Role of cytochrome epoxigenase (CYP2J2) in the pathophysiology of coronary artery disease in South Indian population

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ABSTRACT

Background: The cytochrome P-450 2J2 (CYP2J2) is known to be one of the major enzymes of epoxigenase pathway of arachidonic acid in extralobar tissues, which produces series of regioisomeric cis-epoxyeicosatrienoic acids (EETs) such as 5,6-, 8,9-, 11,12-, and 14,15-EETs. In the present study, we analyzed the impact of a genetic variant in CYP2J2 on coronary artery disease (CAD) in the Telangana region of Indian population.

Material and methods: The case–control study consisted of 100 CAD cases and 110 healthy controls. The deoxynorbornicoleic acid was extracted using the salting out method. Genotyping and gene expression were performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism and real-time PCR methods.

Results: In the present study, the percentage of smokers, alcoholics, hypertensive patients, and diabetics was high. Increase in fasting glucose, urea, creatinine, fasting triglycerides, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), total cholesterol/high-density lipoprotein (TC/HDL), LDL/HDL, homocysteine, and C-reactive protein levels were significantly higher in patients with CAD than in controls (p < 0.001). CYP2J2 G-50T was associated with CAD (p = 0.04). The mRNA expression of CYP2J2 showed altered gene expression in this study among CAD patients in comparison with control (p = 0.01).

Conclusions: A functionally relevant polymorphism of the CYP2J2 gene was independently associated with an increased risk of CAD.

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1. Introduction

Notable progresses have been observed in the treatment of coronary artery disease (CAD). Hence, CAD mortality faced a progressive decline in the past two decades. Strong case exists for the efficacy and safety of primary prevention of CAD through lifestyle changes. Further awareness and primary prevention efforts need to be extended to both public health and clinical arenas. Our previous study showed that essential changes in life leading to preventive measures include smoking avoidance or cessation, lowering alcohol consumption, modifying intakes of foods and nutrients, weight control, and physical activity. 1 Despite these progresses, incidence of new and recurrent CAD remains elevated. 2 The functional role of cytochrome P-450 2J2 (CYP2J2) gene have been identified as targets for investigating the role of CYP2J2 variants in the risk of cardiovascular diseases, such as hypertension and CAD, paying particular attention to the functional polymorphisms. 3–5 In the present study, we aim to investigate the relation between CYP2J2 gene and CAD in South Indian population.

Arachidonic acid (AA) is a polyunsaturated omega-6 fatty acid which is released from the sn2 position of membrane phospholipids by the activity of phospholipases (PLs), and among them, the role of cytosolic PL-2 is noteworthy. Free AA can be metabolized to eicosanoids through three major pathways: (i) the cyclooxygenase pathway, which generates prostanooids; (ii) the lipoxigenase pathway, which generates leukotriene and hydroxyeicosatetraenoic acids; (iii) the cytochrome P450 (CYP) pathway, which includes CYP epoxigenase and CYP 6-ω-hydroxylase enzymes. CYP epoxigenases, such as members of the CYP2C and CYP2J families, metabolize AA to four biologically active

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epoxyeicosatrienoic acids (EETs; 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET). The CYP epoxygenase inserts an oxygen atom on a carbon atom attached to one of the double bonds of AA, and the double bond is reduced as the epoxide forms.6 Epoxygenase enzymes are located in endothelial and vascular smooth muscle cells, and also in astrocytes and cardiomyocytes.7–9 Many evidences suggest that alteration in EET pathway contribute to the pathophysiology of hypertension, including blood pressure elevation, endothelial dysfunction, and end-organ damage. The infusion of angiotensin II, a potent vessel constrictor, elevates blood pressure in various animal models.10 Recently, a protective role of CYP2J2-derived EETs in heart failure was found.11 Thus, CYP2J2-derived EETs may be a target for the development of drugs to prevent cardiac hypertrophy and cardiomyocyte apoptosis in heart failure.

