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CRP Gene (1059G>C) Polymorphism and Its Plasma Levels in Ischemic Stroke and Hemorrhagic Stroke in a South Indian Population

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Abstract—In the present study, we evaluated the association of 1059G>C polymorphism in C-reactive protein (CRP) gene with the risk of ischemic and hemorrhagic strokes. We did not find a significant association of this polymorphism with stroke. However, 2 % of mutants were observed in hemorrhagic stroke patients with a 0.01 frequency for the C allele. We also estimated the high-sensitivity C-reactive protein (hsCRP) levels in hemorrhagic stroke and compared the levels with our already published data on ischemic stroke. The hsCRP level in hemorrhagic stroke was found to be significantly elevated in comparison with that in controls ($p<0.001$). However, there was no difference in the mean value of hsCRP levels between types of stroke. In conclusion, the G>C polymorphism in the promoter region of the CRP gene is not abundant in the population and cannot be connected with different hsCRP levels and stroke prediction. The CRP level is a useful marker in stroke, but cannot help in differentiating between types of stroke.

KEY WORDS: C-reactive protein; 1059G>C; ischemic stroke; hemorrhagic stroke; inflammatory marker.

INTRODUCTION

Stroke has been recognised as a heterogeneous multifactorial disorder. Cardiovascular events and stroke are largely due to rupture of atherosclerotic lesions and inflammation-mediated destabilisation [1, 2]. In India, stroke is a major health problem, and ischemic stroke is the most common type of stroke [3, 4]. Several studies have confirmed that high blood pressure, poor diet, smoking, obesity, and lack of physical activities are common risk factors associated with 80 % of all strokes [5]. In addition to these, genetic factors have also been proposed to influence stroke. Several case-control association studies have suggested inflammatory molecules to be responsible for stroke risk [6]. C-reactive protein (CRP) is a glycoprotein released by the liver and is an acute inflammatory-phase reactant. It is a marker for systemic inflammation [7], influences direct proinflammatory effects [8, 9] and is rapidly upregulated by inflammatory cytokines [10]. It has a long half-life (19 h) with no diurnal variation [11] and has thus emerged as a sensitive indicator of inflammation and a marker for atherosclerosis [12]. It is hypothesised that the variation in the CRP gene influences the levels of CRP in plasma [13, 14]. CRP genetic variants like +1059G>C, +1444C>T, −757A>G, −717A>G, −286C>T>A and +2147C>T have been studied earlier in ischemic and hemorrhagic strokes [15, 16].

In the present study, we evaluated the association of CRP genetic variant, 1059G>C, with ischemic as well as hemorrhagic stroke in a South Indian population from Andhra Pradesh. We have already established the association of high-sensitivity CRP (hsCRP) level with ischemic stroke and its subtypes and with stroke outcome. In this study, we estimated the hsCRP levels in hemorrhagic stroke and compared the levels with controls and ischemic stroke.

MATERIALS AND METHODS

Two hundred ischemic stroke patients (males/females=168:32) and 200 hemorrhagic stroke patients

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(males/females=168:32) presenting with new stroke evaluated in the Neurology Department of Nizam's Institute of Medical Sciences, Hyderabad (A.P, India), between September 2007 and July 2013 were included in the study. The study was approved by the ethical committee of the study hospital and by the institutional ethics committee. All the patients were examined by a qualified stroke neurologist. Ischemic and hemorrhagic strokes were differentiated by computed tomography (CT) scans and magnetic resonance imaging (MRI). All the patients underwent CT scan as well as MRI. Patients with major cardiac, hepatic, renal, endocrinological and skeletal disorders and cancerous diseases were excluded from this study. As a control group, 200 healthy individuals (males/females=168:32) matched for sex and age were recruited from the same demographic area for comparison with ischemic stroke patients and hemorrhagic stroke patients. The controls had no clinical evidence of any cerebrovascular diseases (CVD). Information on demographic features and risk factors was collected by using a structured questionnaire. Hypertension, alcohol use, diabetes, and smoking were defined as reported previously [17]. Subjects included in the study were above the age group of 18 years, and all the samples were collected only after obtaining the written informed consent.

