Original article

Identification of genetic variant in the low density lipoprotein receptor gene causing familial hypercholesterolemia

Amber Hassan[1], Sadia Khan[1], Riffat Mehboob[2], Syed Amir Gilani[3], Maryam Altaf[1], Fridoon Jawad Ahmad[4], Humaira Waseem[5] and Riffat Rehman[6]

[1]Senior Lecturer, Research Unit, Faculty of Allied Health Sciences, University of Lahore, Lahore – Pakistan, [2]Professor, University Institute of Physical Therapy, The University of the Lahore – Pakistan, [3]Professor, Faculty of Allied Health Sciences, The University of Lahore, Lahore – Pakistan, [4]Professor, Biomedical Sciences, King Edward Medical University, Lahore, Pakistan, [5]Department of Health Professional Technologies, The University of Lahore, Lahore, Pakistan, [6]Lecturer, University Institute of Radiology Research Section, The University of the Lahore, Pakistan

Correspondence to: Ms. Sadia Khan, Email: sadiakhan0607@gmail.com

ABSTRACT

Background: Familial hypercholesterolemia (FH) is an autosomal-dominant genetic disorder characterized by mutation in three known genes. High serum low density lipoprotein (LDL) cholesterol levels results in the excess deposition of cholesterol in the tissues leading to atherosclerosis and great possibility of premature coronary heart diseases. The most common genetic aberration is seen in low density lipoprotein receptor (LDLR) gene located on chromosome 19. The *ApoE*, *ApoB*, *LDLR* and *LPL* genes are associated with lipid metabolism. Current study aimed to characterize the Exon 4 of the *LDLR* gene in Familial Hypercholesterolemia.

Materials and methods: After clinical evaluation, 20 samples were collected under sterile conditions from patients of different hospitals of Lahore having history of familial hypercholesterolemia and myocardial infarction. Organic method for DNA extraction was used. Primers were designed for *LDLR* Exon 4 and amplification of the Exon was carried out.

Results: The mean age of the patients was 47.6 years. All of the patients were tested for their lipid profile. The results indicate a mean total cholesterol of 356.3 mg/dl, LDL cholesterol of 251.1mg/dl, HDL cholesterol of 60.2mg/dl and triglycerides of 204.2 mg/dl. The genomic DNA was extracted from all 20 blood samples and was detected through agarose gel electrophoresis. A single band of 470bp with maximum concentration was obtained at 61°C. No SNPs were detected in the amplified regions of the exon 4 of *LDLR* gene in the selected samples

Conclusions: The current stud conclude that the early identification allows the individuals changes in lifestyle that includes dietary intervention, followed by drug treatment. This study will help the early detection and treatment of such cases and may ultimately reduce the incidence of mortality due to myocardial infarction.

Keywords:

Hypercholesterolemia, LDLR, mutations

INTRODUCTION

Familial hypercholesterolemia (FH) is because of the raised serum LDL cholesterol level resulting in the excess deposition of cholesterol in tissues, leading to atherosclerosis and high risk of premature coronary heart diseases.[1, 2] Hypercholesterolemia has an association with high risk of premature cardiovascular complications. It is a genetic caused by abnormality in low density lipoprotein receptor (LDLR) function, premature atherosclerosis, elevated plasma cholesterol levels due to mutations in three known genes.[3] The families with FH showed elevated LDL-cholesterol (LDL-C) levels that is accurate finding of FH and myocardial infarctions.[4] In FH elevated LDL-C form plaque in the arteries,[5, 6] resulting in the increased risk of coronary artery disease.[5, 6] High level of cholesterol sometimes result in the deposition of cholesterol deposits in the tendon also known as xanthoma or around the eye also known as xanthelasmas.[7] The cardiovascular disease (CVD) is most common coronary heart disease (CHD) resulting as angina, myocardial infarction (MI) and stroke because of high level of cholesterol.[8]

Heterozygous FH (HeFH) accounts 60-80% due to mutation in *LDLR, APOB and PCSK9* genes. Its occurrence is 1:500. Homozygous FH (HoFH) is rarer than the heterozygous FH.[3, 9] Its prevalence is 1:160,000 to 1:1,000,000. In mid-20s mostly HoFH

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Table 1. The oligonucleotide primers of exon 4 of *LDLR* gene

