Study of C-Reactive Protein and Myocardial Infarction in the Indian Population

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Abstract To analyse the association of high sensitivity C-reactive (hsCRP) protein levels and –717A/G single nucleotide polymorphism of CRP with acute myocardial infarction (AMI) in the Indian population. Study population included 100 MI cases wherein 32 patients had experienced previous MI (MI-Group-1), 68 MI cases were recruited at presentation (MI-Group-2) and equal number of age and gender matched healthy individuals. hsCRP levels were determined by ELISA and genotyping of –717A/G was carried out by polymerase chain reaction-based restriction digestion method. The –717A/G genotypes did not influence hsCRP level and their distribution did not differ between groups. However, in the present study hsCRP demonstrated significant correlation with BMI in controls of both the genders and with triglycerides in females of AMI at presentation who otherwise are with low risk profile. Identifying traditional risk factors associated with inflammation may help in controlling the acute event.

Keywords Myocardial infarction · C-reactive protein · Single nucleotide polymorphism · Indian population

Introduction

Atherosclerosis leading to myocardial infarction (MI) is the most common and severe clinical manifestation observed in cardiovascular diseases. MI usually results from the rupture of the atherosclerotic plaque with thrombus formation and occlusion of the coronary vessel, resulting in an acute reduction of blood supply to a portion of the myocardium. The average age for acute MI (AMI) attack in Indians has decreased by 20 years and about half of the reported MI cases are below the age of 50 [1].

C-reactive protein (CRP) is a phylogenetically highly conserved plasma protein that participates in the systemic response to inflammation. It is an excellent biomarker for acute-phase response and has emerged as an important, powerful and characteristic predictor of future cardiovascular disease and metabolic abnormalities in ostensibly healthy men and women [2–4]. People are risk stratified based on amount of CRP in blood. There are three groups; less than 1 mg/l of CRP is low risk group, between 1–3 mg/l is classified as the moderate risk group and more than 3 mg/l is the high risk group on the basis of the American Heart Association guidelines [5]. Apart from this, baseline CRP level has also been demonstrated as predictor of cardiac events during hospitalization [6–8] as well as of worse outcome after coronary [9–11] and peripheral vascular surgical procedures [12]. Studies on animal models indicate that CRP might have a pathogenic role in vascular disease [13–15]. CRP plays a proatherogenic role in the process of atherosclerosis by up regulating and stimulating the release of several cytokines and growth

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factors and by down regulating nitric oxide, a potent vasodilator [16].

Studies have shown that CRP genetic polymorphisms exist and that certain of these influence steady-state blood CRP level. This has led to a proposition that genetic predisposition to high baseline CRP might exist due to these genetic variations and account for a significant proportion of people with a higher than average risk of coronary artery disease and MI [17–20]. There are various SNPs identified for *CRP* at various positions [21–29]. The SNPs in the promoter region might account for an increase or decrease in the overall gene expression. Recent studies have shown that the Guanine (G) to Adenine (A) exchange at the site –717 resulted in an increased transcriptional activity of the promoter of *CRP* gene [29, 30]. Studies for –717A/G polymorphism and its association with hsCRP levels have been studied in various populations [23, 27, 30] with some showing significant association. However similar studies have not been reported in the Indian population. Thus the objective was to analyse the hsCRP levels, its relationship with the traditional risk factors and association of –717A/G SNP of *CRP* with MI in the Indian population.

Materials and Methods

Study Groups and Protocols

Patients who suffered MI with prolonged chest pain for more than 30 min, ST elevation more than 0.5 mv on at least two adjacent ECG leads, with elevated cardiac enzymes and admitted in the emergency department of cardiac unit with clinical diagnosis of AMI were recruited. They were sub-grouped under two categories based on the day of their blood collection. Patients under MI-Group-1 ($N = 32$) were with recent event of MI on treatment and their blood sample was collected on the day of the coronary angiography before coronary intervention which was on an average 4.5 days from the stabilization of their symptoms. The blood sample of patients under MI-Group-2 ($N = 68$) was collected at presentation of MI before administration of any thrombolytic therapy for routine analysis such as electrolytes, cardiac enzymes, complete blood count and prothrombin time. The remaining serum was stored at -80°C and EDTA blood sample at 4°C for further analysis. These patients were with first episode of chest pain, had no past history of such clinical symptoms and were not on any known medications for the same. Exclusion criteria were: diabetes valvular heart disease, known cardiomyopathy, malignancy, renal or liver diseases, current use of anti-inflammatory (except Aspirin or Statin) or immunosuppressive drugs.

