Genetics of *LDLR* Gene in Pakistani Hypercholesterolemia Families

Akhtar Ali[1], Ros Whittall[2], Masroor Ellahi Babar[1], Tanveer Hussain[1], and Steve E. Humphries[2]
[1] Virtual University of Pakistan, Lahore, Pakistan
[2] Center for Cardiovascular Genetics, University College London, United Kingdom
Email: {akhtar.ali, masroor.ellahi, tanveer.hussain}@vu.edu.pk, {r.whittall, steve.humphries}@ucl.ac.uk

***Abstract*—Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the three known genes. Low density lipoprotein receptor (LDLR) is considered as main contributor. We recruited clinically diagnosed 21 hypercholesterolemia families from Punjab-Pakistan. High resolution melting analysis used to screen the *LDLR* gene (all exons and promoter region) and variations were confirmed by restriction fragment length polymorphism and sequencing analysis. The mean of total cholesterol and LDL-cholesterol in the FH patients was 7.5±1.4mmol/l and 5.2±1.5mmol/l respectively. Seven patients showed synonymous variations in the sequence at position c.81T>C, c.993C>T, c.1413G>A, c.1617C>T, c.1725C>.T, c.1959T>C and c.2232A>G while one carried the non-synonymous change c.1171G>A resulting in the non-pathogenic p.(A391T) amino acid change. One common non-pathogenic variant c.1060+10C>G was found in the intronic region. In-silico analysis predicted c.1725C>T, c.1959T>C and c.2232A>G to be affecting the LDLR protein, by altering splicing sites as predicted by Human Splicing Finder and Mutation Taster software. Our findings suggest that ~15% (3/21) of FH patients in Pakistan with no detectable mis-sense mutation may carry pathogenic splicing variants in the LDLR gene sequence.**

***Index Terms*—LDL-cholesterol, synonymous, polymorphic, splicing site**

I. Introduction

Cardiovascular diseases are among the leading causes of mortality in the developing world. Cholesterol metabolism is considered as the leading risk factor. Family members having increased serum contents of Total Cholesterol (TC) or Low Density Lipoprotein Cholesterol (LDLC) and history of premature coronary heart disease in the first degree blood relatives are known affected with Familial Hypercholesterolemia (FH). Autosomal dominant inheritance pattern is observed in FH (MIM#143890). The TC contents in FH are above 7.5mmol/L and LDLC 4.9mmol/ (Marks *et al.* 2003).

Monogenic factor of FH is the mutations in three genes including *PCSK9 (proprotein convertase subtilitsin kexin type 9), APOB (apolipoprotein B) and LDLR (low density lipoprotein receptor)* (Hobbs *et al.* 1992; Abifadel *et al.* 2003; Rader *et al.* 2003). LDL molecule is a transmemberane structural protein located on the cell surface which provides cholesterol to the cells by facilitating the uptake and metabolism of low density lipoprotein molecules (Goldstein and Brown 2009). *LDLR* gene mutations are the predominant in the prevalence of FH.

The *LDLR* gene is located 19 chromosome comprising of 18 codons and genetic defect leads to maladaptive activity which execute the decreased metabolic clearance of LDL particles from the circulation which leads to the development of premature atherosclerosis (Jensen *et al.* 1999). Online data (http://www.ucl.ac.uk/fh/) updated in January, 2011 indicate that over 1741 genetic variations in the *LDLR* gene have been identified. These variations occur in promoter, introns and exonic regions of *LDLR* gene. About 20% of mutations have been identified in exon 4 than any other region of *LDLR.* Exon 4 is the longest exon and encode for ligand binding domain. Any missense variant in this exon is likely to be pathogenic (Leigh *et al.* 2008). We aimed to identify the genetic variants in the *LDLR* gene in the Pakistani hypercholesterolemia families.