DNA Isolation and Genotyping

Five millilitres of blood was collected in EDTA coated tubes, and genomic DNA was extracted from blood samples using standard phenol-chloroform method. The 1059G>C polymorphism in the CRP gene was analysed using PCR and RFLP techniques. The primers used for the amplification of the gene are forward 5′ GATCTGTGTGATCTGAGAAACCTCT 3′ and reverse 5′ GAGGTACCAGAGACAGAGACGTG 3′. The amplified 744-bp PCR product was digested with MaeIII restriction enzyme (Fermentas Fast digest) by incubating it at 37 °C for 5 min followed by separation of fragments on 2 % agarose gel. The most common homozygous genotype (GG) shows the presence of fragments of 310, 233 and 210 bp; heterozygous genotype (GC) shows 434-, 310-, 233- and 201-bp bands; and rare homozygous genotype (CC) was detected as 434- and 310-bp bands.

CRP Assay

All the blood samples used for the assessment of CRP were collected within 24 h after qualifying for stroke. Blood was subjected to centrifugation after the collection, and hsCRP levels were estimated in fresh serum samples. Commercially available ELISA kit from Diagnostics Biochem Canada was used to calculate the CRP levels in micrograms per millilitre. The enzyme immunoassay test follows a two-step capture or sandwich-type assay, which makes use of two highly specific monoclonal antibodies. A monoclonal antibody specific for CRP is immobilised on a microwell plate, and another specific for a different region of CRP is conjugated to horseradish peroxidase (HRP). Both patient and control samples along with standards are allowed to bind to the plate, washed and subsequently incubated with the HRP conjugate. The absorbance is measured by a microtiter plate reader, and the intensity of the colour formed is directly proportional to the concentration of CRP in the samples. A set of standards is plotted to get a standard curve from which the amount of CRP in test samples can be directly read. This assay was done only in blood samples of hemorrhagic stroke patients and controls. For ischemic stroke, the data has already been published and that data set was used for comparison with hemorrhagic stroke hsCRP levels [18].

Statistical Analysis

The difference in demographic features between ischemic stroke cases, hemorrhagic stroke cases and controls was evaluated by Student's t test. Statistical significance was defined as $p<0.05$. Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. The enzyme assay data were expressed as mean ± SD value. The association of hsCRP levels with hemorrhagic stroke was examined by odds ratio with 95 % confidence interval (CI) and chi-square analysis using Open EPI6 software (Open Epi Version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). Since the distribution of CRP values was not normal and it was positively skewed, the Mann-Whitney U test (nonparametric test) was used for comparison of mean values of hsCRP in hemorrhagic stroke patients and controls. For comparison of mean values of hsCRP levels between hemorrhagic stroke patients and ischemic stroke patients (already published data), the Student's t test was used.

RESULTS

A total of 200 ischemic stroke cases and hemorrhagic stroke cases along with 200 controls from the same

Table 1. Clinical Characteristics of Ischemic Stroke Patients, Hemorrhagic Stroke Patients and Controls

Characteristics	Ischemic stroke patients (n=200)	Controls (n=200)	p value	Haemorrhagic stroke patients (n=200)	p value
Age	49.4 (17.4)	49.08 (16.9)		53.8 (3.9)	
Male/female	168:32	168:32		168:32	
Systolic BP [mmHg] (mean ± SD)	141.8 (17.1)	120.2 (12.2)	<0.001	142.02 (16.8)	<0.001
Diastolic BP [mmHg] (mean ± SD)	88.2 (20.6)	74.3 (10.2)	<0.001	87.5 (19.5)	<0.001
Total cholesterol (mean ± SD)	198.2 (40.7)	194.8 (42.9)	NS	197.7 (39.24)	NS
Triglycerides (mean ± SD)	179.1 (39.9)	128.2 (44.3)	<0.001	174.84 (58.8)	<0.001
Random glucose (mean ± SD)	130.3 (7.4)	119.03 (21.9)	<0.001	128.7 (6.4)	<0.001
HDL cholesterol (mean ± SD)	53.45 (20.97)	59.3 (23.3)	NS	53.7 (19.1)	NS
Hypertension	57.8 %	41.1 %	<0.001	59.85 %	<0.001
Diabetes	44.7 %	27.9 %	<0.001	43.04 %	<0.001
Smokers	43.81 %	29.8 %	0.001	58 %	<0.001
Alcohol use	33.1 %	27.8 %	<0.001	52 %	<0.001
Family history of stroke	26.8 %	2 %	<0.001	5 %	<0.001