LDLR gene	Primers	Length	Tm	GC content	Product size
Exon 4	Forward primer CACACGGTGATGGTGGTCT	19bp	60.45	57.9%	470bp
	Reverse primer CCAGGGACAGGTGATAGGAC	20bp	59.38	60.0%	

patients suffer from CHD.[3, 9] Heterozygous FH increased 50% risk of CHD in men by the age of 50 and 30% in women by the age of 60%.[10] An autosomal recessive FH results because of mutation in *LDLRAP1.* A form of autosomal recessive FH that is caused by mutations in *LDLRAP1*, encoding for protein in clathrin-mediated internalization of the LDL receptor by liver cells.[11] Current study aimed to characterize the Exon 4 of the *LDLR* gene in Familial Hypercholesterolemia.

MATERIALS AND METHODS

After the ethical approval from Punjab Institute of Cardiology and University of Veterinary and Animal Sciences (UVAS), Lahore a structured questionnaire was used for personal record containing demographic data (age, sex and residency), clinical manifestations, diseased history and family history of patients.

Sample collection and DNA extraction: The FH already diagnosed patients on routinely based criteria and already done with complete lipid profile were selected. The lipid profile was only assessed to evaluate the clinical status. The peripheral blood samples about 3 ml of blood was collected in the EDTA tubes containing EDTA from all participating individuals and were stored at -20°C before DNA extraction. The DNA was extracted from all the blood samples. DNA extraction was done by organic method.[12] DNA template concentration and purification are the critical factors in polymerase chain reaction (PCR). The optimal concentration of template/µl for PCR is 15 to 50ng/µl. Therefore, after DNA extractions, both the concentration in ng/µl and 260/280 ratio for each sample were measured with Nano drop 2000 spectrophotometer (Thermo-Scientific).

Amplification of the *LDLR* gene and sequencing: Primers for the amplification of exon 4 were designed using Primer 3 software (Table 1).[13] For the amplification of *LDLR* gene from DNA samples by using the Polymerase Chain Reaction (PCR) the various annealing temperature (61-55°C) were tested using gradient PCR[14] on control samples. To analyze the polymorphic sites of exon 4 of *LDLR* gene, fragments were amplified in DNA thermal cycler (BIO-RAD, T100T^{M}). PCR amplifications were performed to detect the mutations on these sites and amplified products were electrophoresed on 1.2% agarose gel.[15] Invitrogen 1Kb plus DNA ladder (Catalog # 10787018) was used for the DNA quantification. After gel elution, PCR amplicons were sequenced Sanger Chain Termination method. To analyze the sequencing results, CHROMAS software was used after converting to FASTA format. These FASTA format sequences were aligned by using online BLAST software to detect any single nucleotide polymorphisms (SNP's).

RESULTS

Total 20 patients with a clinical diagnosis of heterozygous FH and family history of myocardial infarction were recruited from the Punjab Institute of Cardiology, Lahore. The selection criteria were based on clinical symptoms of FH showing the elevated cholesterol contents (5-10% chance of having *LDLR* gene mutation), coronary heart disease history in first degree relatives (40% chance of having mutation in *LDLR* gene) and tendon xanthoma (95% chance of having *LDLR* gene mutation)in male and female patients.[16] Control individuals were healthy normal lipidemic volunteers. The mean age of the patients was 47.6 years. All of the patients were tested for their lipid profile. The results indicate a mean total cholesterol of 356.3 mg/dl, LDL cholesterol of 251.1mg/dl, HDL cholesterol of 60.2mg/dl and triglycerides of 204.2 mg/dl. Table 2 summarizes the results of lipid profile of the study participants.

