Cardiology and Cardiovascular Research
2017; 1(2): 62-66
http://www.sciencepublishinggroup.com/j/ccr
doi: 10.11648/j.ccr.20170102.16

Role of Polymorphism in P*LA2G7* Gene in Pathogenesis of Atherosclerosis in Patients of Kashmir Valley India

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To cite this article:

Nazia, Tehseen Hassan, Showkat Ahmad Bhat, Ishraq Hussain, Muneeb U Rehman, Sabhiya Majid, Roohi Ashraf, Sheikh Bilal Ahmad. Role of Polymorphism in PLA2G7 Gene in Pathogenesis of Atherosclerosis in Patients of Kashmir Valley India. *Cardiology and Cardiovascular Research*. Vol. 1, No. 2, 2017, pp. 62-66. doi: 10.11648/j.ccr.20170102.16

Received: April 29, 2017; **Accepted**: May 6, 2017; **Published**: June 15, 2017

Abstract: *Background*: Coronary artery disease (CAD) also known as atherosclerotic heart disease. Atherosclerosis is a multifactorial progressive disease manifested by the focal development within the arterial wall of lesions the atherosclerotic plaques. The *PLA2G7* gene polymorphism is related to enzyme activity which is related to the pro atherosclerotic abilities. This study was designed to investigate an association of this polymorphism with coronary artery disease (CAD) and healthy subjects. *Material and method*: In this study, the *PLA2G7* genotype of 30 patients with coronary artery disease, and 20 healthy subjects were assessed by Polymerase chain reaction polymorphism (PCR-RFLP) method. On genetic level the role of polymorphism of *PLA2G7* gene has been widely studied across the world in different ethnic populations. *Results*: Our study shown 66% (20/30) of the Coronary Heart Disease cases showed homozygous and 26% (8/30) of the cases however showed heterozygous condition. Almost all 80% (16/20) of the normal samples showed heterozygous condition except in four cases where *PLA2G7* gene was found to be Homozygous Mutant two & Homozygous normal two. The allelic association of this polymorphism with Coronary Heart Disease was evaluated by χ^2 (Chi square) test and was found to be significant (P=0.006).Homozygous mutant condition of PLA2G7 gene was found to be certainly higher in Coronary Heart Disease Cases of above 60 years of age (80%), than ages below 60 years and in controls (16.6%) and was significant as p=0.005, compared to below 60 years of age (33.3%) and in controls (0%) and association was insignificant as p=0.4667. *Conclusion*: Our study suggests that the polymorphisms of *PLA2G7* gene act synergistically to increase the risk of Coronary Heart Disease. Furthermore, it should be noted that the sample size was relatively small in the studied population and so large-scale prospective studies are needed to confirm these findings.

Keywords: Coronary Artery Disease, Heterozygous, Homozygous & *PLA2G7 Gene*

1. Introduction

Atherosclerosis, largest killer in the world is the principal cause of coronary artery disease (CAD). CAD is a progressive disease generally begins in childhood and (manifests clinically) during the mean and the elderly ages. The term atherosclerosis derives from the Greek and refers to the thickening of the arteries (sclerosis, indurations) and the accumulation of lipids (ather, jelly, fatty material) that characterized the lesions. The sites most affected are the aorta and its main branches, the vessels of the cerebral circulation, lower limb and the coronary arteries, where the disease affects epicardial arteries causing reduced blood flow reserve. Atherosclerosis is a multifactorial progressive disease manifested by the focal development within the arterial wall in response to various deleterious insults (Millonig., *et al* 2002 & Kruth., *et al*, 1984). Diseases such as coronary heart disease (CHD) may be thought of as resulting from failure of adequate homeostasis within a physiological system. This mayoccur at the genetic level (e.g. gene

transcription) or due to an environmental exposure (e.g. smoking) or due to an imbalance between the two (Doll., *et al* 1966 & Talmud., *et al* 2000). Rarely CHD result from a single mutation in a single important homeostatic gene, such as those regulating lipoprotein syntheses. More often, a subject with CHD will have modest input from minor mutations in several different genes that modulate risk. These may control the expression of other genes or affect the structure or function of the transcribed protein. These functional gene polymorphisms account for much of the biological diversity in homeostatic systems. In their absence all humans would respond in an identical manner to an environmental challenge, and the risk of developing disease would be directly proportional to the environmental stimulus. We know that this is not the case. For example, some individual's exposed to cigarette smoke with an otherwise identical risk factor profile will go on to develop CHD, whilst others will not. Therefore, the well accepted view is that CHD is a multifactorial disorder, with both environment and genetic factors. (Hokanson., *et al* 1996 & Sandhu., *et al* 2008)Lipoprotein lipase plays a central role in lipid metabolism, hydrolyzing triglyceride-rich particles in muscle, adipose tissue and macrophages and generating free fatty acids and glycerol for energy utilization and storage (Talmud., *et al*, 2002&Beisiegel., *et al* 1991). It also has a key bridging role as a ligand in lipoprotein–cell surface interactions and receptor-mediated uptake of lipo-proteins (Beisiegel., *et al*, 1991).Any mutation resulting in a partial deficiency of LPL would thus result in a modest increase in plasma triglyceride concentration, with the increase being proportional to the degree of deficiency (Fisher., *et al*, 1997). There is need of such Polymorphic studies in genes related to CAD patho-physiology to clearly define attributable genetic risk.

