found to be homozygous major (CC) while 27 patients were found to be heterozygous carriers (CT)

and no recessive homozygous minor (TT) were found. And all the control samples showed CC genotype, which is evident of the fact that CC was protective genotype. Hardy-Weinberg equilibrium gave frequencies of genotype CC 61.78% in HHC and 100% in controls which shows that , genotype CT 24.58% in HHC and 0% in controls and genotype TT 0% in HHC and 0% in controls, respectively. Frequency for C allele was found to be 0.86 in HHC patients and 1% in control subjects. Similarly frequency of T allele was found to be 0.13 and 0% in HHC patients and controls respectively. Statistical analysis that P value and Chi square value were found to be greater than significant level of P = 0.05 hence it has been concluded that, rs1799853 has no significant association with HHC in current study group. The SNP of CYP2C9*2 was analyzed by evaluating association with body mass index. BMI is a strong risk factor for HHC. In this study, values of BMI were different for all three genotypes. Mean BMI 37.79 and 30.7 for CC and CT genotypes respectively, showed significant results. Therefore, CYP2C9*2 may be associated with BMI in selected population.

3. Discussion

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular diseases. Patients with higher level of homocysteine in their blood may suffer from different mortal CVD's like ischemic stroke, coronary heart diseases, hypertensive heart disease, cardiomyopathy, rheumatic heart disease and many others. Cytochrome P450 system is a vital set of genes responsible for the regulation and metabolism of many biochemical pathways and have a remarkable role in the drug metabolism. CYP2C9 is a significant member of CYP family that is involved in metabolism of many drugs such as warfarin. Different polymorphic variants of CYP2C9 exist like CYP2C9*1, CYP2C9*2, some variants are poor metabolizers. Under these considerations, we performed this research study to investigate the association of CYP2C9*2 variant of CYP2C9 with elevated homocysteine level in cardiovascular patients.

In current study, CYP2C9*2 showed no significant association with HHC as the calculated P value was greater than 0.05. No association was found between CYP2C9 genotype and stroke etiology with P value of 0.8 that was greater than 0.05 (20). In a study, it was reported that CYP2C9*3 is prominent risk factor for stent thrombosis (21). Frequency for C allele was found to be 0.86 in HHC patients and 1% in control subjects. Similarly frequency of T allele was found to be 0.13 and 0% in HHC patients and controls respectively. In a study of Yoon *et al.*, 2001, no mutant CYP2C9*2 allele was found in any of Korean subjects genotyped (22). In this study, mean value of BMI was found to be more than 25 showing that BMI is most vital risk factor for CVD's. In previous study, observed mortality rate was higher in CVDs with the value of BMI at 25 or above (ranging from 22.5 to 24.9).

4. Conclusion:

In conclusion, our data does not supports significant association of CYP2C9*2 variant of CYP2C9, with elevated level of homocysteine in cardiovascular patients. Most interesting finding of this study was that not a single homozygous recessive TT genotype was found in selected population. It may be considered as unique trait related to our geographic region. Although in our population, CYP2C9*2 variant is not associated with enhanced level of homocysteine level but it may be possible that other variants of CYP2C9 may have strong association with elevated level of homocysteine in CVD patients.

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Table 1:

Mean values for the characteristics of HHC

Patient's Characteristics	Mean ± Standard Deviation
BMI	36.96±36.40
Age	50.2±11.69
Systolic	2.17±0.78
Diastolic	2.54±1.07
Uric Acid	6.73±1.55
Folic Acid	5.90±5.86
Homocysteine	16.71±7.91

Table 2: Frequency of Parameters

Parameters	Frequency (%ages)
Age	50.20
Height	165.57
Weight	84.18
BMI	36.39
Waist	105.19
Hip	110
WHR	0.95

Folic Acid	5.90
Uric Acid	6.73
Systolic	2.17
Diastolic	2.54
Homocysteine	**16.56**

Table 3: Genotype Distribution of CC, CT and TT for CYP2C9*2 in HHC Patients and Controls

Genotype for CYP2C9*2			
HHC (N=100)	CC	CT	TT
	73	27	0
Expected H-W Frequency	61.78%	24.58%	0
Controls (N=40)	CC	CT	TT
	40	0	0
Expected H-W Frequency	100%	0	0

Table 4: Chi- Square and P-value Calculated Using SPSS Version 16.0

	Value	**Degree of Freedom**	**P-value**
Chi-Square	0.125	2	0.924

Table 5: Mean BMI of HHC Patients in Effect of CYP2C9*2

CYP2C9*2 Genotypes of HHC	**Mean BMI ± Standard Deviation**
CC	37.79 ± 40.07
CT	30.7 ± 6.42
TT	Nil