The Controls ($N = 100$) were healthy individuals with systolic blood pressure/diastolic blood pressure (SBP/DBP) = 135/85 mmHg or less, with no risk factors of coronary artery disease or clinical symptoms of any other organic disease. Their blood sample was collected after over night 12 h fast. The subjects having fasting glucose levels >110 mg/dl, serum transaminases, blood urea nitrogen (BUN), Creatinine levels beyond normal range, abnormal ECGs and abnormal Carotid Doppler were excluded from the Control group. As per the selection criteria in each group, subjects were recruited in study only after obtaining their informed consent. Information regarding their demographic status, clinical history, family history and medications were noted down in detail. The ethical committee of Sir H. N. Hospital and Research Centre approved the study protocol.

Biochemical Parameters

Lipid profile involving total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides and very low density lipoprotein cholesterol (VLDL-C) were tested on routine biochemical analyzer. The hsCRP levels were analyzed by sandwich ELISA technique using hsCRP kit from Biocheck Foster, USA with minimum detectable level of 0.1 mg/l. The intra and inter assay coefficient variations were 4.25 and 5.95% respectively.

Genotyping of Polymorphisms

Genomic DNA extraction was carried out from peripheral blood leukocytes using the modified salting out method of Miller et al. [31]. Analysis of –717A/G genotype was carried out by polymerase chain reaction (PCR)-based restriction digestion method using specific primers [23]; FP: 5'ACT GGA CTT TTA CTG TCA GGG C 3', RP: 5' ATT CCC ATC TAT GAG TGA GAA CC 3'. Fifty μl of PCR reaction mixture contained 100 ng genomic DNA, 1.5U Taq DNA polymerase (Fermentas-MBI), $1\times$ Taq Buffer, 2 mM MgCl_2 , 200 μmole dNTPs and 40 pmol of each specific primers. PCR was carried out in thermal cycler of Applied Biosciences 2710 with an initial denaturation step at 95°C for 5 min followed by 30 cycles, each consisting of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a cycle of extension at 72°C for 10 min. PCR product of 136 bp obtained after amplification was subjected to digestion with *SacII* enzyme which recognizes the sequence 5'CCGC ∇ GG3'. The digested fragments of approximately 110 and 24 bp were observed on 12% polyacrylamide gel electrophoresis (PAGE). The results were interpreted based on the size and number of bands obtained (Fig. 1). For homozygous wild type

Fig. 1 Restriction digestion pattern of $-717A/G$ polymorphism of CRP gene. *Lane 1:* O' Range Ruler 20 bp DNA Ladder. *Lane 2:* Cut PCR product showing one band of 110 bp of homozygous mutant (G-717G). *Lane 3:* Uncut PCR product showing one band of 136 bp of homozygous wild type (A-717A). *Lane 4:* Partially cut PCR product showing two bands of 136 bp and 110 bp of heterozygous mutant (A-717G). *Lane 5:* Undigested PCR product of 136 bp

(A-717A) genotype and homozygous mutant (G-717G) genotype, a single band of 136 and of 110 bp respectively was obtained. For the heterozygous genotype (A-717G) two fragments of 136 and 110 bp were obtained. One sample of each genotype was confirmed by sequencing the PCR product (Fig. 2).

Statistical Analysis

Results are expressed as frequency and percentages, Mean $\pm$ SD for parametric variables and Median with 25 and 75% quartile ranges for non-parametric variables. For parametric variables analysis of significance of difference between two groups was performed by student's unpaired *t*-test. Median levels of hsCRP were compared between the groups by non parametric test such as Mann–Whitney *U* test. Correlations were evaluated by Spearman's rank correlation test. Genotype frequencies were estimated by the gene-counting method. Genotypes were tested for deviations from Hardy–Weinberg equilibrium. The Chi square statistics with Yates correction was used to determine whether allele or genotype frequencies were significantly different between MI patients and the control subjects. A *P*-value <0.05 was considered statistically

significant. Analyses were performed using statistical software SPSS (version 16.0, Chicago, IL).

