Original Article

A Rare Missense Mutation and a Polymorphism with High Frequency in *LDLR* Gene among Iranian Patients with Familial Hypercholesterolemia

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Abstract

Background: Familial hypercholesterolemia (FH) is a disorder that is inherited by autosomal dominant pattern. The main cause of FH disease is the occurrence of mutations in low-density lipoprotein receptor (LDLR) gene sequence, as well as apolipoprotein B and proprotein convertase subtilisin/kexin type 9 genes, located in the next ranks, respectively. **Materials and Methods:** Forty-five unrelated Iranian patients with FH were screened using a high-resolution melting (HRM) method for exon 9 along with intron/exon boundaries of *LDLR* gene. Samples with shift in resultant HRM curves were compared to normal ones, sequenced, and analyzed. **Results:** Our findings revealed a missense mutation c. 1246C>T and a known variant IVS9-30C>T (rs1003723) that was recognized in 71% of the patients (22%: homozygous and 49%: heterozygous genotypes). *In silico* analysis, predicted the pathological effect of the c. 1246C>T mutation in LDLR protein structure, but IVS9-30C>T variant had no predicted effect on splice site and branch point function. **Conclusion:** FH is a hereditary type of hypercholesterolemia that leads to premature cardiovascular disease and atherosclerosis, and early diagnosis is needed. We detected a rare missense mutation (1246C>T) and a common single nucleotide polymorphism (SNP) in the Iranian population. These reports could help in the genetic diagnosis and counseling of FH patients.

Keywords: *Familial hypercholesterolemia, high-resolution melting, Iran, low-density lipoprotein receptor, missense mutation*

Introduction

The level of the cholesterol is affected by many factors, including lifestyle, diet, and heredity factors.[1] Familial Hypercholesterolemia (FH) (OMIM 143890) is a hereditary type of hypercholesterolemia that is generally transmitted in an autosomal dominant pattern.[1,2] FH is categorized as a lipoprotein metabolism disorder with a frequency of 1:500 in the heterozygote form in most population; but its homozygote form is infrequent (1:1000000 in a live born), extremely severe and usually causes death in the early decades of life.[3]

The main pathophysiology of FH is generally the mutation in low-density lipoprotein receptor (*LDLR*) gene,[4] and less frequently in apolipoprotein B (*APOB*) and proprotein convertase subtilisin/kexin type 9 genes.[5] *LDLR* gene is located on chromosome 19 and has 18 exons with length 45 kbps. Up to now, over 1288 unique mutations and allelic variants have been identified in *LDLR* gene.[6] The mutation distribution and frequency in *LDLR* gene are not balanced through the gene sequence. According to the study of Palacios *et al*.,[7] some exons such as four and nine have the highest frequency through the *LDLR* exons among different populations worldwide.

In physiological conditions, LDLRs regulate cholesterol metabolism by up taking LDL-cholesterol (LDL-C) from blood circulation[8] and this process is mediated by ApoB protein; which is a fundamental part of the LDL particle. Mutations in *LDLR* gene result in lack of functional *LDLR* on the surface of hepatocytes that subsequently increases the plasma LDL level.[9] The raised levels of LDL-C and total cholesterol (TC) in plasma are the most important biochemical characteristics of FH.[10] The deposited cholesterol in peripheral tissues causes xanthomas, xanthelasma, arcus senilis, and increases the risk of premature atherosclerosis, coronary heart disease, and eventually myocardial infarction (MI).[11] Early diagnosis and clinical management of the elevated cholesterol will decrease the risk

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of atherosclerosis, premature cardiovascular condition and improve the prognosis in patients with FH.[12] The genetic mutation screening in FH patients has been found to be cost-effective and could help early diagnosis and management.[13] Genetic studies of patients with FH around the world were mostly performed in non-Iranian population, and there are few studies have investigated Iranian population.[14-16] In these limited studies, mainly, the genetic alteration profiles of exon four of *LDLR* gene were investigated and to the best of our knowledge, there is only one study exploring the exon 9 of *LDLR* in the Iranian FH population.[17] Thus, according to high frequency of mutation that has been reported in this exon among many population around the world and the limited studies on it in Iran, we screened this part of *LDLR* gene in 45 unrelated Iranian patients suspected to FH.

