Polymorphisms in the *IL6* gene in Asian Indian families with premature coronary artery disease – The Indian Atherosclerosis Research Study

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Summary

Inflammation plays a major role in coronary artery disease (CAD). We investigated the polymorphisms in the interleukin 6 (*IL6*) gene and their effect on the expression of acute-phase proteins in premature CAD in Asian Indian families. One hundred and ninety affected sibling pairs (ASPs) were genotyped for three tag single nucleotide polymorphisms (SNPs) in the *IL6* gene for linkage analysis. We observed suggestive logarithm of odds (LOD) score for one SNP (rs2066992) in a subset of 62 ASPs with the age at onset less than 45 years (LOD score = 1.114, p = 0.011 in linkage analysis; pi = 0.55, p = 0.008 in identity by descent; LOD score = 1.06, p = 0.014 in quantitative trait locus for plasma levels of high sensitivity C-reactive protein, hsCRP). This was followed by sequencing of the promoter region and haplotype analysis in 46 probands and 40 controls. Five out of the eight previously reported promoter SNPs were found to be polymorphic (rs1800797, rs1800796, rs7802307, rs7802308, rs1800795). Two novel sequence variants were also found. One promoter haplotype (GGAAG) was detected with an odds ratio (OR) of 3.676 (p = 0.0017, 95% confidence interval [CI]: 1.68 – 8.045) and population attributable risk of 21.1% (95%CI: 9.2%-31.5%). The plasma levels of both hsCRP and fibrinogen exhibited significant association with these promoter SNP genotypes (p < 0.001). In conclusion, *IL6* gene polymorphisms appear to be important genetic factors in premature CAD, and in the regulation of key atherogenic markers in Asian Indian families.

Keywords

Asian Indians, CAD, IL6, CRP, fibrinogen

Introduction

Recent advances in cardiovascular research have led to the identification of the fundamental role played by inflammation in atherosclerosis (1, 2). Investigations on the pathophysiology of atherosclerosis have highlighted the importance of inflammatory markers in all stages of the disease process (3). Interleukin-6 (*IL6*) is known to play a pivotal role in inflammation by provoking a broad range of cellular and physiological responses (4). It upregulates the hepatic synthesis of acute phase proteins like C-reactive protein (CRP) and fibrinogen (5–7). The presence of elevated levels of IL6 mRNA in the atherosclerotic tissues and the association of high plasma IL6 levels with increased risk for cardiovascular diseases (CVD) have indicated an important role played by this inflammatory marker in the pathogenesis of atherosclerosis (8, 9).

Asian Indians have been reported to have a high susceptibility to premature coronary heart disease (CHD) (10, 11). Recent evidence suggests that differences exist between the patterns of coronary artery disease (CAD) in men of Indian and European
origin living in the UK (12). Asian Indians have a higher prevalence of risk factors associated with CHD, particularly type 2 diabetes, than individuals belonging to the populations of European ancestry (13). Multiple studies conducted on healthy Indian participants, living in the Indian subcontinent and abroad, have detected the presence of elevated plasma levels of the proinflammatory markers like IL6 and CRP, compared to their European counterparts (14, 15).

Thus, an increased prevalence of an elevated proinflammatory state, CHD and its associated risk factors in the Asian Indians emphasizes the importance of studying the molecular biology of the inflammatory genes in this population. Most of the existing reports on the role of IL6 gene polymorphisms in the regulation of plasma IL6 and CRP levels in CHD have been based on patients of European ancestry. Additionally, some of these studies have reported contradictory findings (16–18). Hence, there is an emergent need to elucidate the putative involvement of the IL6 gene and its genetic variants among Asian Indians, a population that carries an inherent high susceptibility to CVD and its comorbidities.

The Indian Atherosclerosis Research Study (IARS) is an ongoing study, initiated and conducted by the Thrombosis Research Institute (TRI), located in Bangalore, India, to elucidate the involvement of the genetic and non-genetic factors contributing to the etiopathology of CAD among Asian Indian families affected with premature heart diseases. As a part of the IARS, the present study was undertaken to explore the importance of the polymorphisms of the IL6 gene and their effect on the expression of acute-phase proteins in Asian Indians affected with premature CAD.