2. Materials and methods

2.1. Study population

A total of 100 CAD patients as defined by angiographically documented coronary artery stenosis of >50% severity and 110 healthy controls were recruited from Mahavir Hospital and Research Centre, Hyderabad. All patients gave an informed consent that explicitly provided permission for DNA analysis and collection of relevant clinical data. Three milliliters of blood was drawn from an arm vein into a sterile tube containing ethylenediamine tetraacetic acid. At the same time, a complete clinical history, including cardiovascular risk factors, was obtained from all study patients. The study protocols were approved by the ethics committee of Bhagwan Mahavir Hospital and Research Centre, Hyderabad, India. Estimation of total cholesterol (TC), triglycerides (Triglycerides; Fossati and Lorenzo, 1982), and high-density lipoprotein (HDL)12 was also determined. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald's formula, LDL-C mg/dl = TC - HDL-C - VLDL-C. VLDL cholesterol was calculated using the following formula mg/dl = Triglycerides x 5. For blood analysis, the following cutoff values were defined as abnormal: TC > 200 mg/dl, triglycerides > 180 mg/dl, HDL-C < 40 mg/dl, and LDL-C > 150 mg/dl.

2.2. Genotype analysis

Genomic DNA was extracted using a standard salting out extraction method.15 The proximal promoter of CYP2J2 G-50T was analyzed for genetic variants by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). A 373-bp fragment of CYP2J2 G-50T was amplified by primers shown in Table 1. The CYP2J2-50T allele has an Alu I cleavage site, whereas the wild type CYP2J2-50G allele does not. As such, the homozygous GG genotype was detected by the presence of a single 373-bp fragment, whereas the heterozygous GT genotype yielded three digested fragments that were 99, 274, and 373 bp in length (Fig. 1). Homozygous TT genotypes were also observed in the study cohort.

Lane 1 shows the molecular weight marker (ladder); lane 2, 3, 4 shows GG genotype; lane 5 and 7 shows GT genotype; and lane 6 shows TT genotype.

2.3. Real-time PCR

Gene expression for CYP2J2 gene was performed in 20 CAD cases and 20 controls using real-time polymerase chain reaction (RT-PCR). Total RNA was extracted using a RNeasy Mini kit (Qiagen, Germany). The concentration and purity of extracted RNA were determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer. All samples with a minimum concentration of 100 ng/µl at 260/280 between 1.8 and 2 were included in the study. Reverse transcription was performed in a personal Master Cycler (Bio-Rad CFX 96), using 1 µl of total RNA in the presence of random hexamer (50 ng/µl) and reverse transcriptase (50 U/µl) in a total volume of 20 µl, including also 10 X TaqMan RTBuffer, MgCl2 solution (25 mM), deoxyribonucleotide triphosphates (dNTPs) mixture (10 mM), an RNase inhibitor (20 U/µl), and nuclelease-free water. The reaction mixture was incubated for 10 min at 25°C, 60 min at 42°C, heated for 5 min at 95°C, and then at 4°C for a minimum of 2 min. The resulting cDNA was stored at −20°C until further use. RT-PCR was performed, with 1 µl of cDNA, 12.5 µl EvaGreen, and with specific primers (Table 1) synthesized from Bioserve Biotechnologies Ltd (Hyderabad, India). A three-step PCR was standardized using a Bio-Rad thermocycler and carried out with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. A final extension at 72°C for 5 min was carried out. Gene expression levels were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Amplification products were visualized by ethidium bromide staining after separation by run of 2% agarose gel and the respective bands are analyzed using GelDoc.

3. Results

Clinical characteristics of the two groups are described in Table 2. The CAD patients were younger in age (mean age 48.3 ± 7.4 years vs 55.4 ± 10.5 years, p value = 0.001) and had a higher proportion of men (69% vs 56.4%, p value = 0.06) as compared with controls. Most of the CAD patients were in the age group 45–50 years or 50–55 years (34% and 25%, respectively). The onset of disease was most common in the age group 41–45 years, suggesting that most of the patients develop CAD after 40 years of age.
Table 2
Demographic details of CAD patients and controls of this study.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients with CAD (n = 100)</th>
<th>Patients without CAD (n = 110)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>48.2 ± 7.4</td>
<td>55.4 ± 10.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Male</td>
<td>89 (69%)</td>
<td>62 (56.3%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Age male (mean ± SD)</td>
<td>47.3 ± 9.5</td>
<td>45.9 ± 9.3</td>
<td>0.75</td>
</tr>
<tr>
<td>Female</td>
<td>31 (31%)</td>
<td>48 (43.63%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Age female (mean ± SD)</td>
<td>52.4 ± 8.7</td>
<td>57.3 ± 10.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Smokers</td>
<td>65 (65%)</td>
<td>40 (36.4%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Alcoholic</td>
<td>20 (20%)</td>
<td>5 (4.55%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35 (35%)</td>
<td>15 (13.6%)</td>
<td>0.001</td>
</tr>
<tr>
<td>DM</td>
<td>30 (30%)</td>
<td>9 (8.2%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Family history of DM</td>
<td>24 (24%)</td>
<td>13 (11.8%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; DM, diabetes mellitus; SD, standard deviation.