Materials and Methods

Sample collection

Dutch Lipid Clinic Network (DLCN) criteria were used for screening FH patients among people with hypercholesterolemia.[18] Finally, 45 unrelated patients of 500 suspected FH patients were selected from hospitals/clinics Shahid Sadoughi University of Medical Sciences (Yazd, Iran). The sampling protocol was reviewed and accepted by Shahid Sadoughi University of Medical Sciences Ethics Committee.

Lipid profile

Lipid profile containing TC, LDL-C, high-density-lipoprotein-cholesterol (HDL-C) and triglyceride (TG) of all the subjects were obtained with BT3000 PLUS automated system by using commercial kits cholesterol-L (CHOD-PAP) Cat. No. 135 L, cholesterol-LDL direct-L Cat. No. 145 L, cholesterol-HDL direct-L Cat. No. 143 L and TGs-L (GPO-PAP) Cat. No. 315 L (Biotecnica Instruments, Rome, Italy).

Primer designing

Polymerase chain reaction (PCR) primers for amplifying exon 9 and the exon-intron boundaries of it were designed using the GenRunner software (http://www.generunner.net) and Oligo7.5 (http://www.oligo.net). NCBI primer blast[19] was used to check the primers for specificity against *LDLR* gene.

DNA extraction and measurement

Genomic DNA (gDNA) was extracted from whole blood by AccuPrep® gDNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instruction. The purity and quality of DNA samples were measured by Thermo Scientific™ NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

Polymerase chain reaction and high-resolution melting

PCR and high-resolution melting (HRM) were accomplished by Rotor-Gene Q 2plx HRM (Qiagen, Hilden, Germany) using Type-it HRM PCR Kit (Qiagen, Hilden, Germany) with 60 ng of gDNA and 13 pmol of each primer in a final volume of 25 μl, in accordance with manufacturer's instruction. The amplified segment of exon 9, including coding region and exon-intron boundaries of *LDLR*, was screened by HRM as described in Whittall *et al.* procedure[20] and the samples with a shift in HRM and melting temperature curves were then sequenced.

Sequencing

The amplified products were purified using the ExoSAP-ITzreg Purification Kit from Affymetrix/USB and sequenced by ABI3730XL (Applied Biosciences, ABI) sequence system in the MacroGen company (Macrogen, Seoul, Korea).

In-silico analysis

For predicting the mutation effects on the function of LDLR protein *in-silico*, web-based tools including PolyPhen 2 (http://genetics.bwh.harvard.edu/pph 2/) sift (http://sift.bii.a-star.edu.sg/) and Mutation-t@ ster (http://www.mutationtaster.org) were used. These software programs predict the possible impacts of an amino acid substitution on the structure and function of a human protein by considering evaluating its physical and molecular characteristics in compared to the database.[21-23] Furthermore, HOPE tool (homotopy optimization using perturbations and ensembles)[24] was employed to determine the mutation effects on the three-dimensional (3D) structure of protein. T-coffee tool was also used for evaluating and manipulating multiple alignments of protein sequences and structures.[25] The possible effects of nucleotide changes (in intronic conserved site) on splicing process were investigated using Splice Port, NetGene2 Server and Human Splicing Finder software programs.[26-28] These programs provide predictive scores for splicing site and branch point sequence for wild and modified sequence types.

Results

Clinical characteristics of the patients

The medical history and clinical examination of 45 possible FH subjects that they gained DLCN diagnostic criteria revealed that 82.2% of them had family history of coronary artery disease and MI, 46.6% had combined xanthelasmata with corneal arcus, 24.4% had combined xanthomata and corneal arcus, 13.3% had corneal arcus alone, 8.8% had corneal arcus with xanthelasmata, and 4.4% had xanthelasmata alone [Table 1].

A rare missense mutation was found in low-density lipoprotein receptor gene among Iranian patients

PCR-HRM technique was employed for nucleotide alteration screening in exon 9 (coding region and exon-intron boundaries) of the *LDLR* gene. The missense

mutation, c. 1246C>T; p.(R416W) was detected in exon 9 in one patient after DNA sequencing of modified HRM profile in comparison to normal ones [Figure 1].