Materials and methods

Study participants and samples
All participants included in the study were recruited between February 2004 to June 2005, in the phase I of IARS, by obtaining the informed voluntary consent, from the Narayana Hrudayalaya Hospital, a specialty hospital for cardiac care, and other hospital and clinics in Bangalore city and from the Asian Heart Institute, Mumbai, India. The samples were collected from the participants who were above 18 years of age as per the IARS protocol approved by the institutional ethics committee of the Thrombosis Research Institute, Bangalore and the Indian Council of Medical Research (ICMR) guidelines on bioethics (19). One hundred ninety affected sibling pairs (ASP) were selected for analysis from 130 families. The ASPs consisted of 239 men and 45 women. One hundred and ten families had single ASPs while 20 families had three or more ASPs. The families were recruited through the probands who had a history of premature CAD, which included stable and unstable angina and myocardial infarction (MI) diagnosed by echocardiogram (ECD) and treated based on the catheter lab availability with standard medication or coronary angiography followed by percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass graft (CABG). The inclusion criterion for the probands was age at onset of CAD of 60 years or less for men and 65 years or less for women. The affected siblings were included on the basis of their confirmed CAD status without the age criterion. Additionally, samples were collected from 40 healthy controls consisting of individuals who did not have any personal or family history of CAD, diabetes mellitus or hypertension. Genomic DNA was isolated from the whole blood using a salting out procedure described previously and quantitated by real time polymerase chain reaction (PCR) (20). Plasma was isolated from the blood samples and stored at −70°C.

Biomarker assays
Plasma IL6 levels were measured using the Quantikine human IL6 ELISA kit (R&D systems, Minneapolis, USA), plasma high sensitivity CRP (hsCRP) levels by an immunoturbidimetric assay on the COBAS Fara using the Tina-quant latex hsCRP kit (Roche Diagnostics, Basel, Switzerland) and plasma fibrinogen levels by clotting assays on the Automated Coagulation Analyzer (ACL 300, Instrumentation Laboratory, Milan, Italy). Pooled normal human plasma prepared in-house from the samples obtained from 30 healthy volunteers was used as a control with every assay. The calibrators and controls for the hsCRP and the fibrinogen assays were obtained from the BioRad Laboratories (Hercules, CA, USA) and the Instrumentation Laboratory (Milan, Italy) respectively. The inter-assay coefficient of variation (%CV) for the plasma IL6, hsCRP and fibrinogen assays were 4.3%, 7.85% and 5.9%, respectively.

SNP genotyping and sequencing
Initially, three single nucleotide polymorphisms (SNPs) rs2066992, rs2069845 and rs2069849 were short-listed from a list of tagging SNPs located in the IL6 gene based on information available in the HAPMAP database, as per the following criteria – minor allele frequency greater than 0.1 in the HAPMAP populations, pairwise r² values of less than 0.8, and their reported linkage disequilibrium (LD) status with the other IL6 SNPs studied till date (21). The genomic DNA samples from one hundred and ninety ASPs were genotyped for these three SNPs using the TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) in a 7500 Real Time PCR instrument (Applied Biosystems). The promoter region of the IL6 gene was sequenced for 46 proband samples selected from the ASPs in which at least one member had an age at onset of 45 years or less. The promoter region was also sequenced in 40 samples from unrelated healthy controls. The PCR-based amplification of the promoter region in the genomic DNA samples was performed with published primers and True Allele PCR Mix using a 9700 PCR instrument (Applied Biosystems) (22). The PCR products thus obtained were purified by ExoSAPit digestion (Amersham Biosciences, Piscataway, NJ, USA) and sequenced with the PCR primers as well as two additional internal primers designed in house (MF: CGTGATGACTTCAGCTTTACT; and MR: CAGTGACCAGATTACGCGCTAG) and Big Dye Terminator v3.1. cycle sequencing kit (Applied Biosystems). The sequencing reactions were analyzed in a 3130XL Genetic Analyzer (Applied Biosystems). The assembly and the genotype analysis of the sequence data were performed using the SeqScape v2.5 software (Applied Biosystems). The reference sequence of the IL6 promoter region used for analysis was downloaded from the Ensembl Genome Browser release 44-April 2007 (23). Haplotype analysis was performed using the Haploview v3.32 software (24). The QA/QC of the genotype and the
sequence data obtained was performed by testing independent replicates of random samples. Additionally, quality values of the individual base calls in all the generated sequence traces and assemblies were used to assess the quality of the sequence data and the genotypes.

