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IDENTIFICATION AND ANALYSIS OF NOVEL MUTATIONS IN NFKBIL1 GENE IN PATIENTS WITH MYOCARDIAL INFARCTION (MI) IN SOUTH INDIA

Cardiology

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ABSTRACT

NF-kappa-B inhibitor-like protein 1(NFKBIL1) gene located on chromosome 6p21.33 is a transcription factor of importance in normal inflammatory and immune responses.NFKBIL1gene regulates the transduction pathway in immune response, in atherosclerotic plaques and is implicated in the pathogenesis of atherosclerosis and myocardial infarction (MI). The NFKBIL1 gene is regulated through IkB kinase proteins and any variations in this delicate balance can interfere with normal NFKBIL1 functions. Mutations in the NFKBIL1 gene result in improper functioning of transcription factor which is associated with increased susceptibility to MI. Hence we studied 100 patients diagnosed with MI and 100 apparently healthy control subjects to identify the mutations in NFKBIL1 gene. The genomic Deoxyribo Nucleic Acid (DNA) was isolated from the MI patients and control subjects and exon 2 active site of NFKBIL1 gene was polymerase chain reaction (PCR) amplified and variations observed in the Single-strand conformation polymorphism (SSCP) gel of PCR products were sequenced. The sequence analysis of exon 2 of NFKBIL1gene showed novel mutations in 4 patients c.283G>A; p.(Asp95Asn), c.293G>T; p. (Gly98Val), c.323A>G; p.(Gln108Arg) and c.331G>T; p.(Asp111Tyr) respectively. Further, structural analysis showed mutations in exon 2 of NFKBIL1 gene affects the functioning of ankyrin repeat regions and Adenosine triphosphate (ATP) binding domains, resulting in non-functional NFKBIL1 protein in these patients and resulting increased risk of MI.

KEYWORDS

NFKBIL1 gene, Myocardial infarction, ATP binding domain, PCR, SSCP.

Introduction

Myocardial infarction (MI) is a manifestation of coronary artery disease[1]. MI is a complex disease with a strong genetic component. The pathogenesis of coronary atherosclerosis involves inherited, behavioral and environmental factors. Prevalence of MI is higher in individuals with > 65 years of age, but younger individuals are also reported to suffer from MI. This condition associated with heritability. Thus, MI in young is a promising phenotype for genetic mapping[1,2]. The single-nucleotide polymorphisms (SNPs) are used as markers and candidate gene analyses and genome-wideapproaches have exposed multiple susceptibility loci for Mi[3].

The NFKBIL1 gene is a key transcription factor in volvedin regulation of immune responses. This gene is located in the human leukocyte antigen (HLA) class III region on the short arm of chromosome 6 and encodes a protein, inhibitor of kB-like protein (IkBL), which shows a partial homology to inhibitor of kB (IkB)[4].The various studies have demonstrated the association between genetic variations inNFKBIL1 gene and susceptibility to inflammatory diseases like MI. Inflammatory processes are thought to promoteatherogenesis from early lesion formation to unstable plaquerupture[5]. This gene mediates a large variety of inflammatory and antiviral responses in the secondary lymphoid organs during development and activates signalling pathways for cell survival, differentiation and death[6, 7]. This also has multiple functions in regulating the immune system which plays a critical role in the pathogenesis of plaque rupture which precedes acute Mi[8]. Diseases attributable to atherosclerosis and other vascular etiologies, such as coronary artery disease, aortic aneurysm formation and cerebral infarction carry a high risk of death. In these instances, a family history has been identified as an independent predictor of disease development. Knowledge of genetic risk factors mediating the healing response to injury and inflammation, and those contributing to atherosclerosis has influenced our understanding of the pathophysiology of vascular and cardiovascular disease[9].