The arginine (Arg), amino acid in this site in different species, is extremely conserved [Figure 2] and moreover, our prediction by polyPhen-2 ("Sift" and "Mutation Taster" were also used, and their results are shown in Table 2) also showed that this change could be "Probably Damaging" (score 0.998). The change in ionic charge, hydrophobicity, and size of the amino acid side chain when Arg to tryptophan substitution happens is expected to bring about an alteration in the protein conformation. This expectancy was also illustrated by protein modeling in Figure 3.

High frequency of rs1003723 polymorphism among Iranian patients

The PCR-HRM analysis and DNA sequencing of the patients showed 32 (CT: 22 and TT: 10) of them has a polymorphism that has not been previously reported in Iran [Figure 4], and its allele frequency was about 71.1% among investigated patients. This polymorphism, IVS9-30C>T (rs1003723), was detected in intron 9 of *LDLR* gene. As for, the site of this polymorphism were analyzed by different programs, including "NetGene2," "Splice port," and "Human splicing finder" to predict the potential effect(s) of it on splicing procedure of LDLR transcript (produced results has been shown in Table 2). All of them predicted that rs1003723 has no effect on splice sites, branch point, or alternative splice sites.

Table 1: Lipid profile of the studied patients

Parameters	Mean±SD		
	Total	Men	Women
n	45	23	22
Age	40.1±9.5	40.5±9.3	39.6±9.9
Triglycerides	150.1±65.7	153.7±62.2	146.3±70.4
LDL-cholesterol	210.6±78.6	211.4±74.8	209.9±84.3
HDL-cholesterol	50.5±11.1	48.7±10.8	52.4±11.4
Total cholesterol	300±119.7	308.6±142.1	289.5±93.1

SD: Standard deviation, LDL: Low-density lipoprotein, HDL: High-density lipoprotein

Discussion

LDLR protein has different domains and any modification in their function can result in a spectrum of hypercholesterolemia with various severities. The epidermal growth factor (EGF) precursor homology domain which is encoded by exons 7–14 of *LDLR* gene is located near the ligand binding domain and includes two EGF-like motifs, a series of six YWTD repeats and again EGF-like motif. Second and third EGF-like motifs accompanied by YWTD repeats create six bladed β-propeller that is responsible for the release of LDL particle at low pH of endosome and recycling of the receptor to the cell membrane.[29] Mutations in these repeats and C-terminal of the third EGF-like motif can cause defects in releasing of the receptors from the LDL particle in the endosome by interruption of the β-propeller configuration.[29,30] The recently recognized mutation, c.1246C>T; p.(R416W), is close to the YWDT repeats. Therefore, the defective recycling of the receptor

Table 2: *In-silico* analysis of nucleotide changes and the predicted impacts

Nucleotide change	Amino acid change	Previously reported in Iran	Protein prediction			Splice prediction			Overall *in-silico* prediction pathogenic
			PolyPhen-2	Sift	Mutation Taster	NetGene2 Server	Splice Port	Human Splicing Finder (branch point)	
c. 1246C>T	p.(R416W)	No	Probably damaging	Tolerated	Disease causing	-	-	-	Yes
IVS9-30C>T	-	No	-	-	-	No difference	No difference	Variation	No

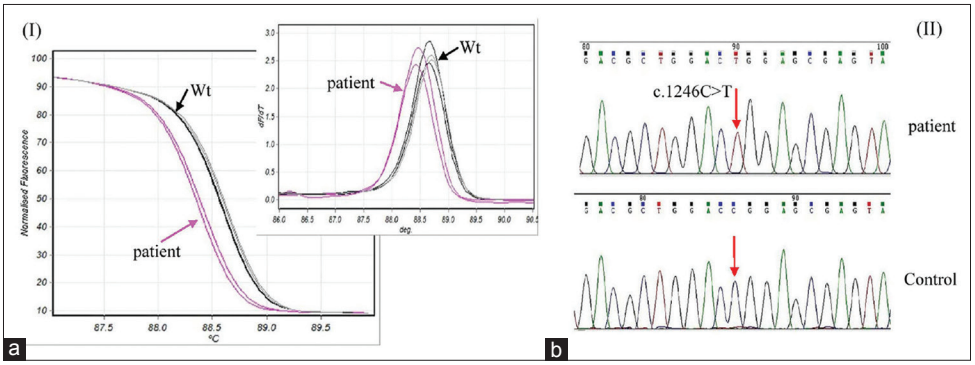

Figure 1: High-resolution melting and sequencing of low-density lipoprotein receptor exon 9 showing c.1246C>T; p.(R416W) in a patient. (a) High-resolution melting. The wild type profile and shift in patient are shown. (b) Sequencing. The homozygous sequence of the variant in patient is shown in the upper panel and the wild type sequence in the bottom panel. The relevant bases are arrowed

