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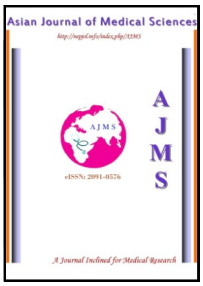

Mutation Analysis of the LDL Receptor Gene in Indian Families with Familial Hypercholesterolemia

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Abstract

Objective: Familial Hypercholesterolemia (FH) is a metabolic disorder inherited as an autosomal dominant trait characterized by an increased plasma low-density lipoprotein (LDL) level. The disease is caused by several different mutations in the LDL receptor (LDLR) gene. Several mutations have been reported in this gene in patients from several ethnic groups. Early identification of individuals carrying the defective gene could be useful in reducing the risk of atherosclerosis and myocardial infraction by the available therapeutic methods. The techniques available for determining the number of the functional LDLR molecules are difficult to carry out and expensive. Our study presents mutation analysis of the LDLR gene in 24 Indian families with FH.

Material & Methods: *Peripheral blood samples were obtained form individuals after taking informed consent on the condition that each of these individuals had at least one first-degree relative affected with FH. Genomic DNA was isolated, exon-specific intronic primers were designed and used to amplify DNA samples from individuals.PCR products were directly subjected to automated DNA sequencing to detect the mutations. Along with the affected individuals, ten ethnically matched controls were also analyzed to determine the presence of the same mutations. Patients with Nephrotic Syndrome admitted to hospital were excluded from the study.*

Results: *All the 24 patients had total cholesterol level above 300 mg/dl and LDL cholesterol level above 200mg/dl. Sequence analysis of the LDL receptor (LDLR) gene showed 3 novel mutations which have never been reported elsewhere. In exon 10 we reported g.29372_29373insC, which was found in all the 24 patients and was missence mutation coding for C (cysteine) instead of V (valine).*

Conclusion: *Our study reported 3 novel mutations in 24 Indian families. These novel mutations are predicted to produce change in the amino acid and thus leading to the conformational changes in the structure of LDLR protein. Change in the LDLR protein makes the LDL receptor unable to transport the cholesterol in to the cell and hence cholesterol starts accumulating in the blood stream and leads to FH.*

Key Words: *Familial Hypercholesterolemia; Mutation analysis; LDL Receptor gene*

1. Introduction

Familial hypercholesterolemia (FH) is a disease caused by a mutation in the gene (Entrez Gene ID: 3949) specifying the receptor for a plasma cholesterol transport particle called Low Density Lipoprotein (LDL). In patients who inherit one or two copies of this mutant gene, the reduction in the number of LDL receptor causes LDL particles to accumulate to high levels in plasma. The elevated plasma LDL level inturn produces atherosclerosis and eventually death.[1] FH is a very common autosomal dominant disease with the frequency of 0.2%world wide.[2,3] In India still the exact estimate of the FH is unknown.

Premature coronary heart disease, elevated LDL-cholesterol (LDL-C) and tendon xanthomas are the common clinical features of the FH.[2,4] Sometimes, in early stage of occurrence of FH, these clinical features are absent especially in children and thus do not allow predicting FH in early stage. If, FH is diagnosed in early onset of its occurrence, it can be controlled by appropriate diet and drug treatment and thus would prevent development of coronary heart disease in future. Molecular profiling and mutation analysis of the LDLR gene would be an effective tool in diagnosis of FH

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especially where the clinical features are totally absent. Early identification of individuals carrying the defective gene could be useful in reducing the risk of atherosclerosis and myocardial infarction. The available techniques for identifying the number of properly working LDLR molecules are difficult and expensive too. Many studies have shown different LDLR gene polymorphisms in different ethnic groups.[5-8] India is known for its diverse population and many ethnic groups, hence development of ethnic group specific biomarker would be of great importance and also cost effective.

In the present study, we report the mutations in the LDLR gene which are observed in 24 South Indian families by direct automated sequencing method.