II. Methodology

Families having hypercholesterolemia and cardiovascular diseases were identified and screened for lipid profile after written informed consent. FH was diagnosed based on Simon's Broom Register Criteria and characterized by increased plasma contents of TC 7.5mmol/L 7.5 mmol/L or LDL-cholesterol 4.9 mmol/L and premature coronary disease history in first degree relative (Marks *et al.* 2003). Twenty one families were identified and collected from different regions of Punjab province of Pakistan after their willingness. Detailed family history was taken out from each family. Blood samples were collected in serum collection tubes (company) from each willing member of the families. Serum samples were analyzed using the UV-spectrophotometry methodology for estimation of serum total cholesterol, triglycerides and high density cholesterol contents. LDLC was determined by the standard formula (Friedewald *et al.* 1972).

A. DNA Extraction and Amplification

All the samples were processed for leukocyte based organic method of DNA extraction (Sambrook and

Manuscript received March 6, 2019; revised August 12, 2019.

doi: 10.18178/ijpmbs.8.4.143-146

Russell 2001). DNA samples were quantified quantified using NanoDrop (Eppendrof, USA) and brought at the same concentration at 30ng/uL.

On the basis of pedigrees and lipid profiles, FH proband from each of 21 families were selected and analyzed using high resolution melting technique (HRM). HRM melting analysis of all was done using R-Corbett (Qiagen, Ltd). Primers from exon/ intron boundaries and promoter region of the *LDLR* gene were used for screening of FH. Reported primers and protocol for reaction mixture for HRM analysis was used (Whittall *et al. 2010)*.

B. RFLP Digestion

Restriction enzymes were selected using restriction mapping. The genotypes identified by high resolution melting and sequencing were confirmed by RFLP (restriction fragment length polymorphism) analysis. Polymorphic sites in exon 7, 10, 12 and 13 were digested with restriction enzymes. The restriction enzyme SmaI for rs12710260 (c.1060+10C>G) in the exon7, BsmaA1 for rs5930 (c.1413G>A) in exon 10, HincII for rs688 (c.1773C>T) in exon 12 and AvaII for rs5925 (c.1959C>T) were used following the manufacturer's protocol (New England, BioLabs inc. UK).

C. Data Analysis

Sequence results were aligned and analyzed by FinchTV and BioEdit software.

Conformance of allele frequency and level of significance for Hardy-Weinberg Equilibrium was tested by Chi-square test. Sequence variations were analyzed by SIFT, Polyphen2 and Mutation Taster to predict their significance.

III. Results

Lipid profiles of family members with strong family history of coronary artery disease or hypercholesterolemia were determined for this study. Mean values determined as given in the Table I.

TABLE I. Lipid Contents in FH Patients

Lipid	Mean	Standard deviation
Total cholesterol	7.4	±1.2
Triglycerides	2.1	±0.4
HDL- cholesterol	1.1	±0.1
LDL- cholesterol	5.8	±1.2

Probands of twenty one FH families were amplified and detected for heterozygous. Heterozygous DNA strands showed a different melting curve from homozygous. Melting curve analysis revealed deviation in exon 2, 7, 8, 10, 11, 12, 13 and 15 region showed deviation as given in the figure1 and confirmed by sequencing.

Heteroduplex in exon 13 suggested the polymorphic pattern. HRM results were confirmed by sequencing the selected samples with deviated curve and showed single nucleotide T>C change in exon 13 of *LDLR* gene (Fig. 1).

Sequencing of the selected deviated samples confirmed the changes as mentioned in the Table II. Exon 2, 7, 8, 10, 11, 12, 13, 15 showed polymorphism at c.81c.478, c.993, c.1171, c.1413, c.1617, c.1725, c.1959, c.2232. While one intronic polymorphism c.1060+10C>G identified.