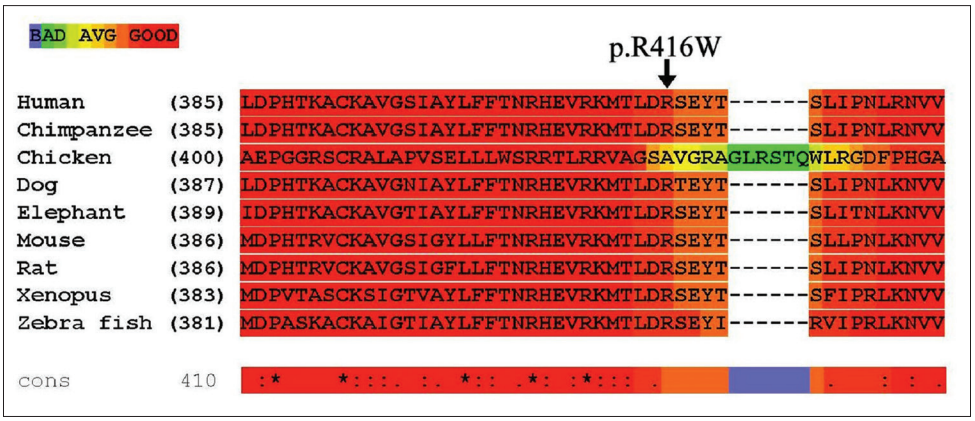

Figure 2: Amino acid conservation in different species. The low-density lipoprotein receptor protein of the species indicated was aligned using ClustalW (T-Coffee Server). The relevant amino acid, p.R416W, is arrowed showing that the amino acid is highly conserved across species

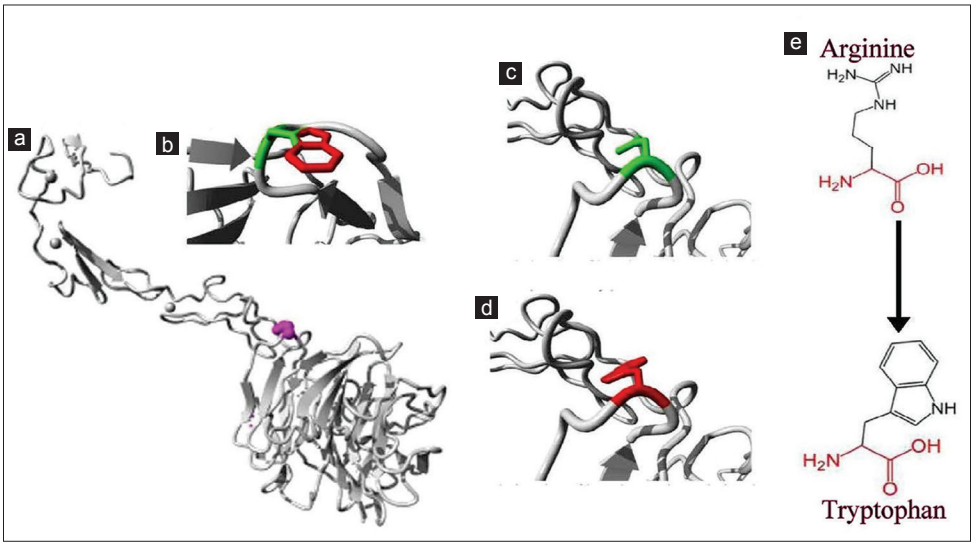

Figure 3: The three-dimensional structural effect of the low-density lipoprotein receptor mutation. (a) Three-dimensional structure of the protein with purple area representing the site of mutation. (b) Close-up of the mutation. The protein is Gray, the side chains of both the wild-type and the mutant residue are shown in green and red respectively. (c) The wild-type structure with the arginine side chain. (d) The substitution of Tryptophan results in the change of the side chain thereby affecting the charge, hydrophobicity, and size of the amino acid. (e) The structural representation of arginine and tryptophan

