Clinical Biochemistry 49 (2016) 669–674

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Molecular analysis of the *LDLR* gene in coronary artery disease patients from the Indian population

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ARTICLE INFO

Article history:
Received 26 December 2015
Received in revised form 22 February 2016
Accepted 24 February 2016
Available online 27 February 2016

Keywords:
Coronary artery disease
Familial hypercholesterolemia
High resolution melt analysis
LDLR
UK-Simon Broome criteria

ABSTRACT

Background: Cardiovascular disease is a leading cause of mortality in Indian population. Mutations in *LDLR*, *APOB* and *PCSK9* genes may lead to Familial Hypercholesterolemia, an autosomal dominant disorder which in turn leads to cardiovascular diseases. The primary objective of this study is to analyze these genes in CAD patients of Indian population.

Methods: A total of 30 patients were selected out of 300 CAD patients based on UK-Simon Broome criteria from South India. The gDNA was isolated by organic extraction method and the exons and exon-intron boundaries of *LDLR* gene, *APOB* (exon 26) and *PCSK9* (exon 7) were screened by PCR-high resolution melt analysis. The amplicons showing shift in melting pattern were sequenced to find out the variation.

Results: This study reports three novel variations, an intronic deletion c.694 + 8_694 + 18del in intron 4, a synonymous variation c.966 C>T [p. (N322=)] in exon 7 and a deletion insertion c.1399_1340delinsTA [p. (T467Y)] in exon 10, two recurrent variations c.862G>A [p. (E288K)] in exon 6 and a splice site variation c.1845 + 2T>C in exon–intron junction of exon 12 in *LDLR* gene and *PCSK9* gene had c.1180 + 17C>T change in intron 7. However there are no pathogenic variations in *APOB* and *PCSK9* genes in Indian population. In silico analysis predicted all the variations as pathogenic except the synonymous variation.

Conclusion: This report adds five new variations to the spectrum of *LDLR* variations in Indian population. This study also suggests that UK Simon Broom criteria can be followed to categorize FH patients in Indian population.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by elevated plasma LDL cholesterol (LDL-C) caused by mutations mainly in *LDLR* [1], *APOB* [2] and *PCSK9* genes [3] and the FH condition may lead to cardiovascular diseases. In the general population the frequency of heterozygous FH is 1 in 500 and homozygous is 1 in a million. The estimate may vary with respect to the population, country and origin. Some countries have varied frequency distribution compared to the general distribution worldwide. Estimation of an accurate frequency distribution and recurrent mutations in a country may be helpful in the diagnosis and treatment of the disease. The Indian subcontinent has a diverged population with closed inbred communities and has a prevalence of consanguinity in most regions which indicates that extensive studies may be needed for a proper estimate of the distribution of mutations in FH associated genes. Earlier reports in Indian population illustrated that the mutations are commonly seen in exons 3, 4, 9 and 14 [4]. There are no standard procedures or guidelines established for plasma LDL-C or TC levels for a clinical diagnosis of heterozygous FH in the Indian population. This is the first study to select subjects for a FH study based on UK Simon-Broome criteria and Dutch lipid Clinic score [5], which are standard guidelines for the clinical diagnosis of FH.

The LDL receptor is involved in clearing LDL-C from the blood, ApoB and LDL form LDL-C particles which binds to LDL receptor and endocytosed through clathrin coated vesicles. The LDL-C is hydrolyzed further whereas the LDL receptor is recycled through vesicles to the cell surface for further uptake of LDL-C or degraded based on the need of cell [6]. A heterozygous mutation in the *LDLR* gene may lead to decreased uptake of LDL-C by liver cells and results in elevated plasma LDL-C, whereas homozygous mutation in the gene leads to severe FH due to lack of functional LDL receptors. In the latter case the subjects may experience MI in their early ages compared HeFH individuals. There are more than 1100 variants found in the *LDLR* gene worldwide [7]. There are about 12 mutations in the LDLR gene reported in the Indian population [4,8–10].

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http://dx.doi.org/10.1016/j.clinbiochem.2016.02.009
0009-9120/

Apolipoprotein B aids in binding of LDL cholesterol to LDLR and the mutation in *APOB* gene causes defective receptor binding and leads to elevated plasma LDL cholesterol. Previous reports suggest that the mutation, p. (R3527Q) in exon 26, affects the functional binding domain leading to disease and emphasized the crucial role of arginine at this position for normal binding of LDL-C to receptor [11]. There is no report, so far, of any mutation in the *APOB* gene in the Indian population.

PCSK9 is involved in the degradation of LDL receptors [12] and, in turn, influences the LDL cholesterol level in plasma. Gain of function mutations in *PCSK9* leads to uncontrolled degradation of *LDLR* and may result in severe FH whereas loss of function mutation leads to decreased levels of LDL-C [13,14]. Till date there are no reports of mutation in the *PCSK9* gene in the Indian population.

This is the first study to screen for mutations in the *LDLR*, *APOB* and *PCSK9* genes in CAD patients from the southernmost part of India, a region known for inbred communities and consanguineous marriages. The mutations reported in this study have not been reported before in the Indian population but have been seen in other populations.

2. Materials and methods

2.1. Blood sampling and DNA isolation

Blood samples were collected from 300 unrelated South Indian CAD patients including patients with acute coronary syndrome and chronic stable angina as diagnosed by ACC/AHA guidelines from SRM Medical college hospital, Chennai, India after getting their written consent. Serum samples were collected from 300 patients and lipid profiles were analyzed and categorized with respect to UK Simon-Broome criteria for the diagnosis of FH and 30 patients with elevated levels of total cholesterol and LDL-C with possible FH were considered for the study (TC > 6.5 mmol/L and LDL-C > 4.0 mmol/L). Tendon Xanthoma was not observed in any of the patients considered in this study. DNA was isolated from whole blood by phenol chloroform extraction and quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific).