Data analysis
The Chi²-test was used to detect any significant departure from the Hardy-Weinberg equilibrium in the genotype data. In the first phase, the genotype data of the SNPs rs2066992, rs2069845 and rs2069849 were analyzed for linkage and identity by descent (IBD) using the SAGE release 5.3 software (25). The analysis for the quantitative trait loci (QTL) was performed with the genotype data of these SNPs and the plasma IL6 and CRP levels in the MERLIN software (26). Subsequently, the ASPs were ranked by the age at onset of CAD and analyzed by the ordered subset analysis previously described (27). In the second phase of the study, differences between genotype frequencies of the IL6 promoter SNPs in the probands and the controls were tested by the Cochran-Armitage trend test in XLSTAT software (Addinsoft, New York, NY, USA). The normality of the distribution of all quantitative data was tested by the Kolmogorov-Smirnov test and the Q-Q plots in the SPSS v10 software (SPSS Inc., Chicago, IL, USA) and the data was log transformed for normalization. The analysis of variance was performed for the plasma IL6, CRP and fibrinogen levels in the SPSS v10 software (SPSS Inc.). Adjustments were performed for the clinical covariates of age, gender, body mass index (BMI), smoking status and the use of statins. A p-value of 0.05 or less was considered statistically significant for all analysis and tests performed in this study.

Results
Clinical characteristics of study participants
The clinical characteristics of the affected individuals and the controls included in the present study are provided in Table 1. The mean age at recruitment for the affected men and women were similar. Among the affected individuals, 68% had unstable angina or had suffered an MI.

Genotyping of tagging SNPs
There was no significant departure from the Hardy-Weinberg equilibrium in all the genotypes under investigation (p > 0.05). The log of odds (LOD) scores obtained by multipoint and single-point linkage analysis of the SNPs rs2066992, rs2069845 and rs2069849 in 190 ASPs did not attain significant levels. In the subsequent ordered subset analysis, a LOD score of 1.114 (p = 0.011) was obtained for one of the tagging SNPs (rs2066992) in a subset of 62 ASPs in which at least one member had an age at onset of 45 years or less (Table 2). Significant deviation from the expected proportion of allele sharing was also observed for this subset of ASPs by IBD analysis (pi = 0.55, p = 0.008). Similar evidence of linkage was also observed for this SNP with the plasma hsCRP levels by QTL analysis (LOD score = 1.06, p = 0.014).

Table 1: Summary of clinical information of samples. Clinical characteristics of patients and controls included in this study. Angiotensin converting enzyme (ACE), body mass index (BMI), coronary artery disease (CAD), standard deviation (SD), waist hip Ratio (WHR). Probability values for continuous variables are from two-sample t tests and for categorical variables from Chi²-tests.

<table>
<thead>
<tr>
<th>Continuous variables, mean (SD)</th>
<th>Cases (n=284)</th>
<th>Controls (n=40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57.17 ± 9.22</td>
<td>45.53 ± 5.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>25.76 ± 4.73</td>
<td>24.91 ± 3.38</td>
<td>0.282</td>
</tr>
<tr>
<td>WHR</td>
<td>0.93 ± 0.12</td>
<td>0.97 ± 0.06</td>
<td>0.063</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>129.02 ± 16.51</td>
<td>120.15 ± 16.03</td>
<td>0.002</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>82.37 ± 8.13</td>
<td>80 ± 7.57</td>
<td>0.083</td>
</tr>
<tr>
<td>Age at onset of CAD (years)</td>
<td>51.87 ± 8.47</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categorical variables, n (%)</th>
<th>Cases (n=284)</th>
<th>Controls (n=40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>...</td>
<td>...</td>
<td>0.363</td>
</tr>
<tr>
<td>Men</td>
<td>239 (84.2)</td>
<td>31 (77.5)</td>
<td>...</td>
</tr>
<tr>
<td>Women</td>
<td>45 (15.8)</td>
<td>9 (22.5)</td>
<td>...</td>
</tr>
<tr>
<td>Smoking</td>
<td>...</td>
<td>...</td>
<td>0.695</td>
</tr>
<tr>
<td>Never</td>
<td>170 (59.9)</td>
<td>26 (65)</td>
<td>...</td>
</tr>
<tr>
<td>Current smoker</td>
<td>111 (39.1)</td>
<td>14 (35)</td>
<td>...</td>
</tr>
<tr>
<td>Diabetes</td>
<td>137 (48.2)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Hypertension</td>
<td>148 (52.3)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Statin</td>
<td>180 (63.4)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>155 (54.6)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>94 (33.1)</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Table 2: Linkage analysis results. Linkage, IBD and QTL analysis was performed for three intragenic SNPs by TaqMan assay on 62 affected sib pairs (46 probands). Log of odds (LOD) score, mean proportion of alleles shared (pi), identity by descent (IBD) and quantitative trait loci (QTL).
Sequencing of promoter region

