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Study of TNF- α Polymorphism (308G/A) and the Role of NF- κ B as an Novel Marker of Severity of Atherosclerosis: A Pilot Study in Indian Population

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Abstract

Background: Biallelic polymorphism of A/G variation at position 308 in the promoter region of TNF-α gene is an important genetic factor causing high TNF-α transcription which could influence the clinical outcome of atherosclerosis. Studies have also implicated the role of NF-κB in atherogenesis by regulating genes involved in the inflammatory response and insulin sensitivity.

Material and Method: 50 cases of angiographically significant atherosclerosis (>50% obstruction in coronary arteries) and 50 age, sex, BMI matched patients with insignificant atherosclerosis (>50% obstruction) on angiography were selected from GB Pant Hospital. Polymorphism was studied by amplifying DNA using PCR and amplified segments were digested by restriction enzyme Nco-I and followed by RFLP. Serum NF-κB, TNF-α levels were estimated by sandwich ELISA.

Results: The mean serum NF-κB and TNF-α levels were significantly (p=0.04, 0.000 respectively) raised in cases as compared to controls. Upon binomial logistic regression analysis, NF-κB emerged as the best predictor of severity of atherosclerosis (Odds ratio=27) among other markers. Our results showed no intergenotypic variation of 308-G/A polymorphism of the TNF-α gene between cases and controls.

Conclusion: Our study establishes NF-κB as an emerging biomarker of severity of atherosclerosis in Indian population. No intergenotypic variation between cases and controls indicates that significantly high levels of TNF-α in the cases is attributed to cause other than polymorphism in Indian population. High prevalence of chronic low grade inflammation in population could be postulated as the possible cause.

Introduction

Over the past few decades, the management of atherosclerosis mainly included reduction of Low Density Lipoprotein-cholesterol by lipid lowering drugs like statins. In spite of success in lowering LDL the epidemic of Ischemic Heart Disease has continued to surge. The focus of research has therefore justifiably shifted from dyslipidemia to inflammatory processes. Studies show inflammatory processes might have a key role in the initiation and progression of atherosclerosis.

TNF-α is a proinflammatory cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, endothelial cells and adipose tissue. It is chemoattractant for neutrophils and increases the expression of cell adhesion molecules on endothelial cells [1]. In liver, it causes insulin resistance by serine phosphorylation of insulin receptor substrate (IRS-1) which further predisposes to atherosclerosis [2].

Because genetic traits contribute significantly to the global risk of ischaemic heart disease (IHD) [3], a number of studies have now addressed the hypothesis that variations in the genetics of the inflammatory system may increase the risk of disease. Differences in the genetic regulation of inflammatory processes might explain why some people develop a greater inflammatory response predisposing them to increased cardiovascular risk [3].

TNF-α POLYMORPHISM-TNF-α gene is located on chromosome 6p21.3.It codes for a 157 aminoacid polypeptide processed from 233 amino acid precursors. The biallelic polymorphism of A/G variation at position 308 in the promoter region of TNF-alpha gene is an important genetic factor predisposing to inflammation and insulin resistance [4]. Previous studies indicate that rare allele A might be associated with high TNF-α transcription causing greater insulin resistance which could influence the clinical outcome of atherosclerosis [5,6].

Rel or NF-kappaB (NF-kB) proteins comprise a family of structurally-related eukaryotic transcription factors that are involved in the immune and inflammatory responses and are implicated in number of disease states, including heart disease.

NF-κB regulates host inflammatory and immune responses [7] by increasing the expression of specific cellular genes encoding at least 27 different cytokines and chemokines, receptors involved in immune

recognition such as members of the MHC, proteins involved in antigen presentation and receptors required for neutrophil adhesion and migration [8]. NF-κB regulation of genes involved in the inflammatory response likely plays an important role in the initiation and progression of atherosclerosis.

Material and Methods

It was Descriptive Observational case control study conducted in the Department of Biochemistry in Lady Hardinge Medical College in collaboration with the Department of Cardiology, GB Pant Hospital after approval by the ethical committee of LHMC, New Delhi. We enrolled 50 non diabetic cases diagnosed with angiographically significant atherosclerosis (>50% obstruction in coronary artery) and 50 age and sex matched controls with insignificant atherosclerosis (<50% obstruction) on angiography after informed written consent. The patients on statins were excluded from the cases. The study was approved by institutional ethical committee of LHMC.

Venous blood sample was collected from the subjects under sterile condition after overnight fasting of 8-12 hrs. The plasma samples were stored at -20°C till subsequent analysis for TNF-α and NF-κB. Special investigation like TNF-α was estimated by ELISA using kit from Diaclone Research (France) while NF-κB was estimated using a kit from Biomedical Medical assay (Beijing, China).

To determine the TNF-α -308 G/A gene polymorphism, we extracted genomic DNA from the leukocyte of whole blood sample using the QI Amp DNA Blood Kit (Qiagen, Hilden, Germany). The extracted genomic DNA was then amplified by PCR using following primers:

Forward Primer: 5'-AGGCAATAGGTTTTGAGGGCCAT-3' (23 bp)

Reverse Primer: 5'-TCCTCCCTGCTCCGATTCCG-3' (20 bp)

PCR was carried out in a final volume of 25 µl. The final concentration of reaction mixture had 1.5 mM $MgCl_2$, 0.4 mM each dNTPs, 0.4 µl of each primer, 5 U of Taq Polymerase and 2 µl of genomic DNA. PCR program comprised of Initial Denaturation 95 for 5 min, followed by Cycle Denaturation 94 for 30 sec, Cycle Annealing 60 for 40 sec, Cycle Extension 72 for 30 sec each for 32 cycles and then Final Extension at 72 for 7 min.