2. Material and Methods

2.1. Patient Selection

All the patients included in this study were admitted and being treated at S.D.M. Medical college, Dharwad, Karnataka, India between 2009-2010. Plasma lipid concentrations were measured while all patients consumed their regular diet and prior to the administration of hypolipidemic drug. Total cholesterol, triglycerides and HDL-C levels were measured enzymatically with commercial kits (Span Diagnostics, India) using automated analyzer (Artos, India). LDLc cholesterol was calculated by Friedewald's formula. After conducting biochemical tests and on the condition that each of these patients had at least one first-degree relative affected with FH, 24 patients agreed to involve in the study. Alongwith this, we also collected blood samples from 10 ethnically matched individuals who are not related to these patient's families and were absolutely normal. Detailed informed consent was obtained from all the 24 patients and controls in vernacular.1ml of blood sample was collected from the patients and controls in EDTA coated vacutainer (BD, USA) stored at -20^{0}C until further process.

2.2. Genomic DNA isolation, PCR amplification and mutation analysis

Genomic DNA was isolated from 300µl of peripheral blood samples using a commercial DNA isolation kit (Bangalore Genei, India).All the isolated DNA samples were quantified using biophotometer (Eppendorf, Germany).

Table 1: Details of the primers designed and used for the amplification of all the exons of LDLR gene

S No.	Exon No.	Primer Sequence	Amplicon Size	Annealing Temperature
1	1F	5'-CTCCTCTTGCAGTGAGGTGA-3'	294	59.4
	1R	5'-CCTCTCAACCTATTCTGGGG-3'		
2	2F	5'-GATTCTGGCGTTGAGAGACC-3'	191	58.4
	2R	5'-ATATCATGCCCAAAGGGGAC-3'		
3	3F	5'-TGGGTCTTTCCTTTGAGTGA-3'	200	56.3
	3R	5'-AGGCTCAATAGCAAAGGCAG-3'		
4	4F	5'-GACTTCACACGGTGATGGTG-3'	521	58.4
	4R	5'-TCCACTTCGGCACCTAAATC-3'		
5	5F	5'-AGGCCCTGCTTCTTTTTCTC-3'	254	55.3
	5R	5'-AATCATTTGCAAGCAGCAAG-3'		
6	6F	5'-TGAATGAGTGCCAAGCAAAC-3'	277	56.3
	6R	5'-TTCCCAAAACCCTACAGCAC-3'		
7	7F	5'-GAGTGACCAGTCTGCATCCC-3'	213	59.1
	7R	5'-TGGTTGCCATGTCAGGAAG-3'		
8	8F	5'-CTTCGAAGGTGTGGGTTTTG-3'	298	57.3
	8R	5'-TTCAGAGGATGAAACTCCCC-3'		
9	9F	5'-GAGGCACTCTTGGTTCCATC-3'	322	60.4
	9R	5'-CTGAGGCAGGAGGAGAGAAG-3'		
10	10F	5'-CTTCTCTCCTCCTGCCTCAG-3'	312	60.4
	10R	5'-GTTCCTGAAGCTCCTTCCTG-3'		
11	11F	5'-ATTCTCTGTCCTCCCACCAG-3'	195	60.2
	11R	5'-GTCTGTCCTCCAGCCTGTG-3'		
12	12F	5'-ATCAGCACGTGACCTCTCCT-3'	271	57.4
	12R	5'-CAACCAGTTTTCTGCGTTCA-3'		
13	13F	5'-TTCCTTGCTGCCTGTTTAGG-3'	257	58.3
	13R	5'-TCAGCTATACCAGAAGATTCCAGA-3'		
14	14F	5'-ATCTCGTTCCTGCCCTGACT-3'	225	58.4
	14R	5'-GACACAGGACGCAGAAACAA-3'		
15	15F	5'-CATTAGGCGCACACCTATGA-3'	326	56.3
	15R	5'-TCCATCTCGTGACCAAAATG-3'		
16	16F	5'-TCTCGCAGACTTGGGAAGTT-3'	277	59.2
	16R	5'-GAGGTCACATAGCGGGAGG-3'		
17	17F	5'-TTATGGTACGATGCCCGTGT-3'	319	58.4
	17R	5'-GAGGATCATATGCCTCCAGC-3'		
18	18A :F	5'-GCTGGACTGATAGTTTCCGC-3'	566	59.4
	18A :R	5'-CAAAGGCTAACCTGGCTGTC-3'		
19	18B :F	5'-GACAGCCAGGTTAGCCTTTG-3'	467	57.4
	18B :R	5'-ATTCATTGACACGGGCTTTC-3'		
20	18C :F	5'-TCTGTCGTGTGTGTTGGGAT-3'	626	58.4
	18C :R	5'-CCTGAGCTCAAACCATCCTC-3'		
21	18D :F	5'-ATGGTGCATCAGCAGCC-3'	558	57.3
	18D :R	5'-TGTCTCTCCGGACATCAGTG-3'		
22	18E :F	5'-ATCGTTTGACGGGACTTCAG-3'	611	57.3
	18E :R	5'-CAGAAGAACGTGCATCGAGT-3'		