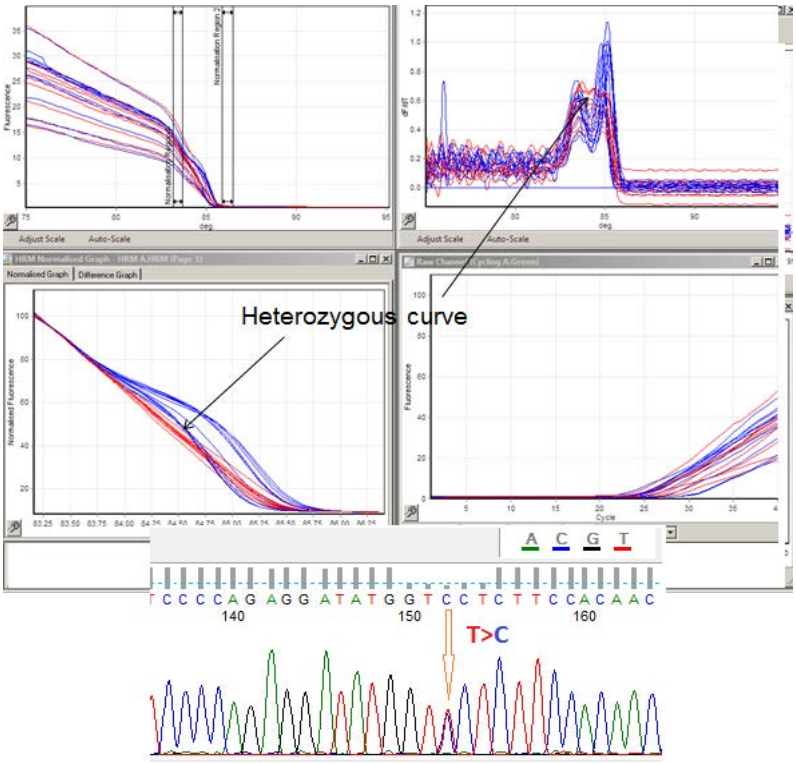

Figure 1. Melting curve and sequencing result of exon 13.

TABLE II. SUMMARY OF GENETIC VARIATIONS IN LDLR GENE IN FAMILIAL HYPERCHOLESTEROLEMIA

Exon	Position	Change	Protein change	Polyphen 2	SIFT	Mutation Taster	HSF
2	c.81	T>C	p.C27=	Benign	Tolerated	Polymorphism	no effect
7	c.993	C>T	p.D331=	Benign	Tolerated	Polymorphism	no effect
7	c.1060+10C>G			Benign			no effect
8	c.1171	G>A	p.A391T	Benign	Tolerated	protein (might) affected, splice site changes	site broken
10	c.1413	G>A	p.R344=	Benign	Tolerated	Polymorphism	no effect
11	c.1617	C>T	p.P539=	Benign	Tolerated	Polymorphism	no effect
12	c.1725	C>T	p.L575=	Benign	Tolerated	protein (might) affected, splice site changes	site broken
13	c.1959	T>C	p.V653=	Benign	Tolerated	protein (might) affected, splice site changes	site broken
15	c.2232	A>G	p.R744=	Benign	Tolerated	protein (might) affected, splice site changes	site broken

One non-synonymous single base change G>A results in codon change GCC>ACC and alanine to threonine amino acid p.A391T at c.117 position in *LDLR* gene. This variation was analyzed for predictive effective. Polyphen2, SIFT and Mutation Taster predicted c.117 G>A as benign, tolerated and polymorphism with possible site broken effect. The exon 10 has also a common polymorphism c.1413G>A (rs5930) which results in AGG>AGA codon and p.R472= amino acid. This polymorphism was genotyped by RFLP. The allele frequency conformation result indicate p=0.201 which suggest this not deviating from Hardy-Weinberg equilibrium.

Three samples showed deviation in HRM curve only in exon 11 region. Sequencing confirmed the single nucleotide C>T change at c.1617 position resulting in a synonymous CCC>CCT at p.539 coding for proline amino acid. Exon 12 melting curve showed a polymorphic pattern and RFLP confirmed c.1773C>T polymorphism. The allele frequency was not significant and hence no deviation from Hardy-Weinberg principle.