Results

Tables 1 and 2 depict the demographic data, lipid profiles and median hsCRP levels of the Controls and MI patients of males and females respectively. The patients and Controls were age and gender matched. The age of the MI patients ranged from 38 to 65 years.

Analysis of hsCRP Levels

hsCRP levels for all groups demonstrated skewed distribution. The levels were therefore depicted in medians and quartiles and further analysis of hsCRP levels between groups was carried out using non-parametric tests such as Mann–Whitney *U* test. Within Control group, three subjects demonstrated hsCRP levels above 10 mg/l and were therefore not included further in the analysis of the data. Moreover, within Control group almost 44.3% (43/97) of the subjects (male = 35, and female = 8) demonstrated hsCRP levels between 1 and 3 mg/l while 32% (31/97) of the subjects (male = 21 and female = 10) demonstrated hsCRP levels between 3 and 10 mg/l suggesting that the basal hsCRP level amongst the present study population was on the higher side. The risk stratification according to hsCRP levels <1 mg/l, 1–3 mg/l and >3 –10 mg/l is demonstrated in Fig. 3. Between male (Table 1) and female (Table 2) of Control groups, females (3 [1/6]) were associated with elevated median hsCRP levels (50%) as compared to males (2 [0.6/4]). Figure 4 depicts hsCRP levels as per BMI groups which shows increasing trend of hsCRP levels with increase in BMI in the Control group and MI-Group-1. Females of all the groups of present study were not associated with risk factors such as smoking, tobacco chewing or consumption of alcohol. In any of the groups as per smoking and alcohol consumption, the difference in the hsCRP levels of males did not reach statistical significance.

Overall hsCRP level were significantly elevated by 3 fold ($P = 0.004$) in the AMI patients at presentation (MI-Group-2), while they were significantly reduced in stable MI patients (MI-Group-1) as compared to Controls ($P = 0.001$) (Fig. 5). Stable MI patients (MI-Group-1) of both the genders showed almost negligible hsCRP levels when compared to Control group (Tables 1, 2). As there were only 4 females under MI Group-1, further statistical analysis for them was not carried out. Within MI-Group-2, when analysed gender wise, males demonstrated a 3 fold while females demonstrated 2 fold increase in median hsCRP levels as compared to the respective Control groups which were statistical significant ($P = 0.001$) (Tables 1, 2).

Fig. 2 Sequencing of $-717A/G$ genotypes

Correlation Analysis of hsCRP Levels with Demographic Data Within the Group

In the Control group, males (Spearman Correlation [r_s] = 0.253, $P = 0.032$) and females ($r_s = 0.441$, $P = 0.04$) demonstrated significant correlation between hsCRP levels and BMI. Male patients of MI-Group-1 demonstrated significant correlation between hsCRP levels and BMI ($r_s = 0.593$, $P = 0.001$) while male patients of MI-Group-2 demonstrated correlation between hsCRP levels and age ($r_s = 0.303$, $P = 0.032$).

Correlation Analysis of hsCRP Levels with Lipid Profile Within the Group

In Control group, hsCRP levels did not demonstrate correlation with any of the lipid parameters. Male patients of MI-Group-1 demonstrated significant correlation of hsCRP with TC/HDL-C ($r_s = 0.381$, $P = 0.045$) and LDL-C/HDL-C ($r_s = 0.4$, $P = 0.038$). Female patients of AMI at presentation (MI-Group-2) showed a significant correlation of hsCRP with triglycerides ($r_s = 0.580$, $P = 0.009$) as well as VLDL-C ($r_s = 0.580$, $P = 0.009$).

Influence of $-717A/G$ Genotypes on hsCRP Levels

The $-717A/G$ genotypes did not show any significant trend for hsCRP levels within Control, MI-Group-1 or MI-Group-2 as seen in Fig. 6.

Distribution of $-717A/G$ Genotypes

For analyzing the distribution of genotypes, the data of MI-Group-1 and MI-Group-2 was combined under common MI-Patients group. Genotype and allele frequencies were tested for Hardy–Weinberg equilibrium and the data met the assumptions of the Hardy–Weinberg Theory. There was no significant difference in the genotype distribution of the $-717A/G$ polymorphism of *CRP* between the MI-Patient and the Control study groups as shown in Table 3.