Age, systolic BP, diastolic BP, total cholesterol, high-density lipoprotein (HDL) cholesterol, random glucose and triglycerides are given as mean (SD). p values were calculated using Student's paired t test (SPSS 18)

demographic area were recruited for the study. All the subjects were of South Indian origin, from Andhra Pradesh, India. The clinical characteristics of stroke patients and controls have been summarised in Table 1. Mean age of ischemic stroke patients and hemorrhagic stroke patients was 49.4 and 53.8 years, respectively, and that of controls was 49.08 years. Risk factor profile of the ischemic stroke patients revealed hypertension in 57.8 %, diabetes in 44.7 %, smoking in 43.81 %, alcohol use in 33.1 % and family history of stroke in 26.8 % subjects. Among hemorrhagic stroke patients hypertension was seen in 59.85 %, diabetes in 43.04 %, smoking in 58 %, alcohol use in 52 % and family history of stroke in 5 % of patients. In the control group, 41.1 % had hypertension, 27.98 % were diabetic, 29.8 % were smokers, 27.8 % were alcoholic and only 2 % had a family history of stroke. The plasma levels of triglycerides and random glucose were significantly elevated (p<0.001) in ischemic stroke patients and hemorrhagic stroke patients as compared with those in controls.

The CRP (1059G>C) genotypes and allelic frequencies for both types of stroke have been summarised in Tables 2 and 3. The results reveal the presence of only GG genotype among ischemic stroke patients and control subjects. The frequency of G allele in ischemic stroke cases and controls was 1.0, whereas in hemorrhagic stroke cases, the frequency of G allele and C allele was found to be 0.99 and 0.01, respectively.

Distribution of normal, moderate and high levels of hsCRP among hemorrhagic stroke cases and controls has been given in Table 4. We found a statistically significant difference in high *vs*. normal levels [χ^2=89.57, p<0.001, odds ratio=13.43 (95 % CI, 7.418–24.32)] and also between moderate *vs*. normal levels [χ^2=84.74, p<0.001, odds ratio=20.32 (95 % CI, 9.993–41.32)]. However, there was no significant difference between high levels *vs*. moderate levels [χ^2=1.95, p=0.081, odds ratio=0.661 (95 % CI, 0.369–1.183)]. The mean hsCRP level was found to be significantly elevated in hemorrhagic stroke patients in comparison with that in controls (p<0.001, Table 5). Table 6 summarises the comparison of mean levels of hsCRP between ischemic stroke and hemorrhagic stroke. No significant difference in the hsCRP levels was observed between the two types of stroke (p=0.07).

Table 2. Distribution of CRP Genotypes and Allelic Frequencies in Ischemic Stroke Patients and Controls

	GG	GC	CC	Total	G	C	Total
Patient *n* (%)	200 (100)	0	0	200	400 (1.0)	0 (0.0)	400
Control *n* (%)	200 (100)	0	0	200	400 (1.0)	0 (0.0)	400

Table 3. Distribution of CRP Genotypes and Allelic Frequencies in Hemorrhagic Stroke Patients and Controls

	GG	GC	CC	Total	G	C	Total
Patient *n* (%)	196 (98)	0	4 (2)	200	396 (0.99)	4 (0.01)	400
Control *n* (%)	200 (100)	0	0	200	400 (1.0)	0.0 (0.0)	400

DISCUSSION

Stroke is a leading cause of morbidity worldwide [19]. It is the third most common cause of death and first leading cause of disability in developed as well as in the developing countries [20]. Evidence suggests that CRP is a peripheral marker of inflammation and for generalised atherosclerosis [21]. The relationship between inflammation and atherosclerosis makes CRP a potential candidate for prognosis of cardiovascular and cerebrovascular events [22]. Further, in cerebral ischemia, neuroinflammatory mechanisms have shown to be responsible for neurological worsening and infarct growth [23]. Therefore, we evaluated the association between 1059G>C promoter polymorphism of CRP gene and hsCRP levels with risk of ischemic stroke and hemorrhagic stroke in a South Indian population from Andhra Pradesh. To the best of our knowledge, this is the first study reporting this variation in CRP gene in association with ischemic stroke and hemorrhagic stroke in a South Indian population. The association of hsCRP levels with ischemic stroke and its subtypes has already been established in this ethnic group (18), but it has not been reported in hemorrhagic stroke. Therefore, we evaluated the hsCRP levels in hemorrhagic stroke and compared it with those in controls and ischemic stroke.