The SNP *–63T >A* in the promoter region of NFKBIL1is linked with the risk of myocardial infarction in Japanese and European population[5,10]. This SNP was reported to affect nuclear factor binding and or transcriptional activity and thus may influence the levels of gene expression and cell physiology. In view of the association between single-nucleotide polymorphisms ofNFKBIL1gene with an increased risk of MI in Japanese and European population[5, 10] and no Indian studies to suggest this association our study aimed to characterize the NFKBIL1gene polymorphisms in patients with MI.

Materials and methods

2.1. Patients and control sample collection

Peripheral blood was collected from 100 patients aged above 18 years diagnosed with acute coronary syndromes (ACS)who were admitted in the Department of cardiology, Sri Venkateswara Institute of Medical Sciences, Tirupati and 100 apparently healthy age and sex matched controls were recruited into the study during the period from October 2015 to March 2016. Patients presenting with ST-Elevation myocardial infarction(STEMI), Non-ST- Elevation myocardial infarction (NSTEMI), unstable angina and chronic stable angina, were included in the study. Patients with co-morbid conditions such as renal, lung, liver, central nerves system (CNS) other systemic illness, pregnant womenand patients unwilling to give an informed consent were excluded. Written informed consent was obtained from all the study

subjects. The study was cleared by institutional ethics committee (IEC Proceedings: Ref Roc No: A&E/08/IEC/SVIMS/09 and IEC No. 486, Dated 14.09.2015).

2.2.Biochemical analysis

Plasma lipid profile including total cholesterol, high density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and triglycerides were assessed Total cholesterol, HDL cholesterol and triglycerides were estimated and LDL & VLDL cholesterol were calculated by Friedewald's formula[11].

2.3. Mutation analysis

2.3.1. Isolation of genomic DNA

Pure, high molecular weight genomic DNA was extracted from peripheral blood of cases and controls using the standard salting-out procedure[12].The isolated genomic DNA was quantified spectroph otometrically and analysed by running in 1% agarose gel electrophoresis[13].

2.3.2. PCR amplification of NFKBIL1 gene

The exon 2 of *NFKBIL1* gene (277 bp) which is located in the active site of *NFKBIL1* gene was PCR amplified by using the Forward primer: 5'-CCCAAGAGTTCCATGGCCTC-3' and Reverse primer:5'-CATCTGGGCCCTGGCGGGCA-3'which were designed from the human genome database. The PCR amplification was performed for control and case samples, using 50 µl mixture containing 100 ρMol of each primer, 100 µM of dNTPsmix, 10 mMTris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 1 U of Taq DNA Polymerase (Merck Biosciences pvt Ltd) and 0.5 µg of genomic DNA. Amplification parameters included an initial denaturation step for 5 min at 94°C; 35 cycles of PCR, 94°C for 40 seconds of denaturation, 60 seconds of annealing at 62°C and 50 seconds of amplification at 72°C which was followed by a final extension step at 72°C for 5 min in a Master cycler gradient Thermocycler (Eppendorf). The amplified products were analysed on 1.5% agarose gel electrophoresis[14].

2.4. Single strand conformational polymorphism (SSCP)

The PCR products of cases and controls were mixed with 8.5 µl of denaturing solution (0.1% sodium dodecyl sulphate (SDS) in 10 mM Ethylenediaminetetraacetic acid (EDTA)) and the electrophoresis was carried out at 10°C at 8 V/cm^2 for 5 hours. The SSCP gel was stained with 0.5 µg/mL ethidium bromide solution for 1 hour at room temperature. This was followed by destaining with autoclaved distilled water and visualization of the bands under UV trans-illuminator. Results were recorded in Vilber Lourmat gel documentation system, France[15].

2.5. Sequence analysis

The variations observed in the SSCP gel of PCR amplified products of cases were purified and sequenced by chain terminating method at Eurofins Genomics India Pvt Ltd, Bangalore[16].

2.6. Screening and identification of mutations

The obtained sequences of exon 2 of *NFKBIL1* gene were compared to the control sequences. Multiple sequence alignment was performed by using ClustalX tool to identify the mutations in the sequences. *NFKBIL1* gene nucleotide sequences were translated into protein sequences and compared to identify the amino acid variations in the protein sequences. The variations observed in exon 2 of *NFKBIL1 gene* sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov /projects/Sequin/)[16].