2. Materials and Methods

Sample Collection: The blood samples of Cardiovascular Disease were collected in EDTA vials from the Department of Cardiology, Government Medical College, Srinagar. Sample containing vials were transported to laboratory on ice and were stored at -20°C for molecular analysis. The control group was apparently healthy volunteers. The information regarding gender, age and residence were collected from the record file of patients present in the hospital.

Molecular analysis:

Extraction of genomic DNA: DNA was extracted from all blood samples with Phenol-Chloroform Method (Joseph Sambrook and David W. Russell. 2001). The DNA samples were then saved and stored at -20°C for future use.

Qualitative and quantitative analysis of genomic DNA: The integrity of the genomic DNA was examined by gel electrophoresis, DNA in the gel was visualized with the help of Gel doc system (Biorad) under UV light and picture was captured by using CCD camera system. The quantity of DNA was determined by U.V. spectrophotometric method. Calculated using the following formula: Quantity of DNA (µg/ml) = (A_{260} x 50 x Dilution factor)

The ratio of 260/280 was calculated and DNA with ratio of 1.7 – 1.9 was considered for the future use. DNA was alliqouted into four tubes so as to protect damage from freeze thawing and stored in -20°C freezer for longer duration of time.

PCR-RFLP: Genotyping was performed by Polymerase Chain Reaction (PCR) amplification of the polymorphic regions found in intron 6 followed by digestion of these amplified fragments with PvuII restriction endonuclease. *PLA2G7* genotypes determined with a PCR-RFLP method used primers for analysis as:

Table 1. *Primer sequence.*

Primers	Sequence	Amplicon size
Forward primer	5'-ATGGCACCCATGTGTAAGGTG -3'	440 bp
Reverse primer	5'-GTGAACTTCTGATAACAATCT -3'	

Table 2. *Volume and concentrations of different reagents used in PCR.*

S. No.	REAGENTS	CONCENTRATION	VOLUME
1	PCR Master Mix	2X	12.5 µl
2	Forward Primer	10pm/µl	1.1 µl
3	Reverse Primer	10pm/µl	1.1 µl
4	DNA	10ng	1.5 µl
5	Nuclease Free Water		8.8 µl
Total			25 µl

PCR Standardization Conditions: The reaction mixture was initially denatured at 95°C for 5min, followed by 35 cycles of 95°C for 1 min, annealing at 49.5°C for 1 min, extension at 72°C for 1 min,with final extension at 72°C for 7 min.

Table 3.*PCR cycle.*

Steps	Temperature °C	Time	Number of cycles
1.Initial Denaturation	95	5 min	1
2.Denaturation	95	1 min	35
3. Annealing	49.5	1 min	
4. Extension	72	1 min	
5. Final extension	72	7 min	1

The PCR products were then allowed to run on 1.5% Agarose gel to check the amplification of our desired product. The ladder (100 bp) was also run along. The amplified product was 440 bp in size.

RFLP (Restriction Fragment Length Polymorphism):The PCR products were digested by the PvuII restriction endonuclease enzyme.

Table 4. *Volume of different reagents used in RFLP.*

S. No.	REAGENTS	VOLUME
1	PCR product	10 µl
2	Nuclease Free Water	18 µl
3	10x Buffer G	2 µl
4	PvuII	2 µl

The prepared reaction mixture was mixed gently and spins down for few seconds & was incubated at 37^0C for 6 hours.

The digested PCR products were then electrophoresed through a 1.5% Agarose gel for at least an hour. Products were visualized by staining with ethidium bromide. The PvuII restriction site (intron 6) yields 330 bp and 110 bp fragments.

3. Results

Genotype determination: Detection of polymorphism in the *PLA2G7* gene was performed by PCR-RFLP analysis according to protocol conditions and primer sequences.