Compared with the controls, the CAD patients had a higher prevalence of smoking (65% vs 36.4%, p value = 0.001), alcohol use (20% vs 45.4%, p value = 0.001), hypertension (35% vs 13.6%, p value = 0.004), diabetes (35% vs 8.2%, p values = 0.001), and family history of premature CAD (24% vs 11.8%, p value = 0.02). Among CAD patients, 25% had single- vessel disease (SVD), 35% had double- vessel disease (DVD), and 40% had triple- vessel disease (TVD) (Table 3).

3.1. Studies on biochemical risk factors

Lipid levels such as TC, triglyceride, HDL-C, LDL-C, and VLDL of the study groups are shown in Table 4. The difference was significant for TC, LDL-C, and triglyceride (TG) when cases of CAD were compared with controls. TC (236 ± 15.5 vs 202 ± 10.3 mg/dl; p < 0.0001), LDL-C (140.8 ± 5.7 vs 102 ± 5.5 mg/dl; p < 0.001), triglycerides (193 ± 24.4 vs 121 ± 2.6 mg/dl; p < 0.0001), CHOL/HDL-C ratio (3.2 ± 1.4 vs 3.8 ± 2.2; p < 0.001), and LDL/HDL-C ratio (5.3 ± 2.3 vs 4.2 ± 1.1; p < 0.001) were higher in patients with CAD, whereas levels of HDL-C (40 ± 2.9 vs 40.2 ± 2.8 mg/dl; p < 0.20) were lower. Fasting glucose levels were also higher in cases (201 ± 10.5) than in controls (122.3 ± 5.4; p < 0.001). Total cholesterol (590 ± 5.6 vs 43.3 ± 2.2) and serum creatinine levels (2.0 ± 2 vs 1.01 ± 0.3) showed significant deviation between the two groups (p < 0.001).

C-reactive protein (CRP) levels were elevated in patients (Table 4). In some of the patients, the CRP levels appeared to reflect disease activity. CRP is used to gauge disease activity in several chronic inflammatory and infectious diseases. There was a significant difference in the levels of CRP in the two groups cases (7.5 ± 2.4 vs controls 6.3 ± 0.9; p = 0.001).

3.2. Genomic results

The frequency of the G/G, G/T, and TT genotypes were present in 72 (72%), 21 (21%), and 7 (7%) of the 100 patients with CAD and 97 (88.1%), 12 (10.90%), 1 (0.90%) of the 110 control patients, respectively (Table 5).

3.3. CYP2J2 gene expression in CAD patients

The relative gene expression of CYP2J2 was quantified in 20 CAD samples by performing RT-PCR. The gene expression levels were determined as a ratio between CYP2J2 and the reference gene GAPDH to correct for variation in the amount of RNA. The relative ratio is presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the control. Among the 20 CAD samples, 10 of 20 (50%) showed CYP2J2 gene—altered expression in CAD patients, whereas all the 20 control samples showed normal expression (Table 6). In 10 samples which showed altered expression, six were upregulated and four were downregulated. The upregulation was six times higher in two samples, five times higher in one sample, three times higher in two samples, and two times higher in one sample, whereas as downregulation showed a sixfold increase in one sample, threefold increase in one sample, and two fold increase in two samples (Fig. 2). In the present study, increased mRNA expression was defined as ≥2.0 folds, normal expression ranging from 0.5001 to 1.9999 folds, and decreased mRNA expression was ≤2.0 folds. The mRNA expression of CYP2J2 was upregulated in this study among CAD patients in comparison with control. The CAD patients group is significantly different from the target gene-control group (p = 0.01).

