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

KN ArulJothi [1], RA Whitthall[2], M Futema[2], SE Humphries[2], Melvin George[3], Elangovan S[3], Devi A[1*]

[1] Department of Genetic Engineering, SRM University, Chennai, India

[2]Centre for Cardiovascular Genetics, Institute of Cardiovascular Science, University College London, London, UK

[3] Department of Cardiology, SRM Medical College Hospital, Chennai, India

*Correspondence to be sent – adevipradeep@gmail.com

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-SB 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.862 G>A [p. (E288K)] in exon 6 and a splice site variation c.1845+2 T>C in exon - intron junction 12 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. Insilico 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], [9] and [10]

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] and [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. Lipid profiles of the 300 patients were analyzed 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 and LDL-C >4.0). 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 the 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 10ul 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 HRM15 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 Finder21 for splice site and silent mutation pathogenicity prediction.

3. Results

In this study, of 300 CVD 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 7.5mmol/L and 4.9mmol/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±0.39mmol /L and 5.9± 0.3 mmol/L respectively (**Figure.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.862 G>A p. (E288K) mutation. The splice site mutation c.1845+2 T>C was observed in patient SInFH 9, the HRM pattern and the sequencing results are shown in **Figure.2.** The splice site mutation was analyzed using in silico tools (Berkley Drosophila Genome Project (BDGP), Human Splice Finder20 (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 (Figure. 2), we found a common polymorphism (c.1413 G>A rs5930) and a deletion-insertion of two bases (c.1399_1340delinsTA) that changes the amino acid at position 467 from threonine to tyrosine (**Figure. 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 in any population SInFH 146 was found to have the mutation c.694+8_694+18del, 11 bases are deleted in the intron 4 of LDLR gene. This mutation was analyzed using BDGP and HSF to see whether it affects splicing. BDGP predicted that the score is lower in the mutant compared to wild type (WT = 0.87, M = 0.6) and ESE finder predicts that the sites for SRSF1 and SRSF2 are lost (**Figure.5**). A novel silent variation c.966 C>T p. (N322=) was observed in SInFH 286 (**Figure. 3**), polyphen2 and SIFT predicted no damage by the mutation whereas Mutation Taster predicted this mutation as disease causing mutation. Since it is a synonymous mutation we looked further to see whether splicing would be affected in any way, using BDGP and ESE tools. BDGP predicted that the synonymous change did not alter the acceptor splice site score compared to that of the wild type allele c = 0.39, t = 0.39. ESE predicted that the sites of SRSF1, SRSF6 and SRSF1 (IgM- BRCA1) is lost and SRSF5 site is gained in the mutant allele.

3.2. PCSK9 and APOB variations

Exon 7 (including the intron/exon boundaries) of PCSK9 was screened by HRM, the sample SInFH 225, showed a shift and the sample was analyzed further by sequencing and found to have a c.1180+17C>T change in intron 7(**Figure .4**). The change was further analyzed using in silico tools, as before, and found this change does not affect splicing in any way (BDGP – no difference in donor site score, ESE predicted the sites of SRSF1, SRSF5 and SRSF1 (IgM-BRCA1) are lost in the mutant. A fragment in exon 26 of APOB was screened and no change was found.

3.3. Lipid levels in the mutation carriers

Table.1 shows the clinical features and lipid profile of the FH patients diagnosed with the mutations. As can be seen in Figure.1, the subjects confirmed with an FH-causing mutation had mean TC and LDLC levels of 7.73±0.39mmol /L and 5.9± 0.3mmol/L respectively, which were significantly higher than the non-mutation group.

