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Research paper

Association of CnB 5I/5D promoter gene polymorphism and serum calcineurin levels in early onset of coronary artery disease of south Indian cohort

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ABSTRACT

Calcineurin, a serine/threonine phosphatase is a calcium dependent protein which on activation triggers transcriptional up regulation of inflammatory genes associated with inflammation in the arteries and progressive formation of plaques in CAD. The present investigation is aimed to study the possible association of Calcineurin encoding gene PPP3R1 (CnB 5I/5D) polymorphism in correlation with serum levels of calcineurin in coronary artery disease (CAD). A total of 300 angiographically documented CAD patients and 300 age, gender ethnicity matched healthy controls were recruited for the study. Serum Calcineurin levels were estimated by enzyme-linked immunosorbent assay (ELISA) and genotypes were determined based on PCR-RFLP. The CnB 5I/5D variation was found to be significantly associated with CAD ($p < 0.03$), correlated to elevated serum calcineurin levels encoded by (< 0.01) 5I/5D allele authenticated by Insilco analysis. Multiple logistic regression analysis also confirmed these findings [adjusted OR for DD genotype was 3.19 (95% CI 1.40–7.24) and $p = 0.001$]. The results suggest that 5-base pair deletion results in increased serum calcineurin levels and may trigger up regulation of calcineurin which mediates vascular inflammation and atherosclerosis in CAD.

1. Introduction

Coronary artery disease is a multifactorial disease characterized by sub endothelial accumulation of lipoprotein particles in the intima of artery wall and clinically presented as deposition of fibrous plaque/atheroma containing smooth muscle cells, fibrous tissue and extra cellular matrix proteins. The formation of atheroma limits the blood flow to myocardium associated with local inflammation leading to plaque rupture and myocardial infarction (Libby et al., 2002).

Recent studies suggest the pivotal role of calcineurin - dependent pathway in the development of cardiac hypertrophy. However its role in vascular inflammatory disease *perse* atherosclerosis still remains elusive (Satonaka et al., 2004). Calcineurin a serine/threonine phosphatase is a calcium dependent protein expressed in diverse range of mammalian tissues like brain, adipose tissue, adrenal cells, heart, osteoclasts, skeletal and smooth muscles (Rusnak and Mertz, 2000; Lakshmikuttyamma et al., 2006). Calcineurin is a heterodimer protein comprising of two subunits *viz*., catalytic subunit A, which contains a Fe^{2+} ion and a Zn^{2+} ion and a subunit B which is involved in the regulatory mechanism of enzyme and calcium- binding protein calmodulin (Rusnak and Mertz, 2000).

Increased intracellular Ca^{2+} results in calcineurin activation leading to subsequent dephosphorylation of transcription factors like NFAT (nuclear factor of activated T cells) in the cytoplasm. Upon dephosphorylation, NFAT translocates to nucleus, binds to the gene promoters of immunomodulatory cytokines like IL-6, IL-10, IL-12, TNF-α and regulates their expression in specific immune cells like macrophages (REA et al., 2012; Schulz and Yutzey, 2004). These macrophages further play a central role in lipid accumulation, secretion of cytokines, release growth factors and thereby promoting additional accumulation of smooth muscle cells (SMCs) which leads to disruption of fibrous cap and formation of plaques and leading to atherosclerosis

PPP3R1 (Protein Phosphatase 3, Regulatory Subunit B, Alpha) is a Calcineurin coding gene located on human chromosome 2p15. Calcineurin plays an important role in both cardiac and skeletal muscle hypertrophy (Wang et al., 1996). Recently, the mechanism of the development of cardiac hypertrophy is the prime locus wherein Tang et al. (2005) suggested that the 5-base pair (bp) insertion/deletion (I/D)

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polymorphism in the promoter region of the PPP3R1 gene to be associated with the high incidence of inappropriately left ventricular mass in severe hypertensives (Tang et al., 2005).

According to Satonaka et al. (2004) calcineurin-dependent pathway mediates MCP-1 expression in VSMCs and vascular inflammation in murine models therefore Calcineurin forms a crucial protein to modulate vascular inflammation in conditions such as atherosclerosis (Satonaka et al., 2004). According to documented medical literature, there are no published reports mentioning PPP3R1 gene polymorphism in relation to atherosclerotic results. Hence the present study was aimed pursued to evaluate the possible association of PPP3R1 gene polymorphism (CnB 5I/D) and assess serum calcineurin concentration to elucidate the risk of developing coronary artery disease (CAD).

2. Materials & methods

2.1. Study population

The study group included 300 patients (20–40 yrs) with angiographically diagnosed clinical presentation of acute myocardial infarction (during the time of hospitalization) admitted at the Department of Cardiology, Gandhi Hospital and Nizams Institute of Medical Sciences, Hyderabad, India. Patients presenting with systemic inflammatory disease, liver disease, malignancy or any other heart diseases were excluded from the study. The control group consisted of 300 age, gender and ethnicity matched healthy individuals with no clinical or family history of CAD or clinical symptoms of any other systemic disease. The epidemiological variables like age, gender, nativity, occupation, life style habits, family history and clinical symptoms were recorded in the form of structured questionnaire. The study was approved from the institutional ethics committee for biomedical research. An informed consent was taken from the patients prior to the study and the objectives of the study were clearly explained.