3.4. Correlation between CYP2J2 G-50T and exogenous factors

CYP2J2 G-50T genotypes (GG, GT, and TT) were correlated with demographic factors such as gender, smoking, and angiographic findings to investigate the effect of genetic polymorphism in modulating the risk of developing CAD. When CYP2J2 genotypes were correlated with gender, it was noted that there was insignificant difference between men [GG, GT, and TT genotypes were in 75.36% (52 patients), 17.39% (12 patients), and 7.24% (5 patients), respectively] and women [GG, GT, and TT genotypes were in 64.51%]
Table 5
Genotypic distribution of CYP2J2 G-50T polymorphism in CAD patients and controls.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>CAD n (%)</th>
<th>Control n (%)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2J2</td>
<td>GG</td>
<td>72 (72%)</td>
<td>97 (88.18%)</td>
<td>0.34</td>
<td>0.16–0.71</td>
<td>0.004</td>
</tr>
<tr>
<td>G-50T</td>
<td>GT</td>
<td>21 (21%)</td>
<td>12 (10.91%)</td>
<td>2.17</td>
<td>1.0–4.08</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>7 (7%)</td>
<td>1 (0.91%)</td>
<td>8.20</td>
<td>0.99–67.90</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>165</td>
<td>206</td>
<td>0.32</td>
<td>0.16–0.61</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>35</td>
<td>14</td>
<td>3.12</td>
<td>1.62–5.9</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

Data are represented as odds ratio, 95% CI, and p-value (p < 0.05 significant).
CI, confidence interval.

Table 6
Relative expression of CYP2J2 in CAD patients' and healthy patients' blood samples.

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Cases (n = 20)</th>
<th>Controls (n = 20)</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2J2 Altered Expression</td>
<td>10 (50%)</td>
<td>0 (0%)</td>
<td>41.0</td>
<td>2.16–77.0</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP2J2 Normal Expression</td>
<td>10 (50%)</td>
<td>20 (100%)</td>
<td>0.02</td>
<td>0.00–0.45</td>
<td>0.01</td>
</tr>
</tbody>
</table>

CYP2J2 expression was expressed as the 2−ΔΔCT.
CI, confidence interval.

Fig. 2. Relative expression of CYP2J2 in coronary artery disease patients and healthy patients.

(20 patients), 29.03% (9 patients), and 6.45% (2 patients), respectively. We also investigated the association between CYP2J2 genotypes versus smoking. In patients, we observed that smokers with CAD were 76.92% (50 patients), 16.92% (11 patients), and 6.15% (4 patients) with GG, GT, and TT genotypes than nonsmokers 22 patients (62.85%), 10 patients (28.57%), and 3 patients (8.57%), respectively. The association between CYP2J2 and angiographic findings showed SVD was found in 18 patients (72%), six patients (24%), and one patient (4%), DVD in 25 patients (71.42%), eight patients (22.85%), and two patients (5.71%), TVD in 29 patients (72.5%), seven patients (17.5%), and four patients (10%) with GG, GT and TT genotypes, respectively (Table 7).

4. Discussion
In the present study, we found a higher percentage of smokers, alcoholics, diabetics, and hypertensive patients. The diagnostic parameters such as fasting glucose, urea, creatinine, fasting triacylglycerides, TC, LDL-C, TC/HDL, LDL/HDL, homocysteine, and CRP levels were significantly higher in patients of CAD than in controls (p < 0.001). Hence, the present study depicts that modifiable risk factors account for much greater share of CAD, than the non-modifiable factors.

Recent data demonstrate that human CYP2J2 gene is highly polymorphic,16–18 and it has been proposed that genetic

Table 7
Relationship of CYP2J2 G-50T genotypes with gender, smoking, and angiographic findings.

<table>
<thead>
<tr>
<th>CYP2J2 genotype</th>
<th>Male (n = 69)</th>
<th>Female (n = 31)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (72)</td>
<td>52</td>
<td>20</td>
<td>OR 1.6, 95% CI 0.6–4.2, P = 0.26</td>
</tr>
<tr>
<td>GT (21)</td>
<td>12</td>
<td>9</td>
<td>OR 0.5, 95% CI 0.1–1.3, P = 0.10</td>
</tr>
<tr>
<td>TT (7)</td>
<td>5</td>
<td>2</td>
<td>OR 1.13, 95% CI 0.2–6.1, P = 0.88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CYP2J2 genotype</th>
<th>Smokers (n = 65)</th>
<th>Nonsmokers (n = 35)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (72)</td>
<td>50</td>
<td>22</td>
<td>OR 1.9, 95% CI 0.80–4.82, P = 0.13</td>
</tr>
<tr>
<td>GT (21)</td>
<td>11</td>
<td>10</td>
<td>OR 0.5, 95% CI 0.19–1.35, P = 0.17</td>
</tr>
<tr>
<td>TT (7)</td>
<td>4</td>
<td>3</td>
<td>OR 0.6, 95% CI 0.14–3.31, P = 0.65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Angiographic findings</th>
<th>SVD (n = 25)</th>
<th>DVD (n = 35)</th>
<th>TVD (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (72)</td>
<td>18</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>GT (21)</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>TT (7)</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