2.7. Structural analysis

2.7.1. Preparation and processing of control and mutated NFKBIL1 structures

The complete X-Ray crystallographic structure of NFKBIL1 was not available in the protein data bank (PDB) and therefore the NFKBIL1 structure was built by using homology modeling method. The built X-Ray crystallographic structure of NFKBIL1was subjected to initial elegance such as removal of water molecules and heteroatoms, addition of polar hydrogens, and finally the protonation of the structure. Then it was put for energy minimization in Gromacs5.0 (http://www.gromacs.org/)software [17]. The mutations identified in the NFKBIL1gene were introduced into the respective positions in the energy-minimized NFKBIL1 structure and again energy minimization was carried out. After energy minimization, the coordinates of the mutated NFKBIL1structure was saved in PDB file.

2.7.2. Structural superimposition of control and mutated NFKBIL1 structure

The comparative structural prediction of control and mutated NFKBIL1structures was carried out to identify the variation in the domain and non-domain regions of structure. The PyMOL program was carried out which defines the structural similarity score as the log-odds of two probabilities using a scheme similar to Dayhoff's amino acid substitution score. An alignment of superimposed structures and similarities was predicted by root mean square deviation (RMSD) values. The positions of alpha helix, beta sheets, interacting residues and active site were studied through PyMOL[18].

2.7.3. Molecular Dynamics simulation

The energy-minimized control and mutated NFKBIL1 structures were subjected to Molecular Dynamics (MD) simulations individually by using the Gromacs5.0 (http://www.gromacs.org/)[17]. Based on the generated trajectory, a residue fluctuation profile (mean-square-fluctuation) was calculated.

2.7.4. PDBSum conformational analysis

The energy stabilized control and mutated NFKBIL1 structures were analyzed for comprehensive structural variations through PDBSum analysis[19]. The conformational variations were measured and correlated for control and mutated NFKBIL1structures in terms of strands, helices, helix–helix interactions, β-turns, sheets, β-α-β units, hairpins, β-bulges and γ-turns.

Results

3.1. Baseline Characteristics

In the present study,100 cases and 100 healthy controls were studied. Demographic data collected from all the participants is summarized in Table1 for cases and controls.

Mean age of patients group was [51.9±10.1] years and control group was [51.2 ±10.4]years. In patients group 77 were males and 23 were females and, in control group also 77 were males and 23 were females. By chance majority of the cases were males (77%). Alcohol addiction was the most prevalent risk factor in CAD group followed by diabetes mellitus (21%), hypertension (18%) and obesity (6%) (Table 1).

In the study group(CAD) most of the patients presented with ST-segment elevation myocardial infarction (STEMI: 54%) whereas Non-STelevation myocardial infarction(NSTEMI) and unstable angina (USA) were present in 31% and 15% of the patients respectively.

In our cases, single vessel disease (SVD), double vessel disease (DVD), triple vessel disease (TVD) and normal coronaries were observed as 70%, 18%, 4% and 8% of the patients respectively. Left anterior descending artery (LAD) was the most frequently found infarct related artery in the study group.

Comparison of lipid profile parameters showed significantly higher levels of total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol and significantly lower level of HDL-cholesterol in patients group compared with control group ($p \leq 0.05$).

3.2. Mutation analysis

The PCR products of patients and controls were analysed by running SSCP gel and variation observed in SSCP gel, were sequenced (Fig. 1). The exon 2 of *NFKBIL1* gene (277 bp) was PCR amplified from 100 patients and 100 controls. Sequence analysis of *NFKBIL1* gene showed novel missense mutations in 4 patients (P75, P87, P92 and P96). Patient P75 showed novel c.283G>A mutation which resulted in p.(Asp95Asn) protein variation (Gen Bank accession no. MF477890), P87 showed novel c.293G>T mutation which resulted in p.(Gly98Val) protein change (Gen Bank accession no. MF477891), P92 showed novel nc.323A>G mutation resulted in p.(Gln108Arg) protein change (Gen Bank accession no. MF477892) and P96 showed novel c.331G>T mutation leading to p.(Asp111Tyr) protein variation (Gen Bank accession no. MF477893) compared to control (Gen Bank accession no. MF477889) (Table2 and Fig. 1).