IV. DISCUSSION

High resolution melting and sequencing of 21 familial hypercholesterolemia probands revealed that monogenic cause of FH is less frequent in Pakistani families while it is about 40% in the Caucasian (Taylor *et al.* 2010). One missense detected mutation c.1171G>A (p.A391T) in FH female patient which suffered with coronary artery disease at 42 years of age. Algorithmic simulation predict splicing effect of this variant and Caucasian studies report it as a polymorphism (Gudnason *et al.* 1995). One polymorphic variant c.1060+10C>G was observed and predicted to be non-pathogenic. Other synonymous variations c.81T>C, c.993C>T, c.1413G>A, c.1617C>T, c.1725C>T, c.1959T>C, c.2232A>G were detected in *LDLR* gene are not FH causing (Tejedor *et al.* 2010). However, c.1725C>T, c.1959T>C, c.2232A>G variant were predicted with protein effect by intron splicing. LDLR is an important cell surface molecule which acts as a regulator of low density lipoproteins. Uptake of LDL-cholesterol from blood stream is dependent on the performance of LDLR molecules (Austin *et al.* 2004). LDL-cholesterol absorption is mediated by isoform of apolipoproteinB (ApoB-100) present in the intestine and it is about 70% of the blood cholesterol (Goldstein *et al.* 1985). Endosomes mediate the ApoB-LDL-LDLR complex formation. At low pH, LDL is released from the LDL-receptor (Jeon *et al.* 2001). N276T and G77S are non-pathogenic variants which do not exhibit any impact on the LDLR performance (Jiang *et al.* 2017) while many variants have been identified in different populations resulting in malfunction of the molecule. Elevated levels of LDL-cholesterol and total cholesterol are usually associated with LDLR defectiveness. These are the major factors of coronary artery disease and complication (Lusis *et al.* 2004).

We have not sequenced all the regions of *LDLR* gene so might be a chance of disease causing mutation in the introns of *LDLR* gene or APOB or *PCSK9* genes. Low rate of mutation detection could also be due to lack of tendon xenthoma a hallmark sign of familial hypercholesterolemia. Patients with tendon xenthoma have mutation detection rate between 60-80% (Humphries *et al.* 2006b; Taylor *et al.* 2010). Rare mutations in signal transducing adaptor family member 1 *(STAP1)* gene have also been reported as linked with familial hypercholesterolemia (Fouchier *et al.* 2014). Screening of rare mutation causing genes could add in the diagnosis of FH. Relative of FH individuals is the cost effective method for early prediction of FH risk and this cascade screening program has already begun in the developed countries (Humphries *et al.* 2006a).

Cardiovascular disease due to coronary artery blockage takes decades in development. Clinical, biochemical and molecular methods are the common reliable tools for genetic diseases prevention. Genetic risks for atherosclerosis development are evident in families with hypercholesterolemia have more trend of cardiovascular disease risk. It is very important to investigate FH individuals at an early age to prevent the consequences of vasculature changes. Early diagnosis and management would delay the thermogenesis and definitely will increase the life expectancy.

ACKNOWLEDGMENT

They extend their thanks to British Heart Foundation Laboratories for providing facilities. Higher education commission of Pakistan is also acknowledged for providing financial assistance.

REFERENCES

[1] D. Marks, M. Thorogood, H. A. W. Neil, and S. E. Humphries, "A review on the diagnosis, natural history, and treatment of familial hypercholesterolaemia," *Atherosclerosis*, vol. 168, no. 1, pp. 1-14, 2003.

[2] D. J. Rader, J. Cohen, and H. H. Hobbs, "Monogenic hypercholesterolemia: New insights in pathogenesis and treatment," *The Journal of Clinical Investigation*, vol. 111, no. 12, pp. 1795-1803, 2003.

[3] H. H. Hobbs, M. S. Brown, and J. L. Goldstein, "Molecular genetics of the LDL receptor gene in familial hypercholesterolemia," *Human Mutation*, vol. 1, no. 6, pp. 445-466, 1992.

[4] M. Abifadel, *et al.*, "Mutations in PCSK9 cause autosomal dominant hypercholesterolemia," *Nat Genet*, vol. 34, no. 2, pp. 154-156, 2003.

[5] J. L. Goldstein and M. S. Brown, "The LDL receptor," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 4, pp. 431-438, April 2009.

[6] H. K. Jensen, L. G. Jensen, H. Meinertz, P. S. Hansen, N. Gregersen, and O. Færgeman, "Spectrum of LDL receptor gene mutations in Denmark: Implications for molecular diagnostic strategy in heterozygous familial hypercholesterolemia," *Atherosclerosis*, vol. 146, no. 2, pp. 337-344, 1999.