4. Discussion

The study was aimed at finding novel and recurrent mutations in LDLR, APOB and PCSK9 genes of CVD patients from southern part of India. There are very few reports about the mutations in these genes in the Indian population, and the sample selection criteria or threshold limits for selection were also not scrutinized elsewhere. Though this study started with 300 CVD patients, based on the UK SB criteria only 30 samples were screened for FH mutation testing in these three genes. An FH-causing mutation was found in four out of the 30 clinically classified FH patients, resulting in 13% mutation detection rate. The cut off values were lowered in the study and the average TC and LDLC were 6.53±0.9mmol /L and 4.9± 0.75 mmol/L respectively and the average TC and LDL-C levels of patients diagnosed for the FH causing mutation was 7.73±0.39 mmol /L and 5.9± 0.3 mmol/L respectively. The patients diagnosed with an FH-causing mutation had their cholesterol (TC and LDL-C) levels within the SB criteria, which suggests that the UK SB criteria can be followed to clinically diagnose FH in individuals from Indian Subcontinent. The sample size is too low to draw a conclusion on setting the cut off values to categorize patients, but the study suggests that the UK Simon Broome cut-offs can be considered for the Indian population to diagnose FH patients.

The mutation detection rate was 13.3% after lowering the cut off points by 1mmol/L from SB criteria, the detection rate may increase considerably if the exact SB criteria is adopted to select patients for the diagnosis of mutation. Whereas when the exact UK-SB (TC-7.5mmol/l and LDL-C-4.9mmol/L) criteria was applied, the mutation detection rate was 57.7% (4/7), which suggests it is an appropriate detection cut-off for FH for the Indian population.

4.1. Detected Mutations

The mutation c.862 G>A p. (E288K) observed in SInFH225 was not previously reported in the Indian population, but has been seen previously in European subjects (Germany, New Zealand and Netherland) [24], [25], [26]. The splice site mutation c.1845+2 T>C was previously reported in a Japanese patient and for the first time in Indian subjects. This mutation may result

in alternate splicing and the prediction made by in silico tool BDGP suggested that the mutant allele resulted in a significant change in the splice site score (allele; wild type T= 0.5; mutant C = 0.0) that would affect splicing.

The sample SInFH 258 had a novel insertion/deletion mutation (c.1399_1340delinsTA) that would result in threonine to tyrosine change at 467 position of the receptor. The patient also had features compatible with a diagnosis of heterozygous FH (TC = 8.07mmol/l, LDL C = 6.44mmol/l), which supports the mutation as pathogenic. Since there are two base pairs altered in the same subject, we may expect a compound homozygous mutation whereas the clinical feature doesn't show homozygous features.

SInFH146 had mutation (c.694+8_694+18del) in the intron which is predicted to affect the splice site by the in silico tool ESE Finder (Figure.5). It was found that the proteins SRSF1 or ASF1/SF2 which is involved in discriminating the cryptic and authentic splice site 27and SRSF2 that interacts with the splicing complex lose their binding site which is at closer proximity to the splice site28. The patient was clinically diagnosed with heterozygous FH and we did not find any other significant variation in LDLR, APOB and PCSK9 genes. Hence the heterozygous phenotype could be conferred by the above mentioned change.

A novel silent mutation c.966C>T, p. (N322=) was found in the patient SInFH286, although synonymous variants are less likely to have an effect on the protein's function, the Mutation taster predicted this variation to be "disease causing". This nucleotide change does not insert any splice enhancing sites, according to the in silico tools used.

There were no mutations observed in exon 7 of PCSK9 and exon 26 of APOB genes in the studied Indian population. No other reports have found any mutations in these genes in the Indian population supporting our findings.

5. Conclusions

Previous reports on Indian population suggested that the mutation were distributed mostly in exons 3, 4, 7, 8 and 14 with this study the distribution extends to exons 6, 10 and 12. These exons can also be given equal importance while screening for the LDLR mutations in the Indian population. The UK Simon Broome criteria can be recommended to screen the patients for FH in future studies. The principle limitation to this study is the relatively small sample size. It is also possible that the methods used to screen for FH-causing mutations may have missed some for technical reasons.

Acknowledgements

The study was supported by a grant from the British Heart Foundation (grant RC 93008). SEH and JAC acknowledge BHF support (RG 2008/008) and also funding from the Department of Health's NIHR Biomedical Research Centers funding scheme.

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