Genetic Analysis

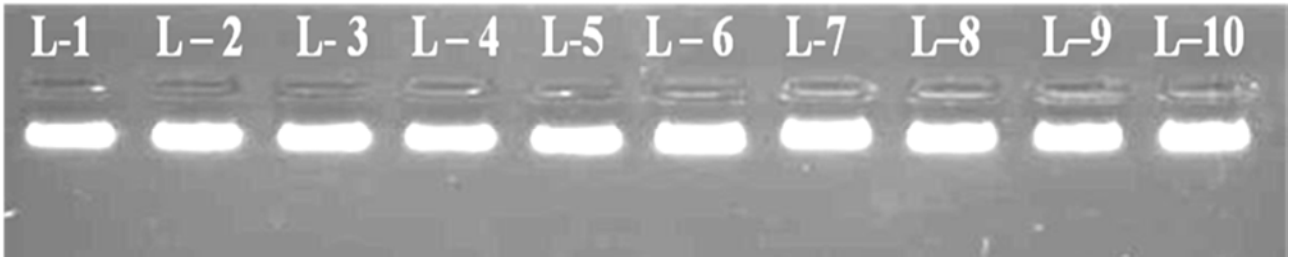

Figure 1. *Lane 1-10 showing the isolated DNA of case samples, run on 1% agarose gel.*

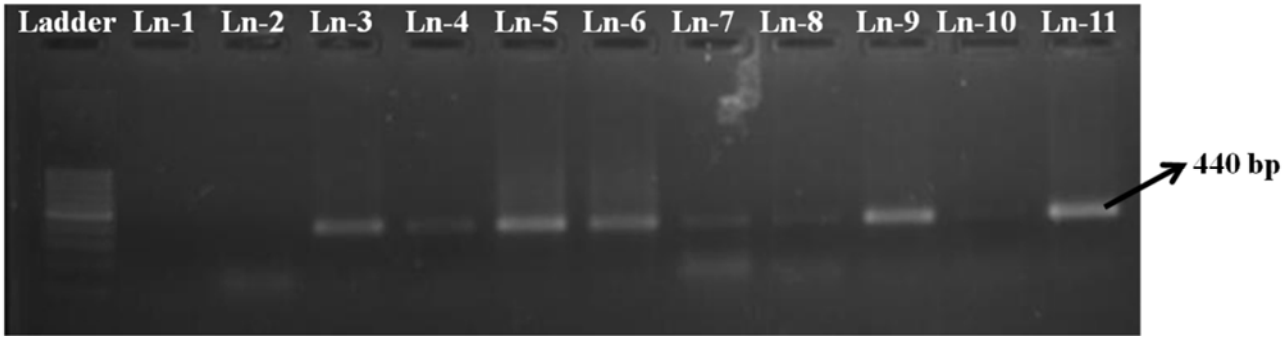

Figure 2. *(L1-L11)-PCR amplified product of coronary heart disease samples run on 1.5% agarose gel (Ladder0100bp).*

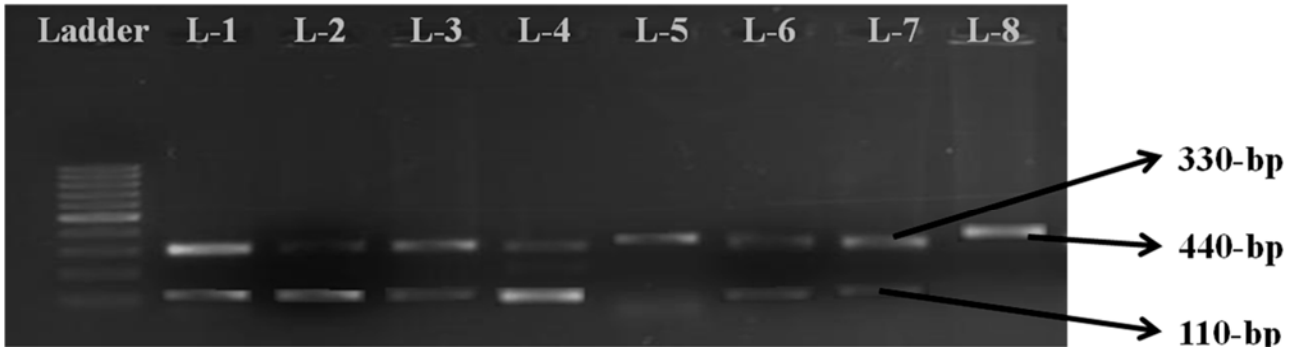

Figure 3. *PLA2G7-Genotype Analysis: The polymorphism of PLA2G7 gene was determined using polymerase chain reaction and Restriction Fragment Length Polymorphism.*