Exon specific intronic primers were designed (Table-1) to cover full length of exon (Reference Sequence: NC_000019.9), keeping the amplicon size 300bp to 500bp using Primer-3 (Bioinformatics tool). Overlapping primers were designed to exons which were more than 500bp.All the designed primers were conformed through Insilico PCR (Bioinformatics tool) for the proper amplification and also confirmed in Genome Build 36 (Bioinformatics tool) for specificity of primer binding in genomic DNA. Primers were got synthesized by commercial oligo synthesizer (MWG Biotech, India).

PCR amplification was carried out in a 20µl reaction volume containing 0.5 µl of genomic DNA (75ng/µl to 150 ng/µl), 0.5µl of each primer (5pmol), 0.4µl of dNTP (10pmol), 0.2µl Taq DNA polymerases (3units/ µl), 4 µl Taq Buffer (5X) (BioRad, USA) and total volume was adjusted to 20µl using molecular biology grade water. Amplification was carried out in Mastercycler gradient (Eppendorf, Germany) under the following conditions: an initial denaturation at 98^{0}C for 10sec, followed by 35 cycles at 98^{0}C for 10sec (cycle denaturation), primer annealing temperature was set depending on the annealing temperature of each primer (Table-1) for 10sec, 72^{0}C for 15sec (primer extension) and a final extension at 72^{0}C for 5 min.PCR products were confirmed for their respective amplicon size by gel electrophoresis with standard 100bp ladder.

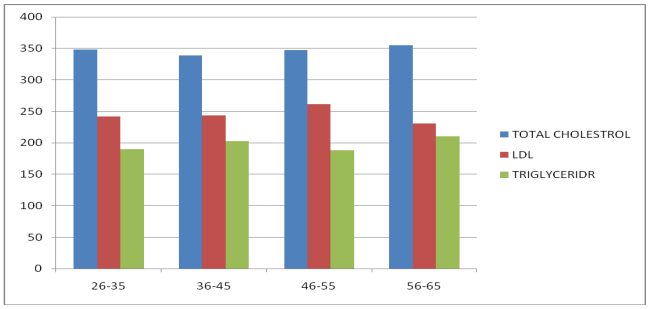
PCR products were directly subjected to automated DNA sequencing (ABI_3500xl).Both forward and reverse reactions were carried out for all the samples and exons to minimize the machine made errors. Electropherograms were obtained and sequence alignment was carried out by DNA Baser (Bioinformatics tool).

3. Results

3.1. Biochemical analysis

The biochemical analysis results are shown in Figure-1, indicates high levels of serum LDL, triglycerides and total cholesterol. It was observed that, across the different age groups the levels remains almost similar.

Figure-1: Biochemical analysis from all 24 patients



3.2. Genetic analysis

After sequencing all the 18 exons, three LDLR mutations were detected which were unique to the South Indian population and never been reported elsewhere [9].Sequence analysis of the exon 3 showed a mutation at position 18298 from A>C (g.18298A>C) (Fig-2) in patients 1,9,18,19 and 20, which introduced a Q(Glutamine) in place of K(Lysine) at amino acid position 69 (p.K69Q) in the reference sequence.

