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Genetic Basis for Diagnosis of Novel Mutation of LDL Receptor Gene

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ABSTRACT

Background: The low density lipoprotein (LDL) receptor is a cell-surface protein that regulates plasma cholesterol by specific uptake of LDL particles from the plasma. Familial hypercholesterolemia (FH) is autosomal dominant hypercholesterolemias that predispose to premature coronary artery diseases. Familial hypercholesterolemia is caused by sequence variations in LDL receptor gene.

Aim & Objective: The molecular analysis of low density lipoprotein for diagnosis of familial hypercholesterolemia (FH), an autosomal dominant disease caused by a multitude of LDL receptor (LDLR) gene mutations and confirmation of these mutations by DNA sequencing.

Methods: Polymerase chain reaction (PCR) amplification of type specific primers allowed the rapid detection of point mutations in exon 3, 4, 9, and 14 of the low density lipoprotein receptor gene in hypercholesterolemia patients. In our study we screened 120 patients with hypercholesterolemia by lipid profiles after twelve hours fasting and with family history of premature coronary heart diseases.

Results: Genomic DNA was extracted from blood samples of an apparently healthy control group and hypercholesterolemia patients with LDL > 160mg/dL and clinical features of FH to detect mutations in exons 3, 4, 9, and 14 of the LDLR gene, with modification in the technique by using type-specific primers.

Discussion/ Conclusions: The frequency of heterozygous FH was noted that 35% were classical and 65% probable cases were observed with mutation at exon 3 and 4. The mutations reported were further confirmed by DNA sequencing.

Keywords: HeFH heterozygous familial hypercholesterolemia, CAD coronary artery diseases, PCR Polymerase chain reaction, LDLR low density lipoprotein receptor, common mutation, Exons 3 & 4

Introduction

Familial hypercholesterolemia, or FH, an autosomal dominant disorder of lipoprotein disorder with a prevalence of about 1 in 500, results from mutations of the LDL receptor gene [1]. No prevalence study for LDLR gene mutation is done for Pakistani population. However, to date ten different LDLR mutations in immigrants from India to South Africa have been reported in the literature [2]. Study by Identification of two LDL-receptor mutations causing familial hypercholesterolemia in Indian subjects by a simplified rapid PCR-heteroduplex method [3]. LDLR gene is located at chromosome 19p13.2 is composed of 18 exons spanning 45 kb [4]. At present number of LDLR mutations are reported (24%) small DNA rearrangements and (11%) large DNA rearrangements [5]. The large DNA rearrangement is associated with Alu element.

When present in a heterozygous form, most of the mutations identified thus far have been reported to cause a typical clinical picture of FH, with grossly elevated serum LDL cholesterol levels, tendon xanthomas and premature coronary heart disease [6]. Common variation of the intact LDL receptor allele may also exert subtle influence on serum cholesterol levels in patients with heterozygous FH. Patients with homozygous FH have been shown to have considerable inter individual variation in serum cholesterol levels as well as in the expression of coronary heart disease [7] which in one study was directly attributed to the nature of the causative mutation. Differences in plasma-cholesterol concentrations are reflected in the severity of coronary heart disease expression [8]. Study by Hill et al shows that only a small number of females with FH had symptoms of CAD; however, the risk of developing CAD in females was significantly increased in the smaller fraction of patients who had

hypertension or elevated triglycerides. In contrast, males had a higher general frequency of disease but were at a much greater risk if they had lower HDL-C values and a history of smoking. In fact, the presence of CAD was almost always associated with one or more of these risk factors [9].

The molecular analysis of familial hypercholesterolemia (FH), an autosomal dominant disease caused by a multitude of LDL receptor (LDLR) gene mutations, is complicated by mutational heterogeneity of the disease in the majority of population studied to date [10,11]. Exceptions occur where the frequencies of specific mutations are increased in a population because of founder effects, or where a mutation has been introduced on many occasions into a small isolated community [11, 12, 13, 14].

The vast majority of the large rearrangements of the LDL-receptor gene are deletions of various sizes along the entire length of the gene. In general different techniques have been used in screening large rearrangements of various genes, including the LDL-receptor gene. However, standard PCR techniques can provide a rapid and reliable screening alternative to Southern-blot hybridization for the detection of various large rearrangements in the LDL-receptor gene in FH patients. We therefore established a screening scheme for large rearrangements of the LDL-receptor gene using multiplex PCR. The exons were PCR amplified [15] using specific oligonucleotide primers. We screened 120 unrelated FH heterozygotes using this scheme and identified two mutations in FH patients in exon 3 and exon 4 common in population of Karachi.