Upon further analysis of the HAPMAP data, the tagging SNP rs2066992 was found to be in LD with the promoter region of the IL6 gene. This region is important for its role in gene expression of IL6 and has been investigated in other populations. Hence, this region was selected for further analysis. The sequencing of the promoter region in 46 probands selected from the 62 ASPs described above and in 40 controls provided the genotypes for eight SNPs as reported in the dbSNP database (NCBI). In the present study, five out of these eight SNPs were found to be polymorphic in the analyzed samples while the remaining three SNPs were invariant (Table 3). There were significant differences between the genotype frequencies in four out of the five polymorphic promoter SNPs in the probands and the controls. One haplotype, GGAAG (rs1800797, rs1800796, rs7802307, rs7802308, rs1800795), was found to be significantly associated with CAD (Fig. 1). An odds ratio (OR) of 3.676 (p < 0.001) (Table 5). No significant association could be detected between the genotype data and the plasma IL6 levels. The association between the promoter SNPs and the plasma hsCRP and the fibrinogen levels was found to be significant (p = 0.0017, 95% confidence interval [CI]: 1.68 – 8.045) and a population attributable risk (PAR) of 21.1% (95%CI: 9.2%-31.5%) were estimated for this haplotype (Table 4). In this haplotype, the SNPs rs1800797 and rs7802307 appeared to be in high LD (pair wise r^2 of 1.0). Similarly, there was significant LD between one of the promoter haplotype SNPs, rs1800796, and the intragenic tag SNP, rs2066992 (pair wise r^2 of 0.9).

Discussion

This is the first study to report an association of the IL6 gene polymorphisms with premature CAD as well as with the downstream hepatic expression of acute phase proteins of CRP and fibrinogen in Asian Indian families. The present findings are in agreement with the existing reports from multiple studies conducted in other populations (28–30). However, some studies have not found significant association of the IL6 gene variants with CHD as well as with the plasma IL6 and CRP levels (31, 32). This discrepancy might be related to the effects caused by differences in the ethnicities of the populations under study and the contributions from multiple environmental factors like lifestyle, diet and other risk factors (33). The success of the present study, even though modest in nature, might be ascribed to several factors. The IARS is based on affected individuals with a well-established family history of CAD, which enhances the proportion of the study cases where the genetic factors, as opposed to the environmental effects, are expected to play an important role. Additionally, since most of the complex disorders are caused by...
small individual contributions from multiple genetic variants, the multimarker-based haplotype analysis has been found to be more effective in detecting such associations as compared to the single variant-based investigations (34).

Asian Indians are known to have elevated levels of the inflammatory markers at baseline (35). Heritability studies conducted in the Phase I of the IARS have shown high scores for plasma levels of IL6 (46%, p < 0.0001), CRP (33%, p < 0.006) and fibrinogen (48%, p < 0.0001) in CAD-affected Asian Indians. These findings indicate that a promoter haplotype of IL6 gene and is in LD with the promoter region of this gene in all the HAPMAP populations as well as in the samples analyzed by sequencing in the present study. The sequencing of the promoter region of the IL6 gene provided genotype types for eight previously reported SNPs. Three of these SNPs did not exhibit any variation in our samples, indicating that they might not be polymorphic in the Asian Indian population. Two minor sequence variants were also observed. These results indicated that there might be differences between the nature of genomic variation in the Indian population and other HAPMAP populations. Since the Asian Indian population consists of multiple ethnic subgroups, these observations require validation.