3.3. Structural analysis

The built mutant NFKBIL1 structure when superimposed with the control NFKBIL1 structure, exhibited extensive variations with an RMSD values in the domain and non-domain regions, with variable number of strands, helices, helix–helix interactions, β-α-β units, hairpins, β-bulges, β-turns, and γ-turns and these results explain the

major conformational changes in the mutant NFKBIL1structure (Fig. 2). Mutations in the NFKBIL1 gene affects the functioning of ankyrin repeat regions and ATP binding domains and results in non-functional NFKBIL1 protein in patients.

3.3.1 MD simulations of wild-type and mutated NFKBIL1 structures

The MD simulated wild-type and mutated NFKBIL1 structures were examined for their energy variations. The wild type NFKBIL1 structure was found to be stabilized at 5000 Kcal/mol, whereas the mutated (P75, P87, P92 and P96) NFKBIL1structures showed variable energy values due to the effect of these mutations in exon 2 of NFKBIL1gene resulting in unstable protein formation in the patients.

3.3.2PDBSum analysis of wild-type and mutated NFKBIL1 structures

The PDBSum conformational analysis of wild-type NFKBIL1 structure showed presence of 8 strands, 12 helices, 6 sheets, 4 β-hairpins, 43 β-turns, 3 γ-turns, 3 β-bulge and18 helix–helix interactions,. Whereas, in the mutated NFKBIL1 structure, variable number of sheets, β-bulges, strands, helices, β- hairpins, helix–helix interactions, β-turns, and γ-turns were observed. These results conclusively elucidate that the mutations in the NFKBIL1 gene causes major structural variations which affects the functioning of NFKBIL1 structure leading to high risk of MI in these patients.

Discussion

Myocardial infarction (MI) is one of the leading causes of death in the world. Its pathogenesis includes chronic formation of atheromatous plaque inside the wall of the coronary artery and acute rupture of the plaque, precipitated by number of inflammation-mediating molecules, including NFKBIL1[5,20]. The genetic polymorphism in the NFKBIL1 gene, potentially leading to lower secretion ofNFKBIL1 transcription factor, would then affect the degree of inflammation, smooth muscle cells and macrophages in human atherosclerotic lesions, expressing through MHC class III region[21].

Mutations in the lymphotoxin alpha gene (LTA),which encodes a cytokine of the immune and inflammatory response, was first identified as a risk factor for myocardial infarct (MI) in the Japanese population by Ozaki et al [10]. Genetic variation in the NFKBIL1 gene located near to the LTA gene on chromosome 6 on a shared haplotype block and having homology to the IkB family of NFkB regulators, exert profound influence on the immune and inflammatory responses in MI patients.In addition, the SNP's within the NFKBIL1 genomic region (6p21.3) have been reported to be linked with the risk of myocardial infarction in Japanese population[5].

Therefore, in the present study exon 2 which encodes active site of NFKBIL1 genes was PCR amplified and sequenced.The sequence analysis of exon 2 of NFKBIL1gene showed novel missense mutations in 4 patients resulting in p.(Asp95Asn), p.(Gly98Val, p.(Gln108Arg), p.(Asp111Tyr) protein change respectively. Similar to our study, Koch et al., 2007[2] included 3657 European patients with myocardial infarction and 1211 control individuals and studied LTA, BAT1 and NFKBIL1 genes. The sequence analysis of BAT1 gene showed23C>G mutation, NFKBIL1 gene showed 63T>A mutation and LTA gene showed 162G>A, 252G>A which resulted in Thr26Asn protein change which was associated with moderately protective effects against myocardial infarction ($P < 0.045$). The genetic analysis of European and Japanese population suggested that homozygosity of the minor alleles of the LTA, BAT1 and NFKBIL1 SNPs conferred increased risks of MI[2,18]. Our results also confer with these reports that mutations in NFKBIL1 gene are one of the causative reasons for increased risk of MI. Further, structural and functional analysis also strongly indicates mutations occurred in active sites of NFKBIL1 protein leading to non-functional protein in MI patients affecting the functioning of pro-inflamatory cytokines and leading to high risk of MI.