Methods

Sample Collection

Samples were collected of hundred and twenty hyperlipidemic cases with high total cholesterol and LDL in the lipid profile (Total cholesterol > 200mg/dL and LDL > 130 mg/dL). All those cases were excluded from this study that had diabetes mellitus, hypertension, renal diseases, hypothyroidism and hypertriglyceridemia. The samples were collected from Dr. Ziauddin Hospital and National Institute of Cardiovascular Diseases, Karachi from June 2008 to June 2010. All these patients were with or without coronary heart disease; all had family history of hypercholesterolemia. The age range was 22 to 60 years in this study. This study was approved by the ethical review committee Ziauddin University. All the participants gave written consent.

The blood samples were collected after overnight fasting for lipid profile and in EDTA tubes for genotyping. The lipid profile parameters were determined through auto analyzer for total cholesterol, LDL-C, HDL-C and triglycerides. Genotyping was done at Dr. Rubina's Pathology and Molecular Biology Laboratory.

DNA Extraction

The genomic DNA was extracted from whole blood collected in EDTA tubes and the DNA extraction was performed, by following the Epicenter DNA Purification Kit (Cat No.MCD85201) [16] procedure. The multiplex PCR was performed in a single tube using mutation specific primers.

PCR conditions

The PCR was carried out in a tube containing 20µl of a reaction mixture made up of the following components: 10pmol of each forward and reverse primer. The type specific primer sequence for different exons used for the identifying the samples with familial hypercholesterolemia are as following (a) Sense (b) Antisense.

Exon3 (a) TGACAGTTCCAATCCTGTCTCTTCTG,
(b) TAGCAAAGGCAGGGCCACACTTAC ----- (162 bp)
Exon 4 (a) CCCCAAGACTGTCTCCAGGACGA
(b) ACGCCCCGCCCCACCTGCCCCGC----- (431bp),
Exon 9 (a) CTGACCTCGTCCCCGGACC
(b) GCCCTCAGCGTCGTGATCG----- (550bp)
Exon 14 (a) AATGTGCGACGTCATCTTCCTTGCTGCCTGT
(b) TATGTCCAGAAACAAGGCGTGTGCCAC----- (496 bp)

500µM of four deoxynucleotides, 2.5U of Taq polymerase (Promega), 10 x PCR buffer containing 1.5mM Mg Cl₂ (Promega, USA). The thermal cycler (Master Gradient PCR System, Eppendorf AG, Germany) was programmed to first incubate the sample for 5 minutes for 95°C followed by 35 cycles consisting of 94° C for 45 seconds, 55° C for 45 seconds and 72°C for 1.5 minute with final extension for 7 minutes at 72°C. The 3 µL of gel loading buffer was added to the amplified products then loaded on a 2% agarose gel. The gel was set at 100 volts for 1 hour and then stained with ethidium bromide. After staining, the bands were visualized under UV light and different mutations were analyzed with the sizes of PCR amplified product were estimated according to the migration pattern of a 100-bp DNA ladder (Gibco BRL Life Technologies) common four mutations of LDL receptor gene. These mutations were further confirmed DNA sequencing.

DNA Sequencing

In the automated DNA sequencing primers are used that are labeled with four different colored fluorescent tags. PCR reactions, in the presence of the different dideoxynucleotides, are performed as described above. However,

next, the four reaction mixtures are then combined and applied to a single lane of a gel. The color of each fragment is detected using a laser beam and the information is collected by a computer which generates chromatograms showing peaks for each color, from which the template DNA sequence can be determined.

Statistical analysis

SPSS (16.0) package was used to analyze data. Mean and Standard deviation determined for lipid profile, total cholesterol, LDL-Cholesterol, triglycerides and HDL-Cholesterol for cases and controls. Student's t test was applied to compare means of total cholesterol, LDL-cholesterol, triglycerides and HDL-cholesterol, p value less than 0.05 was considered significant and 0.001 as highly significant.

Results

Hundred and twenty hyperlipidemics from the Ziauddin Hospital and National Institute of Cardiovascular Diseases with raised LDL-C above 160 mg/dL (table 1).

However this was a concentrated population of hundred and twenty hyperlipidemic patients, as all of these were selected from the cardiovascular unit of the hospital OPD. In these cases, forty two cases were found high LDL-C, xanthelasmas, tendon xanthomas and arcus cornea with exons 3, 4 mutation of LDLR gene. Other patients had high LDL-cholesterol and family history of coronary artery diseases. One of these patients had stroke at 32 years of age.

In addition to cholesterol measurement, clinical signs and family history are the criteria for cases to be classical or probable. Classical are cases with tendon xanthomas in

patients along LDLR gene mutation with hyperlipidemia. Mutation LDLR gene was determined by PCR.