The resulting PCR product (10 µl) was digested with 5 units of NcoI restriction enzyme at 37 overnight and electrophoresed on 10% non-denaturing polyacrylamide gel. Due to 308G/A polymorphism, there are three genotypes, GG (wild type), GA (heterozygous) and AA (mutant type).

Statistical Analysis

It was done by using SPSS (statistical package for social sciences) 20 version. All the data was expressed as mean ± SE of mean. The p value of <0.001 was considered highly significant.

The data obtained was compared between two groups by student t-test. Binomial regression analysis was applied to establish relationship between dependent and independent variables.

Results

In the study the mean plasma TNF-α level in the study group (cases) was 362.20 ± 33.43 pg/ml and in the control group was 186.94 ± 25.07 pg/ml. The difference between the two was statistically significant (p value=0.000*) (Table 1).

The mean serum NF-κB level in the study group (cases) was 0.33 ± 0.10 and in the control group was 0.29 ± 0.11 ng/ml which was statistically significant (p=0.04) (Table 1). Upon binomial logistic regression analysis, NF-κB emerged as the best predictor of severity of atherosclerosis (Odds Ratio=27) among other markers such as IL-6, Adiponectin, HOMA-IR and TNF-α. The RFLP revealed only wild type G/G homozygous genotype in 50 cases and 50 controls.

Discussion

Worldwide there are very few clinical studies evaluating the role of NK-κB in atherosclerosis. In 2001, Tak et al. [9] proposed that nuclear factor-κB (NF-κB) is an important regulator of inflammation and plays a key role in inflammatory diseases. In 2004 Jian Jun et al. [10] reported that NF-κB were raised in cases as compared to controls (p<0.01) in Chinese patients with stable angina. In support of this Gerondakis et al. [7] proposed that NF-κB increases the expression of specific cellular genes encoding for cytokines, major histocompatibility complex (MHC), proteins for antigen presentation, and receptors for neutrophil adhesion and migration [7], thus playing a vital role in intiation and progression of atherosclerosis.

In the study the mean plasma TNF-α level in the study group (cases) was 362.20 ± 33.43 pg/ml and in the control group was 186.94 ± 25.07 pg/ml. The difference between the two was statistically significant (p value=0.000*) (Table 1). With the increasing recognition that inflammatory mechanisms play a key role in the pathogenesis of atherosclerosis [11], attention has been focused on the role of tumour necrosis factor-α in patients at risk for the development of atherosclerosis. Our findings are in accordance to Ross and Skoog et al. [11,12] who have also implicated TNF-α as an important contributor to the development of atherosclerotic lesions by promoting the expression of adhesion molecules on endothelial cells and initiating inflammatory cascade inside the arterial wall.

In 2008, Kosmala et al. [13] also observed significant rise in TNF levels in stable IHD cases. This signifies that TNF-α is an important novel marker of inflammation and has a potential in identifying individuals at high cardiovascular risk.

In my study only wild type G/G homozygous genotype were found in 50 cases and 50 controls. No G/A heterozygous or A/A homozygous were found. Higuchi et al. [14] in a study on 575 Japanese found allele frequency of 98.3% of -308G and 1.7% of 308A. Chu et al. [15] examined 535 Chinese individuals with CHD, out of which 102 heterozygotes for mutant allele A were detected and 5 homozygotes.

Therefore inability to find polymorphism could be attributed to relatively low frequency of A allele as supported by the Chinese studies. In 2011, Lal et al. [16] did the similar 308 G/A promoter region polymorphism of TNF-α gene study in patients with metabolic syndrome in which only 3 heterozygote for mutant allele A and 1 homozygote in 50 cases and 50 controls, the allele frequency being quite similar to that observed in our study. It is possible that the significantly high levels of TNF-α in the cases is attributed to low grade inflammation rather than genetic polymorphism in Indian population.

Our study also shows G allele homozygosity is neither associated with severity of atherosclerosis nor increase in TNF-α levels. Further studies on a larger sample size are required for in depth evaluation role of TNF-α polymorphism in Atheroslerosis.

Parameter	Study Group	Mean	S.E.M	p Value
TNF-α (pg/ml)	Case	362.20	33.43	0.000*
	Control	186.94	25.07	
NF-κB (ng/ml)	Case	0.33	0.014	0.050*
	Control	0.29	0.015	
*p value ≤ 0.05 is considered statistically significant.				

Table 1: Parameters in study population.

Conclusion

Thus, we postulate that, an inflammatory pathology has an important role in atherosclerosis and it can be assessed by biomarkers TNF-α. Hence markers of micro-inflammation can indicate the atherosclerotic risk in an individual. The novel marker NF-κB which is a mediator of inflammation and insulin resistance pathways also has an important role in IHD. We could not find any polymorphism in genotypes of cases and controls as sample size could be a limiting factor in our study.

So, these findings suggest that inflammation promotes atherogenesis in an individual predisposed to cardiovascular risk. This indicates that these novel biomarkers of inflammation need further evaluation in larger sample size for better non-invasive diagnosis of atherosclerosis.

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