[7] S. E. A. Leigh, A. H. Foster, R. A. Whittall, C. S. Hubbart, and S. E. Humphries, "Update and analysis of the University College London low density lipoprotein receptor familial hypercholesterolemia database," *Annals of Human Genetics*, vol. 72, no. 4, pp. 485-498, 2008.

[8] W. T. Friedewald, R. I. Levy, and D. S. Fredrickson, "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clinical Chemistry*, vol. 18, no. 6, pp. 499-502, June 1972.

[9] J. Sambrook and D. Russell, *Molecular Cloning: A Laboratory Manual*, 3rd ed., NY: Cold Spring Harbor Laboratory Press, 2001.

[10] R. A. Whittall, *et al.*, "Development of a high-resolution melting method for mutation detection in familial hypercholesterolemia patients," *Annals of Clinical Biochemistry*, vol. 47, no. 1, pp. 44-55, 2010.

[11] A. Taylor, *et al.*, "Mutation detection rate and spectrum in familial hypercholesterolaemia patients in the UK pilot cascade project," *Clinical Genetics*, vol. 77, no. 6, pp. 572-580, 2010.

[12] V. Gudnason, D. Patel, X. M. Sun, S. Humphries, A. K. Soutar, and B. L. Knight, "Effect of the StuI polymorphism in the LDL receptor gene (Ala 370 to Thr) on lipid levels in healthy individuals," *Clinical Genetics*, vol. 47, no. 2, pp. 68-74, 1995.

[13] M. T. Tejedor, *et al.*, "Haplotype analyses, mechanism and evolution of common double mutants in the human LDL receptor gene," *Molecular Genetics and Genomics*, vol. 283, no. 6, pp. 565-574, Jan. 2010.

[14] M. A. Austin, C. M. Hutter, R. L. Zimmern, and S. E. Humphries, "Genetic causes of monogenic heterozygous familial hypercholesterolemia: A HuGE prevalence review," *American Journal of Epidemiology*, vol. 160, no. 5, pp. 407-420, Sep. 2004.

[15] J. L. Goldstein, M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider, "Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system," *Annual Review of Cell Biology*, vol. 1, no. 1, pp. 1-39, 1985.

[16] H. Jeon, W. Meng, J. Takagi, M. J. Eck, T. A. Springer, and S. C. Blacklow, "Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair," *Nat. Struct. Mol. Biol*, vol. 8, no. 6, pp. 499-504, 2001.

[17] L. Jiang, *et al.*, "Analysis of LDLR variants from homozygous FH patients carrying multiple mutations in the LDLR gene," *Atherosclerosis*, vol. 263, pp. 163-170, Aug. 2017.

[18] A. J. Lusis, A. M. Fogelman, and G. C. Fonarow, "Genetic basis of atherosclerosis: Part I: New genes and pathways," *Circulation*, vol. 110, no. 13, pp. 1868-1873, Sep. 2004.

[19] S. E. Humphries, *et al.*, "Genetic causes of familial hypercholesterolaemia in patients in the UK: Relation to plasma lipid levels and coronary heart disease risk," *Journal of Medical Genetics*, vol. 43, no. 12, pp. 943-949, Dec. 2006.

[20] S. W. Fouchier, *et al.*, "Mutations in STAP1 are associated with autosomal dominant hypercholesterolemia," *Circulation Research*, vol. 115, no. 6, pp. 552-555, Aug. 2014.

[21] S. Humphries, *et al.*, "Mutational analysis in UK patients with a clinical diagnosis of familial hypercholesterolaemia: Relationship with plasma lipid traits, heart disease risk and utility in relative tracing," *Journal of Molecular Medicine*, vol. 84, no. 3, pp. 203-214, Jan. 2006.

Akhtar Ali received his Bachelor degree in Eastern Medicine and Surgery from Islamia University Bahawalpur. One year internship completed in Hijaz Hospital, Gulberg III, Lahore. Then he earned M. Phil in Molecular Biology and Biotechnology from UVAS, Lahore. Now he is doing Ph.D in the same discipline. Since his postgraduation he has been engaged in various research projects in the Institute of Biochemistry & Biotechnology.