Probable are cases with family history of myocardial infarction before 50 years of age or raised cholesterol levels in first degree relatives. All had a positive family history of cardiac diseases.

Out of total one hundred and twenty cases of hyperlipidemia, forty two patients diagnosed with 'classical' FH tested positive for one of the locally defined founder-type LDLR gene mutations. In these cases had raised LDL-C, tendon xanthomas, xanthelasmas and LDL-R gene mutation was determined at exon 3 and 4.

In probable cases of FH their LDL-C was \geq 130mg/dL having premature coronary artery diseases and positive family history of premature coronary artery diseases. Although blood samples of their family members were not available.

Thus study shows a frequency of 35 percent classical and 65 percent probable cases in a concentrated population, as samples were collected from hospital patients.

During our study the common mutations, at exon 3 and 4 were identified as shown Figure 1 in the various ethnic groups of hundred and twenty hyperlipidemia in Karachi.

DNA Sequencing

Total mutation band of exon 3 at 162 bp and exon 4 at 431bp were separated out for sequencing of ten cases of familial hypercholesterolemia. Automated DNA sequencing with coloured probes green for adenine, black for guanine, red for thymine and blue for cytosine was done to screen for mutation sites of exon 3 and 4 as shown in

Figure 2a and 2b. The sequence of exon 4 was found that at 105bp A >T (Fig. 2a) and sequence of exon 3 was found to be at 345 bp G>T (Fig. 2b) at which could be cause of severe hypercholesterolemia in these patients.

Discussion

The frequency of heterozygote FH in most other populations is 1 in 500 except. Fahed et al. confirm previous reports on the higher prevalence of FH in Lebanon [12] Three founder-related gene mutations (FH Afrikaner-1, -2, and -3) of LDL receptor are cause of 90% of the familial hypercholesterolemia (FH) in South African Afrikaners[13] where frequency of heterozygous FH is 1 in 171 and in Quebec [14] this frequency is 1 in 270. The high gene frequency is due to founder effects and high incidence of consanguineous marriages in the above populations. Similar is the case for various ethnic groups in population of Karachi as this is very common culturally to have consanguineous marriages here.

Loubser et al [11] also divided their cases into classical and probable. This study has also shown thirty five percent classical and sixty five percent probable cases of FH in a sample of hundred and twenty hyperlipidemic patients was a multicentre study. Forty two of these cases showed LDLR mutation at the exon 4 from a sample of hundred and twenty hyperlipidemic patients. Classical cases of HeFH will have combination of high LDL-cholesterol levels with xanthomas along with mutation of LDLR gene whereas in probable cases having high total cholesterol and LDL-cholesterol, family history of hypercholesterolemia, without LDLR gene mutations [17].

Descamps et al [18] described LDLR gene mutation which is common cause of familial

hypercholesterolemia in Belgian population. Mutation of exon 4 was most common in a study done with samples of Belgian population of hyperlipidemia cases. Tendon xanthomas was found to be 29.2% in genetically diagnosed HeFH patients [19].

In study done on United Kingdom population mutations of LDLR the greatest number of LDLR mutations were found in exons 3 (10%), exon 4 (28%), exon 10 (10%) and exons 14 (21%). 46% of LDLR mutations were found in the ligand binding domain (exons 3 -6) and 46% were found in the EGF precursor-like domain exons 7 -14 [20]. Most of these mutations have been reported in exons 3, 4, 9, and 14 among Indians settled in South Africa, which suggests an increased frequency of FH in India. Fard-Esfahani et al (21) reported new mutation at exon 4 (445G>T) and it was noted that this exon is the largest and the most common type of mutation till now reported. In study by Chang et al 2003, on Chinese population of 170 hyperlipidemic and two phenotypical FH patients were genotyped for LDLR gene mutations by multiplex PCR, long PCR and single strand conformation polymorphism (SSCP). Two deletions, six point mutations and two polymorphisms were detected [22].

During our study we came across with mutations at exon 3 and 4 which was confirmed by DNA sequencing showing mutation at 105-A >T and 345G>T. This study has proven that the frequency of FH plays an important role in research for the direct identification of some new mutation especially in those patients who are at particularly at high risk of premature cardiovascular disease which is enhanced by environmental factors along with genetic factor. Genetic diagnostic tests assist in the identification of family members while improving cardiovascular risk prediction, prevention of disease and treatment efficacy.

The most cost effective diagnosis for FH is to screen the family members [23, 24, 25].

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Conflict of interest

None to declare

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Table 1: Initial Serum lipids and lipoprotein levels of Cases(120) and Controls.