CI, confidence interval; SVD, single-vessel disease; DVD, double-vessel disease; TVD, triple-vessel disease.
polymorphisms within the gene might contribute to expression and/or activity of the enzyme and, in turn, affect the biosynthesis of EETs. Several single nucleotide polymorphisms (SNPs) have been identified within human CYP2J2 gene; however, only one common SNP (G-50T substitution within the proximal promoter of the gene) was found to be functionally important. The G-50T substitution interrupts a critical binding site for Sp1 transcription factor and, thereby, results in decreased CYP2J2 promoter activity in vitro and reduced levels of CYP2J2 epoxygenase metabolites in vivo.

Few studies about the association between CYP2J2 polymorphisms and the cardiovascular risk have provided inconsistent results. The study by Spiecker et al (2004) showed a functionally relevant polymorphism of the CYP2J2 gene (rs890293) independent associated with an increased risk of CAD. This result was supported by the study of Ping Yin Liu showing the polymorphism of CYP2J2 (rs890293) was an important risk factor for the development of MI in younger groups in Taiwanese. In addition, a low risk of CAD was reported by Lee in African-Americans carrying CYP2J2 variant alleles (rs890293), but no significant association was observed in Caucasians. Our previous study suggested that genetic testing of CYP2C19 may help in prescribing a dose in accordance with genetic makeup and represent the initial steps toward the development of diagnostic tests and therapeutic strategies that will substantially improve human health. Minor abnormalities of chromosomal changes were also found in CAD patients taking clopidogrel drug but were not found to be the cause for CAD.

The frequency of genotypes of CYP2J2 (G-50T) such as G/G genotype were 72% in CAD patients compared with 88% of G/G in controls (Table 5), whereas the results for the G-50T polymorphism showed heterozygous GT and homozygous TT genotypes more in patients than in controls; and the difference between patients and controls was statistically significant (OR 2.17, 95% CI 1.0–4.68, p-value 0.04 and OR 8.20, 95% CI 0.99–67.90, p-value 0.05). Homozygous wild type GG genotypes were more in controls among CAD patients; hence, it is suggested that GG genotype may be protective for CAD (OR 0.34, 95% CI 0.16–0.71, p-value 0.004). Allele G and T in CAD patients compared with controls were statistically highly significant (OR 0.32, 95% CI 0.16–0.61, p-value 0.006 and OR 3.12, 95% CI 1.62–5.9, p-value = 0.006) (Table 5).

Expression studies among the 20 CAD samples showed altered expression of CYP2J2 gene (6 upregulated and 4 downregulated) in 50% of the patients, whereas all the 20 control samples (100%) showed normal expression (Table 6). This study group of CAD patients showed significantly higher expression of CYP2J2 gene than the control group (p = 0.01). G-50T has been earlier shown downregulating the expression of CYP2J2 gene activity. The incidence of G-T polymorphism in our cases was 21%; hence, we decided to further investigate its functional significance by qRT-PCR by taking 10 cases with this polymorphism (G-50T). The other 10 cases were other polymorphisms (GG and TT) of our cases. Our results did not show any elevation of CYP2J2 gene with G-T polymorphism which contradicts with earlier studies. This difference could be due to ethnic population of India which has complex genetic diversity. However, the two polymorphisms (GG and TT) were associated with altered expression of CYP2J2 gene; therefore, the functional investigation of these two polymorphisms needs to be explored. Overall, our results showed that these polymorphisms determine the expression of CYP2J2 gene and plays an important role in the CAD by elevating the expression of CYP2J2 gene.

4.1. Study limitations

This study covered a relatively small number of patients in a single center study, so future studies of larger patient populations are necessary to assess this finding.

5. Conclusion

In conclusion, we found that G-50T may be a novel polymorphism of the CYP2J2 gene associated with CAD in Telangana's (southern region) population of India. Furthermore, CYP2J2 could serve as a useful biomarker for early detection of CAD—which may be confirmed by using more number of samples.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhj.2018.11.011.

References