Genetics of LDLR Gene in Pakistani Hypercholesterolemia Families

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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 or LDL-cholesterol 4.9 mmol/L. Seventy five percent of FH cases are autosomal dominant disorder caused by mutations in 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.

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

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, BsmA1 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>
<thead>
<tr>
<th>Lipid</th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>7.4</td>
<td>± 1.2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.1</td>
<td>± 0.4</td>
</tr>
<tr>
<td>HDL- cholesterol</td>
<td>1.1</td>
<td>± 0.1</td>
</tr>
<tr>
<td>LDL- cholesterol</td>
<td>5.8</td>
<td>± 1.2</td>
</tr>
</tbody>
</table>

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).

Sequence 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.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.](image)
cholesterol from blood stream is dependent on the LDLR is an important cell surface molecule which acts as a receptor for low density lipoprotein. However, c.1725C>T, c.1959T>C, c.2232A>G variant were predicted with protein effect by intron splicing.

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 break effect. The exon 10 also has 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 xanthoma a hallmark sign of familial hypercholesterolemia. Patients with tendon xanthoma 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.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Position</th>
<th>Change</th>
<th>Protein change</th>
<th>Polyphen 2</th>
<th>SIFT</th>
<th>Mutation Taster</th>
<th>HSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>c.81</td>
<td>T&gt;C</td>
<td>p.C27=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>7</td>
<td>c.993</td>
<td>C&gt;T</td>
<td>p.D331=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>7</td>
<td>c.1060+10C&gt;G</td>
<td></td>
<td>Benign</td>
<td></td>
<td></td>
<td></td>
<td>no effect</td>
</tr>
<tr>
<td>8</td>
<td>c.1171</td>
<td>G&gt;A</td>
<td>p.A391T</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>10</td>
<td>c.1413</td>
<td>G&gt;A</td>
<td>p.R344=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>11</td>
<td>c.1617</td>
<td>C&gt;T</td>
<td>p.P539=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>12</td>
<td>c.1725</td>
<td>C&gt;T</td>
<td>p.L575=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>13</td>
<td>c.1959</td>
<td>T&gt;C</td>
<td>p.V653=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
<tr>
<td>15</td>
<td>c.2252</td>
<td>A&gt;G</td>
<td>p.R744=</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Polymorphism</td>
<td>no effect</td>
</tr>
</tbody>
</table>
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REFERENCES


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.