Table 5. *Frequency of coronary heart disease cases among different genotypes.*

CASES – 30	GENOTYPE	FREQUENCY
20	Homozygous Mutant	66% (20/30)
8	Heterozygous	26.6% (8/30)
2	Homozygous normal	6.6% (2/30)
CONTROLS – 20		
2	Homozygous Mutant	10% (2/20)
16	Heterozygous	80% (16/20)
2	Homozygous normal	10% (2/20)

The genotypes were designated pp, Pp and PP corresponding to 440bp, 330bp and 110bp respectively. The prevalence of Coronary Heart Disease was highest among homozygous pp patients and lowest in homozygous PP patients. The prevalence of Coronary heart disease in heterozygous subjects was intermediate between those of the two homozygous patient groups.

Restriction digestion was done to examine the genetic polymorphism in the *PLA2G7* gene. As shown in Figure 4, 66% (20/30) of the Coronary Heart Disease cases showed homozygous and 26% (8/30) of the cases however showed heterozygous condition. Almost 80% (16/20) of the normal samples showed heterozygous condition except four cases where *PLA2G7* gene was found to be Homozygous Mutant two & Homozygous normal two. The allelic association of this polymorphism with Coronary Heart Disease was evaluated by χ^2 (Chi square) test and was found to be significant (P=0.006).

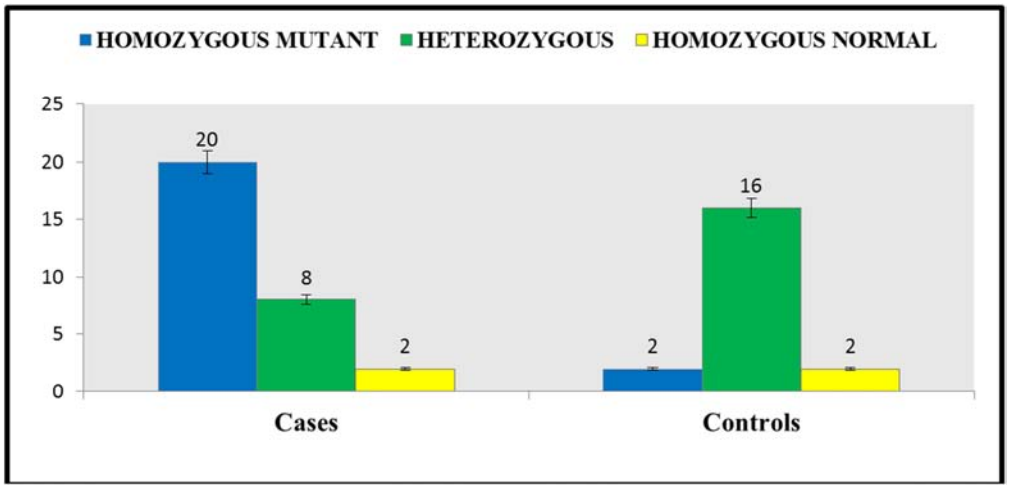

Figure 4. *Histogram representing restriction conditions of cases ofCoronary Heart Disease and normal controls.*

Table 6. *Frequency of coronary heart disease cases among different genotypes with respect to varying age.*

TOTALNUMBER OF CASES (30)			
AGE GROUP (years)	**CASES**	**GENOTYPE**	**FREQUENCY**
30-39	04	2--Homozygous Mutant	50% (2/4)
		1—Heterozygous	25% (1/4)
		1-- Homozygous normal	25% (1/4)
40-49	06	3--Homozygous Mutant	50%(3/6)
		2—Heterozygous	33.3%(2/6)
		1-- Homozygous normal	16.6%(1/6)
50-59	10	7—Homozygous Mutant	70%(7/10)
		3—Heterozygous	30%(3/10)
		0-- Homozygous normal	0%(0/10)
Above-60	10	8--Homozygous Mutant	80%(8/10)
		2—Heterozygous	20%(2/10)
		0-- Homozygous normal	0%(0/10)