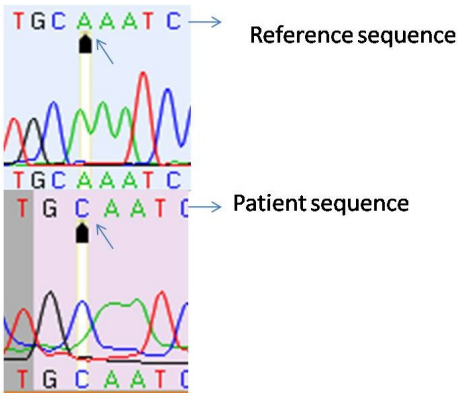


Figure-2:Showing 18298 A>C

In exon 10 we observed insertion C between positions 29372 and 29373 (g.29372_29373insC) in all the 24 patients (Fig-3), which introduced a C (Cysteine) in place of V (Valine) at aminoacid position 527 (p.V527C) in the reference sequence. At position 55, A>G mutation (g.29209A>G) was found and it was predicted as silent mutation (Fig-4).

The sequence analysis of exon 3 and 10 from PCR products of control individuals not shown any of the said mutations (data not shown) according to the previously established studies.[9]

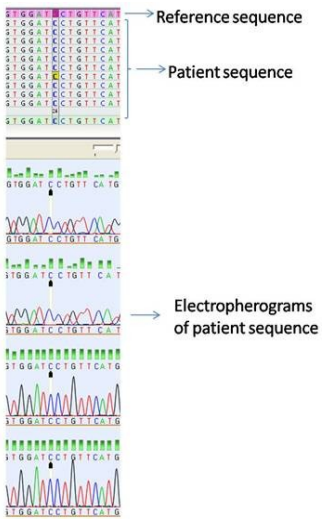


Figure-3:Showing insertion at g.29372_29373insC

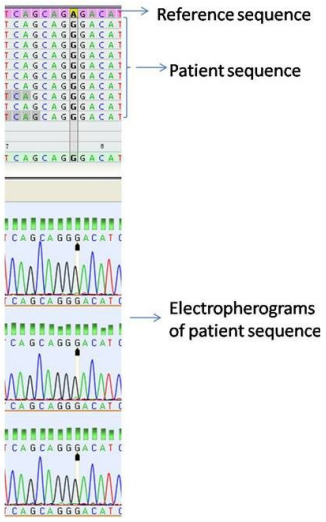


Figure-4:Showing g.29209A>G

4. Discussion

More than 1,100 mutations have been reported in the LDLR gene in different populations.[9-13] This gene is composed by 18 exons that encode a protein involving five domains: the ligand binding domain, the epidermal growth factor (EGF) precursor homology domain, the domain with O-linked carbohydrates, the membrane spanning domain and the cytoplasmic part of the

receptor. The ligand binding domain consists of seven repeats, each containing six cysteine residues, which form disulphide bonds within each repeat.[14]

All the three nucleotide changes reported here fulfilled the criteria of a mutation as these changes were not present in the controls. This is the first report of LDLR gene mutations in South Indian families. The mutations at position 18298 from A>C (g.18298A>C) in exon 3 was found in patients 1,9,18,19 and 20 who were clinically positive for Tendon xanthomas. It is predicted that, this missence mutation replaces K (Lysine) by Q (Glutamine) and hence change in the receptor protein structure of LDLR and thus leading to formation of Tendon xanthomas. A>G mutation (g.29209A>G) was found in exon 10 and was predicted as silent mutation. In all the 24 patients g.29372_29373insC was found in exon 10.This has introduced C (Cysteine) in the place of V (Valine) at p.V527C. The change in amino acid is predicted to cause structural changes in the LDLR protein and hence the receptor fails to bind with the ligand molecule (cholesterol). All the mutations and their corresponding effects on the protein were predicted computationally and was observed that these are responsible for changes in the structure of LDLR protein. Because of the change in the structure of the LDLR protein, it is unable to receive cholesterol molecules and transport inside the cell. Hence, cholesterol starts accumulating in the blood stream and leads to FH.

India is known for high degree of inbreeding. This makes it necessary to screen a large number of patients perhaps within each group in order to get a true picture of contribution of LDLR gene mutation to FH. Even though g.29372_29373insC was found in all the 24 patients, it makes necessary to screen large population to consider this as the biomarker considering frequency of the occurrence of FH, in this regard efforts are going on in our laboratory.

5. Conclusion

In this study, we have reported three novel mutations in which one is silent and two are missence, which have never been reported in LDLR gene previously. The mutation g.29372_29373insC can be a potential biomarker after screening large population in India especially in South India. Once the biomarker is developed, it makes easy for the clinicians in the early diagnose of FH and make the treatment effective considering the cost, time and life of the patients.

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