2.2. Biochemical analysis

Venous blood (5 ml) was drawn from fasting subjects, centrifuged (2000 rpm for 15 min) and assayed for serum concentrations of total cholesterol (TC), triglycerides (TG), LDL-cholesterol, HDL-cholesterol (HDL-C), & VLDL Cholesterol (VLDL-C) using CHEM-7 semi auto analyzer (ERBA Mannheim, Germany).

2.3. Measurement of calcineurin concentrations

Serum Calcineurin levels were estimated using enzyme-linked immunosorbent assay (ELISA) kit (Wuhan EIAab Science Co., Ltd., Catalog number E1323h, China) based on the sandwich principle and the absorbance of the samples was measured by using a micro plate reader at 450 nm.

2.4. Molecular analysis

Genomic DNA was extracted from whole blood by salting out method of Lahari et al. and the (−1059 to 1063) CnB5I > 5D (PPP3R1) genotyping was performed by PCR and RFLP technique. The primers used for the amplification of the gene are Forward: 5′GATCTGTGTGATCTGAGAAACCTCT3′ and Reverse: 5′GCTGGAA GATCACACCAT3′. The 280 bp amplified PCR product was digested with *Ase*I restriction enzyme (Fermentas Fast digest) by incubating at 37 °C for 5 min followed by genotyping on 2% agarose gel. A product of 255, 25 bp indicates a genotype homozygous for insertion (II), 275 bp homozygous for DD and the presence of 275,255 and 25 bp products represents a heterozygous genotype.

2.5. Statistical analysis

The demographic and clinical data were compared between patients and controls, genotypic, allelic frequencies and Hardy-Weinberg equilibrium were calculated by chi-square analysis. The association between genotypes and CAD was evaluated by calculating the odds ratios (OR) at 95% confidence interval using open EPI6 software (Open Epi Version 2.3.1, Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). A two-tailed value of $p < 0.05$ was regarded as statistically significant. The relationship between demographic features, biochemical marker as well as molecular marker with CAD was evaluated by multiple logistic regression analysis with forward stepwise selection (Wald) using SPSS 18 software (SPSS Inc. Chicago, IL, USA). The independent variables were decoded as the following dummy variables: genotype, 0 for II genotype, 1 for ID genotype and 2 for DD genotype; groups; sex, 1 for male and 2 for female; smoking 1 for smokers and 2 for non-smokers; alcoholism 1 for alcoholics and 2 for non-alcoholics; for diabetes 1 for presence and 2 for absence, for Diet 1 veg and 2 for non veg, for CAD family history 1 for presence of history and 2 for absence of history; for Calcineurin Levels 1 for normal levels and 2 for high levels; Statistical significance was defined as $p < 0.05$.

3. Results

The mean age of patients and controls at the time of sample collection was 35.8 ± 3.7 years and 36.4 ± 3.5 years respectively. The differences between CAD patients and control subjects were statistically significant in all measures of established risk factors such as hypertension, smoking, alcohol and family history of CAD. The clinical data on total cholesterol and LDL-C was found to be significantly elevated in patients than in healthy controls ($p < 0.001$) on the contrary HDL-C was higher in controls than their patient counterparts ($p < 0.001$). (Tables 1 & 2) Calcineurin levels were found to be significantly increased in patients when compared to controls ($p < 0.01$), a twofold higher concentration of calcineurin levels was observed in CAD patients suggesting elevated calcineurin levels to be an important determinant factor for developing CAD ($p < 0.01$). (Table 3)

Table 4 represents the distribution of CnB 5I > 5D genotypes and allelic frequencies in cases and controls. The percentage distribution of II, ID and DD genotypes was 64, 14, and 21 in controls and 60, 8.3 and 32 in the cases correspondingly. The genotypic frequencies between the patients and controls differed significantly ($\chi 2$; 10.5 df02; p0.005).However frequencies of I and D alleles were also found to be significant ($\chi 2$; 8.07 df02; p0.004)

In the present study, the frequency of DD genotype was a significant higher in patients than in controls. (Co-dominant model, control *vs* Patients *II vs DD* [odds ratio = 1.626, 95% confidence interval (1.11, 2.36); $p < 0.01$]. In addition to the co dominant model, even recessive model supports the significant association of DD genotype with patients

Table 1
The demographic & clinical characteristics in CAD patients compared to controls.

Characteristics	CAD $n = 300$	Controls $n = 300$	*P*-value
Mean age	35.8 ± 3.7	36.4 ± 3.5	0.3
BMI (Kg/m^2)	25.02 ± 1.40	23.54 ± 1.29	0.157
TC (mg/dl)	214.9 ± 35.3	156.5 ± 28.9	< 0.005*
LDL (mg/dl)	152.2 ± 39.4	93.24 ± 29.4	< 0.001*
HDL (mg/dl)	34.5 ± 9.38	37.12 ± 11.5	< 0.001*
TG (mg/dl)	141.63 ± 22.6	131 ± 23.4	0.54
VLDL(mg/dl)	28.3 ± 4.52	26.2 ± 4.60	0.76
SBP (mm Hg)	129.28 ± 9.48	112.6 ± 9.06	< 0.001*
DBP (mm Hg)	84.63 ± 8.42	79.8 ± 6.58	< 0.001*

P values calculated using student *t*-test.
* $p < 0.05$.