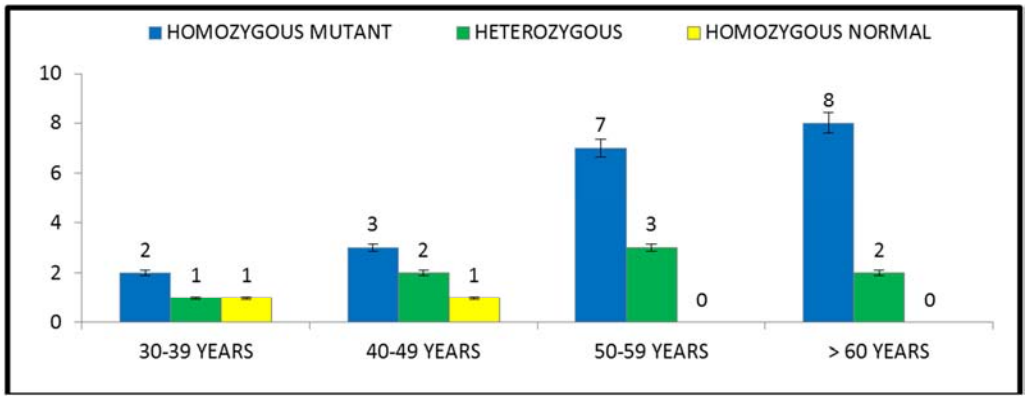

Figure 5. *Histogram representing age group polymorphism of PLA2G7 gene.*

Homozygous mutant condition of *PLA2G7* gene was found to be certainly higher in Coronary Heart Disease Cases of above 60 years of age, 8 out of 10 (80%), than ages below 60 years and in controls 3 out of 18 (16.6%) and was significant as p=0.005, compared to below 60 years of age 1 out of 3 (33.3%) and in controls 0 out of 2 (0%) and association was insignificant as p=0.4667.

4. Discussion

Our study showed a significant association of atherosclerosis in individuals with PLA2G7 polymorphism with the available data of CAD patients in Kashmir valley. Ours is not the first study to examine Lp-PLA2polymorphism associated with coronary artery disease; however it is the first to do so in Kashmir. Our study, similar to another prospective cohort study had results supporting the atherogenic effect of *PLA2G7* Polymorphism in atherosclerosisbut contradicted with few. Ping-Yen Liu, *et al* found that Platelet-Activating Factor Acetylhydrolase (*PLA2G7*) exon 11 gene polymorphism is functionally associated with coronary artery disease severity but not the onset of acute coronary

syndrome. A study by Jianget al, (2012) on 290 coronary heart disease patients, 198 non-CHD patients and 331 unrelated healthy volunteers were studied in case-control study of Han Chinese population and they found this gene to be closely associated as one of the risk factors. The European ancestry study over *PLA2G7*genotyping andlipoprotein-associated phospholipase A2 activity with Coronary heart disease risk I n 10494 cases and 15624 controlsalso showed a clear association with each other with high risk of CHD. Our study is however at variance with a study from MCet al, 2009, Pennsylvania, who found PAF-AH to be least associated with coronary artery disease. Their study also concluded that LP- PLA2 deserve further evaluation as risk factors for CAD.The implication of *PLA2G7* gene in CAD pathogenesis has been extensively studied in several ethnic groups. The actual mechanism of PAF-AH or sPLA2 response to inflammatory or atherosclerotic process, such as MI, is still not clear. Zheng GH *et al* (2012) have reported an in vitro study of inflammatory stimulation on liver cell system. They found that the expression of plasma PAF-AHmRNA and production of plasma PAF-AH protein were increased in the resident macrophages of the liver in response to inflammatory exposure. They also observed an up-regulation response of the plasma PAF-AH expression to be an important mechanism for elevating the local and systemic ability to inactivate PAF, sPLA2or oxidized phospholipids. We thus speculate that the PAF-AH may be responsiveness to ACS with highly inflammatory challenge, and thus modify the expression of oxidized phospholipids in the plasma.The present study is in agreement, undertaken with certain restrictions. Firstly, the sample size in our study was relatively small. Future investigation with more samples needs to be performed to confirm our findings. Secondly, only a single *PLA2G7* genetic marker was selected.Thirdly, although we tried our best to control the confounding factors that may affect the DNA integrity, thereexisted a possibility of an unknown factor that might confound the alteration.Thus, further polymorphic *PLA2G7* markers need to be evaluated, together with the other genetic markers involved in the Coronary artery disease. Coronary artery disease is a complex disorder where environmental and genetic markers both play an important role.

5. Conclusion

In summary, based on present scientific and clinical evidences, Lp-PLA2 appears to be a valuable biomarker for better discriminating patients with moderateor high CV risks.Our data suggest that the *PLA2G7* gene polymorphism is one of the risk factors for Coronary heart disease in Kashmiri patients and the gene polymorphisms may act synergistically to increase the risk. Given the central importance of LP-PLA2 to lipid metabolism and the prevalence of these polymorphisms, additional investigation is warranted. Furthermore, it should be noted prospective studies are to be needed to confirm, whether genotyping will be useful in individual risk assessment of Coronary artery disease.

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