2.2. PCR-high resolution melt

All the exons and exon–intron boundaries of *LDLR* gene, exon 7 of *PCSK9* (p. (D374Y)) and a part of exon26 of the *APOB* gene (p. (R3527Q)) were screened through High Resolution Melt (HRM) analysis, as previously described [15], using the Rotorgene 6000 (Corbett/Qiagen). Accumelt HRM mix (Quanta Bioscience) was used to amplify the fragments in 10 μl reactions with 7.5 ng of genomic DNA and 4 Pico moles of each respective primer.

2.3. Restriction fragment length polymorphism (RFLP)

The polymorphisms in several exons were indicated by a shift in the HRM analysis which are characteristic of heterozygous genotype, which were genotyped using RFLP employing respective restriction enzyme to ensure that they are only known polymorphism not any other mutation. This also used to analyze the prevalence of the polymorphism in the population and to determine its significance. Few of them were sequenced further to confirm the obtained genotype result.

2.4. Sequence analysis

Samples with a shift in melt (HRM) were amplified using the same primers used for HRM [15] and purified by a column purification method (GFX-kit, GE Healthcare). The purified products were sent to Eurofins or Source Biosciences for sequencing.

2.5. In silico analysis

Variations were analyzed using Polyphen2 [16], SIFT [17] and Mutation Taster [18] for pathogenicity prediction and Berkley Drosophila Genome Project (BDGP) [19], Human Splice Finder (HSF) [20] and ESE Finder [21] for splice site and silent mutation pathogenicity prediction.

3. Results

In this study, of 300 CAD patients (baseline characters shown in Table 1), 30 were considered based on UK Simon-Broome criteria for FH setting threshold limits of TC > 6.5 mmol/L and LDLC > 4.0 mmol/L (UK SB criteria are 7.5 mmol/L and 4.9 mmol/L respectively). Since there are no reports of lipid level criteria for FH diagnosis in the Indian population, SB criteria were lowered by 1 mmol/L so that we may not leave out any FH patients for screening. From our study, the subjects confirmed with a FH causing mutation had mean TC and LDLC levels of 7.73 ± 0.39 mmol/L and 5.9 ± 0.3 mmol/L respectively (Fig. 1).

3.1. LDLR variations

This study unveiled five variations in the *LDLR* gene in the studied Indian population (Table 2). The HRM results of exon 6 of patient SIn FH 225 had a characteristic shift of heterozygous nature. It was further analyzed by sequencing and we found c.862G>A p. (E288K) mutation. The splice site mutation c.1845 + 2T>C was observed in patient SInFH 9, the HRM pattern and the sequencing results are shown in Fig. 2. The splice site mutation was analyzed using in silico tools (Berkley Drosophila Genome Project (BDGP), Human Splice Finder [20] (HSF) to suggest whether the change affects splicing. BDGP predicted that there is a significant change in splice site score that may affect splicing (allele; wild type t = 0.5; mutant c = 0.0). HSF predicted that the donor splice site is broken if the mutant c-allele is present and aberrant splicing would be the result.

Exon 10 of SInFH 258 was sequenced after having a characteristic double heterozygous type curve in HRM analysis (Fig. 2), we found a common polymorphism (c.1413G>A rs5930) and a deletion–insertion of two bases (c.1399_1340delinsTA) that changes the amino acid at position 467 from threonine to tyrosine (Fig. 3). The mutation was predicted to be damaging by Polyphen2 and SIFT, while Mutation Taster predicted it as a polymorphism. According to two main LDLR databases UCL LDLR database (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=LDLR) [22] and LDLR UMD (http://www.umd.be/LDLR/4DACTION/WS_SC1) [23] this mutation has not been reported before

Table 1
Baseline characteristic features of CAD patients.

	Whole sample n = 300	Selected patients n = 30	Pathogenic mutation carriers n = 4
	Mean ± SD/frequency	Mean ± SD/frequency	Mean ± SD/frequency
Age	53.78 ± 10.17	57.1 ± 12.1	56.2 ± 11
Gender-male (%)	234 (75.5)	27 (90%)	3(75%)
BMI (kg/m^2)	25.69 ± 3.72	26 ± 3.1	27.3 ± 3.9
Diabetes	155(50%)	14 (46.6%)	1 (25%)
Hypertension	153(49.4)	14 (46.6%)	1 (25%)
Smoking	77(24.8)	10 (33.3%)	1 (25%)
Family history of CAD	76(24.5)	6 (20%)	0
Total cholesterol (mmol/l)	4.719 ± 1.280	6.69 ± 0.92	7.73 ± 0.39
HDL-c (mmol/l)	0.994 ± 0.2674	1.11 ± 0.25	1.22 ± 0.2
VLDL (mmol/l)	0.832 ± 0.609	0.85 ± 0.39	1.05 ± 0.3
TGL (mmol/l)	4.158 ± 1.132	2.1 ± 1.1	2.0 ± 0.5
LDL-c (mmol/l)	3.176 ± 1.296	4.9 ± 0.75	5.9 ± 0.3
Glucose (mg/dl)	171.77 ± 79.19	164 ± 98.0	162 ± 57
Urea (mg/dl)	25.5 ± 11.2	28.3 ± 18.4	22.5 ± 10.8
Creatinine (mg/dl)	0.99 ± 0.3	1.04 ± 0.33	0.95 ± 0.18