In conclusion, novel mutations in the NFKBIL1 gene resulted in structural and functional changes in transcription factor leading to increased risk MI in our study group. Further, studies with larger sample size are needed to establish the impact of NFKBIL1gene polymorphisms as a risk factor of MI.

Table 1. Baseline characteristics of the Study and Control groups

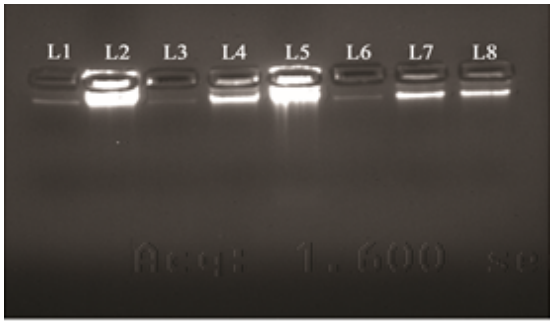
Variable	MI group (n=100)		Control group (n=100)	
	Male	Female	Male	Female
Age, years	50.6±10.0	56.1±10.0	50.5±10.4	53.7±10.2
Male	77	23	77	23
Alcohol addiction, n (%)	34	00	00	00
Diabetes mellitus, n (%)	21	09	00	00
Hypertension, n (%)	18	09	00	00
Family h/o MI, n (%)	18	03	00	00
Obesity, n (%)	6	00	0	0

MI: Myocardial infarction.

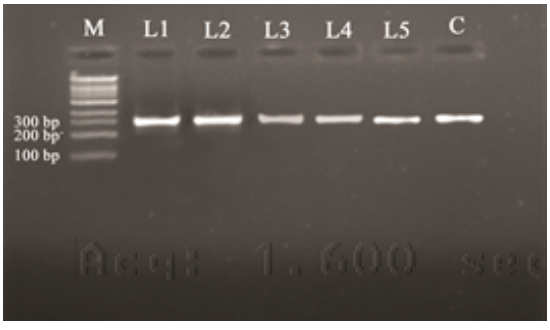
Table 2. Mutations in exon 2 of NFKBIL1 gene

Patient ID	Nucleotide change	Protein change	Novelty	Mutation type	GenBank Accession No.
P75	c.283G>A	p.(Asp95Asn)	Novel	Missense	MF477890
P87	c.293G>T	p.(Gly98Val)	Novel	Missense	MF477891
P92	c.323A>G	p.(Gln108Arg)	Novel	Missense	MF477892
P96	c.331G>T	p.(Asp111Tyr)	Novel	Missense	MF477893
Control	No nucleotide changes were observed in exon 2	-	-	-	MF477889