*LDL-C Low density lipoprotein, *HDL- C High density lipoprotein,*** p<0.001, highly significant

| Variables | Cases(n=120) Mean ±SD | Controls (n=50) Mean ±SD | p-value |
|---------------------------|----------------------------------|-------------------------------------|----------------|
| Serum Cholesterol (mg/dL) | 267 ± 42 | 184 ±27.9 | 0.001*** |
| LDL- Cholesterol (mg/dL) | 187 ± 40 | 105.15 ±22.32 | 0.001*** |
| HDL-Cholesterol (mg/dL) | 37 ± 9.0 | 39 ± 4.28 | 0.33 |
| Triglycerides (mg/dL) | 194 ± 57 | 196 ± 15 | 0.77 |

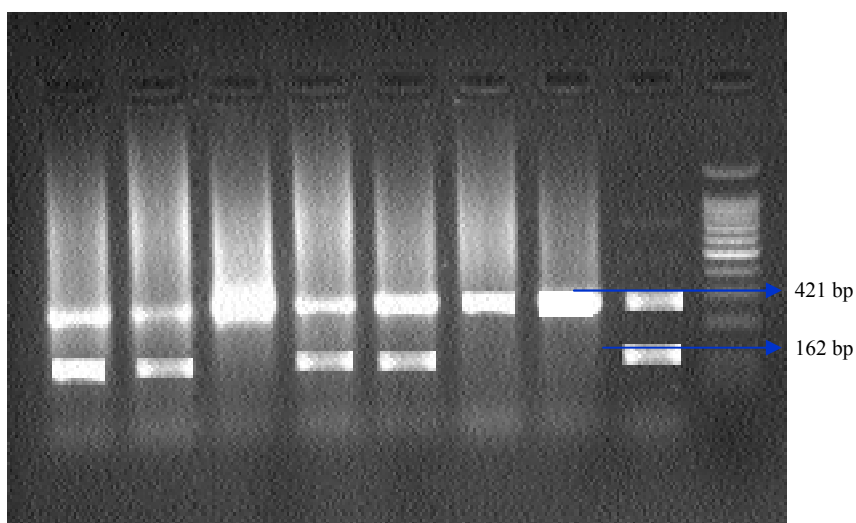


Figure 1: The gel analyzed, lanes- 1, 2, 4, and 5 shows both the mutation exon 3 and exon 4 whereas lanes- 3, 6, and 7 shows mutation on exon 4 of LDLR gene in familial hypercholesterolemia patients. Lane P is the positive control and lane M is the DNA ladder

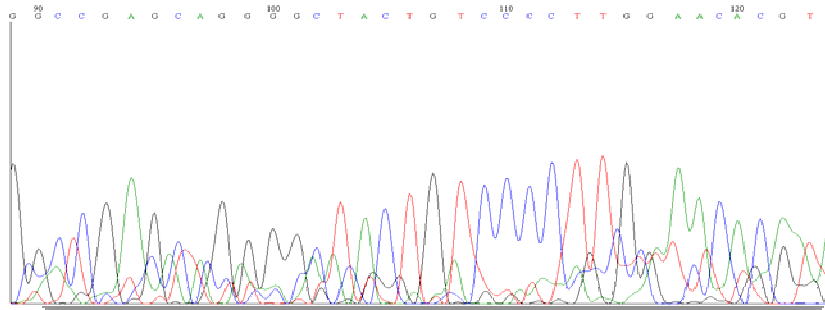


Figure 2a: The DNA Sequencing showing the mutation at the position 105-A >T , and this change is note at exon 4

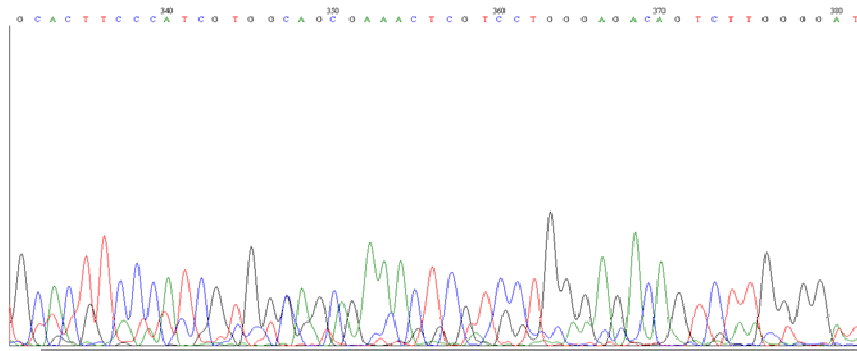


Figure 2b: The DNA Sequencing showing the mutation at the position 345G>T, and this change is note at exon 3