Variants of CRP gene have been studied in ischemic stroke and its subtypes, but with a negligible number of studies in hemorrhagic stroke [15, 16, 24]. A strong association of this variant of CRP was reported in a case-control study in a Japanese population with ischemic stroke [24]. Another study associated the C allele with an increased risk for cardioembolic stroke, a subtype of ischemic stroke, but reported negative association with overall risk for ischemic stroke [15]. The haplotype-based study by Wang *et al.* [2009] reported H3 haplotype (G-C-C) to be an independent risk factor for ischemic stroke and H5 haplotype (A-T-C) as a prognostic marker for hemorrhagic stroke. They also concluded that the −717A>G polymorphism plays a protective role in ischemic stroke [16]. Our results on 1059G>C variant of CRP do not document the presence of any genotypic variation in ischemic stroke patients or controls. However, the frequency of the rare C allele in hemorrhagic stroke patients was found to be 0.01.

A study carried out by Roudbary *et al.* reported that different types of stroke can be related to elevated CRP levels [25]. They found a statistically significant difference in mean hsCRP levels of ischemic stroke and hemorrhagic stroke and concluded the use of this enzyme as a useful tool in the initial diagnosis of different types of stroke. Several other studies from Pakistan, Italy and UK also found elevated levels of the enzyme in 88, 74.2 and 42.1 % of their respective study samples [26–28]. Our earlier study also reports a significant association of hsCRP levels with ischemic stroke and its subtypes [18], which is supported by another study carried out in the same ethnic group [29]. The present study reveals a statistically significant difference in the mean hsCRP levels of hemorrhagic stroke cases when compared to those in controls. However, there was no significant difference in the mean levels between the two stroke types which is in contrast to the results obtained by Roudbary *et al.* who reported a significant difference in the CRP levels between ischemic and hemorrhagic stroke

Table 4. hsCRP Levels in Hemorrhagic Stroke Patients and Controls

Study group	Normal levels	Moderate levels	High levels	Total
Hemorrhagic stroke patients (%)	17 (8.5)	62 (31)	121 (60.5)	200
Controls (%)	117 (58.5)	21 (10.5)	62 (31)	200

Hemorrhagic stroke cases *vs.* controls. For high levels *vs.* normal levels: χ^2 =89.57, p<0.001, odds ratio=13.43 [95 % CI, 7.418–24.32]. For high levels *vs.* moderate levels: χ^2 =1.95, p=0.081, odds ratio=0.661 [95 % CI, 0.369–1.183]. For moderate levels *vs.* normal levels: χ^2 =84.74, p<0.001, odds ratio= 20.32 [95 % CI, 9.993–41.32]

Table 5. Mean hsCRP Levels in Hemorrhagic Stroke Patients and Controls

Group	hsCRP (mean ± SD)
Hemorrhagic stroke patients	4.62±2.30
Controls	2.10±2.06
p value	<0.001

The difference in mean hsCRP levels between hemorrhagic stroke cases and controls was examined by the Mann-Whitney *U* test

[25]. An association of elevated levels of CRP has also been reported in a study from Netherlands in both stroke and myocardial infarction [30].

In a large cohort study of cardiovascular risk, carried out by Carlson *et al.* [2005], an association of several CRP single-nucleotide polymorphisms (SNPs) within the promoter region with CRP plasma levels was reported [31]. Brull *et al.* found a negative association between 1059G>C polymorphism and CRP levels in coronary heart disease [14]. However, some other studies reported a positive association between this polymorphism and enzyme levels [15, 32]. However, an overall association of CRP genotypes or haplotypes with ischemic stroke was not found [15]. In contrast to this finding, Morito *et al.* [2006] suggest rs1800947 SNP and the C-C-C haplotype in the CRP gene to be prognostic markers for ischemic stroke [24].

SNPs such as rs1205, rs1130864, rs3091244 and rs3093077 of the CRP gene have been associated with differences in basal CRP levels [33]. Additionally, lifestyle factors, such as smoking, and BMI have been suggested to influence baseline CRP levels than SNPs, and therefore, the detection of a genetic association between CRP SNPs and CVD becomes difficult [34]. Nevertheless, CRP has emerged as a novel plasma marker for atherothrombotic disease and may reflect the level of inflammatory activity in atherosclerosis and CVD.

Table 6. Mean hsCRP Level Comparison Between Hemorrhagic Stroke Patients and Ischemic Stroke Patients

Group	hsCRP (mean ± SD)
Hemorrhagic stroke patients	4.62±3.72
Ischemic stroke patients	5.38±2.30
p value	=0.07

The difference in mean hsCRP levels between hemorrhagic stroke cases and controls was examined by the Student's *t* test

In conclusion, the present study suggests that the 1059G>C polymorphism in CRP gene is not a significant risk factor for stroke in the study population. Further, hsCRP levels can be used as a general predictor of stroke, but cannot help in distinguishing ischemic and hemorrhagic strokes.

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