Discussion and Conclusion

The present study aimed at analyzing the hsCRP levels in MI, its relationship with the traditional risk factors and association of $-717A/G$ SNP of *CRP* with MI in the Indian population.

Table 1 Demographic data, lipid profile and hsCRP levels in males

	Controls	MI-Group 1	MI-Group 2
<i>N</i>	75	28	49
Age	49.04 ± 9.565	54.07 ± 6.661	47.24 ± 10.057
		NS	NS
BMI kg/m ²	24.64 ± 3.35	24.80 ± 3.15	24.1354 ± 2.99
		NS	NS
SBP (mmHg)	117.92 ± 10.68	133.11 ± 13.48***	126.46 ± 21.37***
DBP (mmHg)	79.49 ± 7.47	83.18 ± 6.34	81.38 ± 15.23
		NS	NS
Smokers	17	12	24
		NS	NS
Alcohol consumption	26	13	16
		NS	NS
Total cholesterol (mmol/l)	5.00 ± 0.88	4.034 ± 1.28***	4.44 ± 0.93***
HDL cholesterol (mmol/l)	1.13 ± 0.21	0.9 ± 0.20***	0.99 ± 0.19**
Total cholesterol/HDL cholesterol	5.11 ± 4.45	4.51 ± 1.14	4.5 ± 0.72
		NS	NS
LDL cholesterol (mmol/l)	3.34 ± 0.644	2.55 ± 1.02***	2.76 ± 0.63***
LDL cholesterol/HDL cholesterol	2.99 ± 0.71	2.85 ± 1.05	2.85 ± 0.59
		NS	NS
Triglyceride (mmol/l)	1.38 ± 0.50	1.4 ± 0.82	1.47 ± 0.79
		NS	NS
VLDL cholesterol (mmol/l)	0.63 ± 0.23	0.61 ± 0.33	0.66 ± 0.37
		NS	NS
Median hsCRP levels (mg/l)	2 (0.6/4)	0 (0/3.75)	6 (2/9)

NS non significant

** $P < 0.01$, *** $P < 0.001$ **Table 2** Demographic data, lipid profile and hs CRP levels in females

	Controls	MI-Group 1	MI-Group 2
<i>N</i>	22	4	19
Age	54 ± 9.39	44.75 ± 6.02	56.42 ± 8.67
			NS
BMI kg/m ²	24.19 ± 6.40	23.437 ± 3.61	27.026 ± 5.05
			NS
SBP (mmHg)	122.48 ± 8.86	123.75 ± 18.04	156 ± 25.91**
DBP (mmHg)	81.81 ± 6.39	75 ± 7.02	94.8 ± 14.12*
Total cholesterol (mmol/l/dl)	5.19 ± 0.87	3.43 ± 1.18	4.84 ± 1.17
			NS
HDL cholesterol (mmol/l)	1.30 ± 0.31	1.03 ± 0.24	1.13 ± 0.32
			NS
Total cholesterol/HDL cholesterol	3.98 ± 0.98	3.65 ± 0.57	4.38 ± 1.12
			NS
LDL cholesterol (mmol/l)	3.38 ± 0.72	2.18 ± 0.78	2.97 ± 1.011
			NS
LDL cholesterol/HDL cholesterol	2.620 ± 0.75	2.073 ± 0.37	2.732 ± 1.02
			NS
Triglyceride (mmol/l)	1.05 ± 0.33	1.31 ± 0.65	1.59 ± 0.57**
VLDL cholesterol (mmol/l)	0.48 ± 0.15	0.7 ± 0.28	0.73 ± 0.26**
Median hsCRP levels (mg/l)	3 (1/6)	0.5 (0/1)	6 (2/11)

NS non significant

* $P < 0.05$, ** $P < 0.01$,*** $P < 0.001$

Fig. 3 Risk stratified hsCRP levels amongst study groups

Fig. 4 hsCRP levels (mg/L) within group as per BMI (kg/m²)

hsCRP Levels

In the present study, our healthy individuals of Control group were screened very meticulously to be free of atherosclerosis and of any other organic disease. In spite of that, 32% of our study population, comprising 31 of total 97 of healthy individuals, demonstrated elevated hsCRP levels. This may be one of the reasons for increase risk of MI in our population. Similar finding has been reported by Rao et al. [32], in Indian population of Bangalore (Southern region of India) and of Mumbai (Western region of India). The females of healthy Controls, in the present study demonstrated increase in hsCRP levels as compared to males. This observation is similar to that reported by Lakoski et al. [33] and by Khera et al. [34]. Similar finding has also been reported by Mahajan et al. [35], in a study amongst Urban North Indians. In the present study, in the Control group, increasing trend of hsCRP levels was observed with increase in BMI in both the genders, which is also in agreement with the study by Mahajan et al. [35].