Table 4: Haplotype analysis. Analysis was performed with the sequence data obtained for five promoter SNPs rs1800797, rs1800796, rs7802307, rs7802308 and rs1800795 of IL6 gene. SNPs are sequentially located in each haplotype. Analysis was performed in Haploview with* or without† permutations. CI, confidence interval.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Unadjusted mean plasma hsCRP (μg/dl)</th>
<th>Adjusted mean plasma hsCRP (μg/dl)</th>
<th>P value†</th>
<th>Unadjusted mean plasma fibrinogen (g/l)</th>
<th>Adjusted mean plasma fibrinogen (g/l)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs1800797*</td>
<td>GG</td>
<td>2.18 ± 2.5</td>
<td>0.000</td>
<td>3.93 ± 1.16</td>
<td>3.88 ± 1.26</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1.08 ± 1.15</td>
<td></td>
<td>3.79 ± 0.83</td>
<td>3.53 ± 0.77</td>
<td></td>
</tr>
<tr>
<td>Rs7802307†</td>
<td>AA</td>
<td>2.18 ± 2.5</td>
<td>0.000</td>
<td>3.93 ± 1.16</td>
<td>3.88 ± 1.26</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>1.08 ± 1.15</td>
<td></td>
<td>3.79 ± 0.83</td>
<td>3.53 ± 0.77</td>
<td></td>
</tr>
<tr>
<td>Rs7802308†</td>
<td>AA</td>
<td>2.4 ± 2.35</td>
<td>0.001</td>
<td>4.33 ± 1.33</td>
<td>4.2 ± 1.6</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>1.8 ± 2.33</td>
<td></td>
<td>3.73 ± 0.96</td>
<td>3.61 ± 0.99</td>
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</tr>
<tr>
<td>Rs1800795*</td>
<td>GG</td>
<td>2.12 ± 2.53</td>
<td>0.000</td>
<td>3.91 ± 1.17</td>
<td>3.83 ± 1.26</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>1.42 ± 1.44</td>
<td></td>
<td>3.88 ± 0.84</td>
<td>3.74 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Phenotype-genotype analysis of promoter SNPs. Promoter SNPs exhibiting significant difference between the adjusted mean plasma levels of hsCRP and fibrinogen between genotypes. †P-values after covariate adjustment. hsCRP, high sensitivity C-reactive protein; SNP, single nucleotide polymorphism.

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in future studies using a large number of samples (39). Four of the five variants in this study exhibited significant differences in the genotype frequencies between the probands and the controls. Earlier studies had shown that one of the variants, rs1800795, exhibited significant association with the baseline CRP levels (40). The variant rs1800796 has also been shown to be an independent predictor of plasma CRP and fibrinogen levels after adjustment for confounding factors among Hong Kong Chinese individuals (41). In the present study, one of the promoter haplotypes, which included these two apparently important SNPs, exhibited a significant OR of 3.676. This haplotype might be playing an important role in the modulation of the IL6-mediated inflammatory response among CAD patients. The actual alteration of the expression of the IL6 and the other downstream acute phase markers effected by this haplotype remains to be confirmed by specific functional assays of gene expression.

There was no significant evidence of linkage or association between the plasma IL6 level and any of the SNPs genotyped, either in the ASPs or in the probands and controls. However, there was significant association between the genotypes and both plasma CRP and fibrinogen levels in the probands. CRP has been proven to be a good surrogate marker for IL6 and might be a better indicator for assessing the extent of the inflammatory response (42). It is a more stable analyte and is less subject to the diurnal variations than IL6 and is synthesized upon direct IL6 stimulus, whereas the plasma IL6 level is regulated by the collective and complex influence of diverse stimuli (43, 44). Plasma IL6 levels have been found to predict coronary stenosis with high sensitivities and specificity. A similar association has been previously reported between the IL6 genotypes with the plasma CRP levels but not with the plasma IL6 levels (45, 46). However, the significant correlation observed among all the three inflammatory markers in the Asian Indian families in the IARS indicates that they might collectively contribute to the sustained systemic inflammatory milieu generally exhibited by CAD patients (IARS communication in preparation).

Since multiple testing is an important issue in genetic analysis, the conservative Bonferroni correction (p = 0.01) was applied to the results obtained with the promoter variants (47). Despite this correction, significant associations (p < 0.01) were retained for the genotypes obtained from two of the promoter SNPs and the promoter haplotype as well as that obtained between the promoter genotypes and the plasma levels of hsCRP and fibrinogen. Extended analysis with a data set substantially larger than that tested presently and the application of more sophisticated correction techniques that account for multiple testing rather than the Bonferroni correction alone, might provide robust information (48). Hence, these genomic variants located in the promoter region of the IL6 gene will be analyzed in future in 4,500 additional affected samples in the IARS in order to elucidate their role in CAD.

In conclusion, the IL6 gene appears to be an important candidate gene for CAD and might play an important role in the modulation of the downstream acute-phase response, especially in a genetically predisposed population like the Asian Indians. The findings of this study provide preliminary evidence of the involvement of promoter variants of the IL6 gene in premature CAD. These results suggest that further large-scale population-based studies as well as functional assays for measuring the effect of IL6 promoter haplotypes on gene expression should be performed to completely understand the role of inflammation in general, and IL6 in particular, in the onset of premature CAD in Asian Indians.

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