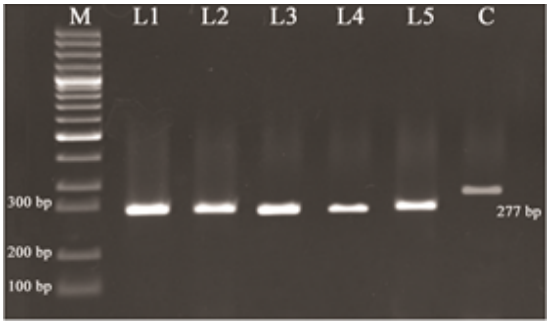
A



B



C



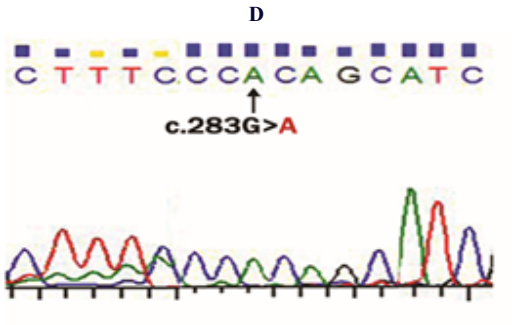
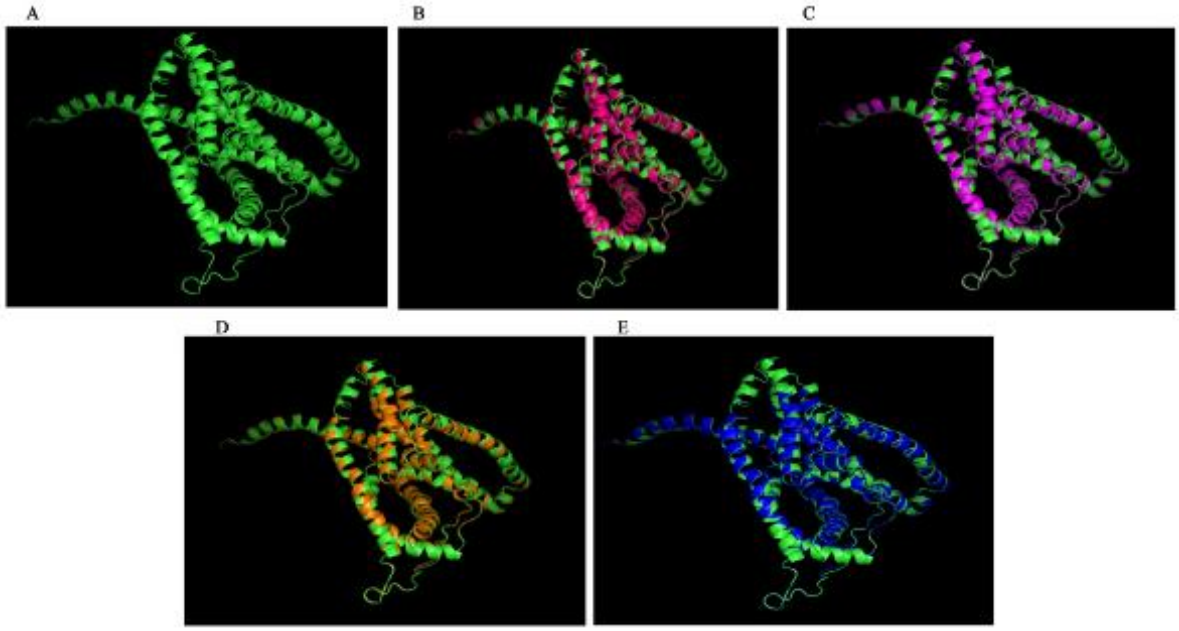


Fig 1A. Genomic DNA isolation from patients and controls, B. PCR amplification of exon 2 of NFKBIL1 gene using FP and RP primers following the method described, Lane M 100 bp ladder obtained from Merck Biosciences Pvt. Ltd, lane L1-L5277 bp PCR amplified products from the patients and lane C PCR amplified product from control. C. The electrophoretogram showing SSCP analysis of exon 2 of NFKBIL1 gene of PCR amplified products, Lane M 100 bp ladder obtained from Merck Biosciences Pvt. Ltd, lane L1-L5 Mutants and lane C control. **D.** The chromatogram showing the variations in the exon 2 of NFKBIL1 gene in MI patients.

Fig 2. Structural analysis of NFKBIL1 gene. **A.** PyMOL view of wild-type NFKBIL1 structure, **B.** Superimposed conformations of wild-type



(green color) with mutated p.D95N NFKBIL1 structure showing RMSD 1.193Å, **C.** With mutant p.G98V NFKBIL1 structure showed RMSD 1.181 Å, **D.** With mutant p.Q108R NFKBIL1structure showed RMSD 1.190Å and E. Mutant p.D111Y NFKBIL1 structure showing RMSD 0.232Å.

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