Fig. 5 Distribution of hsCRP levels amongst the three study groups. 1 = Control group, 2 = MI-Group-1 and 3 = MI-Group-2

In the present study, in male patients of stable MI also, elevated BMI was demonstrated to be the identifiable traditional risk factor for elevated hsCRP.

In the present study, stable MI patients showed decreased hsCRP levels. This could be because all stable MI patients were on standard medication which included Statin which not only reduces cholesterol level but also has an anti-inflammatory activity. This is in accordance with Nissen et al. [36] who have hypothesized & showed that Statins bring down CRP level and reduce the risk of coronary heart disease.

As expected in concordance with the extensive inflammatory phenomenon of MI, three fold increase in the total hsCRP levels in MI patients at presentation was observed as compared to Controls in the present study. In the same group, male demonstrated significant correlation between hsCRP and age. Another, highlighting point of present study was the emergence of strong correlation of hsCRP with triglycerides and VLDL-C levels in female AMI cases at presentation of the MI attack, who otherwise are with low risk profile. In the Women's Health Study, median hsCRP has been reported to be 1.0–1.5 mg/l more among women with elevated triglycerides and with low HDL-C ($P < 0.0001$ for both findings) [37].

At a given BMI, females have a higher percentage of body fat than males, and body fat is a source of IL-6 [38] which in turn induces the production of CRP. Elevated triglycerides may thus be a signal, chiefly of dysfunctional visceral adipose tissue; which promote an inflammatory state by increasing proinflammatory and inflammatory cytokines such as IL-6 and CRP respectively. These cytokines in turn accelerate the pathophysiology of atherosclerosis and thrombosis.

Fig. 6 hsCRP levels as per the three genotypes in all three study groups

Table 3 Genotype and allele frequencies of -717 A/G polymorphism of CRP gene

-717 A/G	Genotype distribution			Allele frequency distribution	
	A-717A	A-717G	G-717G	-717A	-717G
Control	51 (52.57%)	38 (39.1%)	8 (8%)	0.72	0.28
MI-Patients*	44 (44%)	48 (48%)	8 (8%)	0.68	0.32
	$\chi^2 = 1.307$, df = 2, $P = 0.52$, NS			$\chi^2 = 0.814$, df = 1, $P = 0.3$, NS	

Df degree of freedom, NS non significant

* Genotype data of MI-Group-1 and MI-Group-2 combined under MI-Patients

Parenthesis indicates percentage of the total

Association of CRP Levels with Genotype

The -717A/G genotype of *CRP* did not show any significant trend for hsCRP levels. This is in accordance with the research by Miller et al. [21] and Chen et al. [20] who concluded that the -717A/G polymorphism was unrelated to CRP levels. However, it is not in conformance with in vitro studies by Wang et al. [30] who have showed that the exchange of G with A at the -717 site resulted in an

increased transcriptional activity of the promoter of CRP gene.

Association of the -717A/G Genotype with MI

There was no significant difference in the genotype distribution of the polymorphism of CRP gene between the MI-Patients and Controls. This was not in accordance with the studies by Chen et al. [27] who surprisingly, have

reported that the frequency of the A allele was significantly higher in patients than in controls. This may be due to the difference in the ethnicity of the two populations.

Identifying the known traditional risk factor (such as triglycerides and BMI), linked with a molecule of pathophysiological importance in atherothrombosis (such as CRP), may simplify our approach in controlling the acute event such as MI. Moreover, a higher trend of hsCRP levels in healthy individuals observed in our study also strongly suggests the need to carry out large-scale study to determine the hsCRP levels of our population. This in turn may help in defining the steps in controlling the hsCRP levels and reducing the impact of this disease.

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