The genomic DNA was extracted from all 20 blood samples and was detected through agarose gel electrophoresis. The annealing conditions for the primers were optimized using temperature gradient thermocycler. A single band of 470bp with maximum

Table 2. Lipid profile of selected blood samples

Groups	Mean ± SD
Age	47.6 ± 12.2
Total cholesterol (mg/dl)	356.3 ± 96.8
LDL cholesterol (mg/dl)	251.1 ± 85.9
HDL cholesterol (mg/dl)	60.2 ± 47.2
Triglyceride (mg/dl)	204.2 ± 120.5

Table 3. Optimized conditions for the amplification of *LDLR* gene

Step	Temperature (°C)	Time (minutes)	Number of Cycles
Initial denaturation	95	5	1
Denaturation	94	1	35
Annealing	61	1	
Extension	72	1	
Final extension	72	1	1

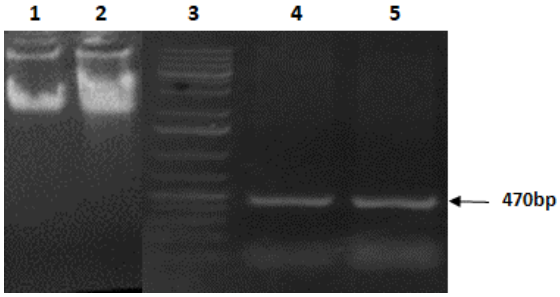

Figure 1. Screening of samples using optimized PCR conditions. Lane 1 and 2 shows genomic DNA; 3) DNA size marker; 4 and 5) amplified product.

concentration was obtained at 61°C, therefore, the temperature was selected for amplification for the patient samples. Table 3 shows the optimized cyclic conditions used for the amplification of *LDLR* gene while Figure 1 shows the gel diagram of genomic DNA and amplified product.

The amplified *LDLR* gene was eluted from the agarose gel and sequenced. The sequences were converted to FASTA format and submitted to NCBI BLAST database for any single nucleotide polymorphism (SNPs), however, no mutation was seen in the selected region of the LDLR gene.

DISCUSSION

Hypercholesterolemia is a widespread problem and a big challenge for healthcare professionals these days. In Pakistan about 10,000 cases of this disease is still under-diagnosed.[17] The present study gave representation of Pakistani data on familial hypercholesterolemia. We established a study for the characterization of the Exon 4 of the *LDLR* gene in familial hypercholesterolemia

During various studies in which mutations were investigated, up to 15% of any group of definite heterozygous FH in patients in the UK showed no mutation in *LDLR* and *APOB* gene.[2, 18] The Portuguese FH study showed that it was not possible to identify a mutation in about 50% of the clinical FH patient studied. In the patients with an unidentified mutation, it could be suggested that some defects in the *LDLR* gene remain undetected or that other genes may be involved.[19] Single nucleotide polymorphisms (SNPs) were not found in the Exon 4 of the *LDLR* gene in the selected population. The study conducted in Lebanon[20] and Spain[21] reported the exon 3 and 4 of *LDLR* involve in the FH.

In the previous reported work functional SNPs were associated with increased total and LDL-cholesterol. Considered together, *LDLR* mutations have been recognized as causing hypercholesterolemia, a common functional *LDLR* SNPs have been detected.[22, 23] Surprisingly, we did not observed xanthoma in patients; either tendon xanthoma are not being well diagnosed in FH patients are less susceptible to the development of tendon xanthoma for some environmental reasons. This study showed no *LDLR* mutations in the patients with hypercholesterolemia. By increasing the sample size and including the more exone regions might increase the possibility of finding common functional *LDLR* SNPs in hypercholesterolemic patients.[24] If such mutations detected, those results might provide insight into the genetic basis of cholesterol homeostasis and might lead to insight in cholesterol-associated diseases.[6]

As Pakistan is a multi-racial nation therefore this heterogeneous population is expected to carry a number of novel LDLR mutations. Recently, a triple nucleotide variant is reported in the LDLR gene that results in the production of a truncated protein lacking the b-propeller region[25], which was predicted to cause the observed cardiac complications including premature coronary artery disease leading to myocardial infarction.

CONCLUSIONS

The current study further defines the LDLR mutation spectrum that will result in timely management of the disease and understanding of the molecular mechanisms involved in disease pathogenesis. In future, this study will be helpful for identification of genetic variations responsible for hypercholesterolemia and cardiovascular disease in Pakistan. Genetic variations that will be found during the analysis of the genes may be already known or novel ones. But this data will be helpful for molecular assessment of cardiovascular disease and association of genetic variations with the other diseases

like diabetes mellitus in this part of world. The early identification allows the individuals changes in lifestyle that includes dietary intervention, followed by drug treatment. This study will help the early detection and treatment of such cases and may ultimately reduce the incidence of mortality due to myocardial infarction.

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