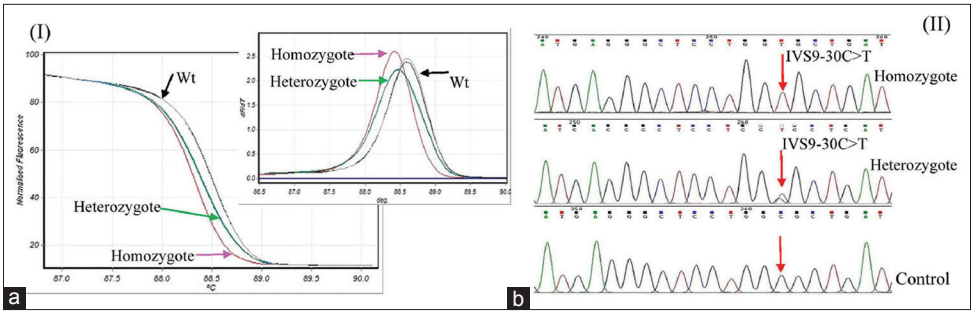

Figure 4: High-resolution melting and sequencing of low-density lipoprotein receptor intron 9 showing IVS9-30C>T (rs1003723) in patients. (a) High-resolution melting. The wild type profile and shifts in patients are shown. The heterozygous and homozygous patients are distinguishable in their curve. (b) Sequencing. The homozygous sequence of the variant in a patient is shown in the upper panel; the heterozygous sequence is shown in the middle panel and the wild type sequence in the bottom panel. The relevant bases are arrowed

by this change may finally decrease the number of cell surface LDLR, and increases TC and LDL-C levels.

Chiou and Charng[31] have previously reported two nucleotide substitutions in this amino acid; c.1246C>T; p.(R416W), similar to our result, and c.1247G>T; p.(R416 L) among the Taiwanese population. The evaluation of R416 conservation reveals that this amino acid residue in this site is highly conserved among different species of

vertebrates [Figure 3]. Furthermore, our predicted effects of the p.(R416W) on protein structure revealed that these changes, i.e., R416W and R416 L can have different effects on LDLR protein. The R416W is potentially able to result in the loss of hydrogen bonds and/or disturbing correct folding, while R416 L causes the loss of interactions with other molecules.

Etxebarria *et al.* have recently acclaimed that this mutation does not affect expression levels of LDLR but can significantly reduce the presence of receptors on the mutant cell surfaces. He ascertains that this phenomenon is related to defects in the returning procedure of receptors in the cell surfaces and locates this mutation in class IV of LDLR pathogenic variants.[32] Hence, the c.1246C>T variant in exon 9 in this study is supposed to be the causal variant involved in elevated serum cholesterol levels in the patients and the other mutation c.1247G>T; p.(R416 L) seems to have a benign effect on 3D structure of LDLR protein.

We also identified an SNP, IVS9-30C>T (rs1003723), with high frequency among investigated patients. The minor allele frequency of rs1003723 in 1000 genome project was reported t = 28.2%. However, the frequency of this allele in investigating patients with FH was 71.1%. Andreotti *et al.* have previously studied this allele in a Chinese population.[33,34] Their results showed that this allele is associated with high TC and LDL.[33] They also claimed that this polymorphism can be associated with a 1.5-fold increased risk of bile duct cancer[34] but its mechanism is not clear. Our *in-silico* analysis did not predict any effect from IVS9-30C>T on splice sites, branch point or alternative splice sites. However, recent *In-silico* investigation for finding mirtrons in human genome has predicted that the sequence of intron 9 consists of mirtron (miR6886)[35] and rs1003723 SNP is exactly placed in the loop on this predicted mirtron.

Conclusion

FH is a hereditary type of hypercholesterolemia that leads to premature cardiovascular disease and atherosclerosis, and early diagnosis is needed. We detected a rare missense mutation (1246C>T) and a common SNP in Iranian population. These reports will help in the genetic diagnosis and counseling of FH patients.

In the future, investigation of other regions of LDLR sequence and different related genes involved in cholesterol metabolism in the patient that has missense homozygous 1246C>T would be able to make us more sure about clinical effect(s) of this mutation on LDLR function. Moreover, further study about the frequency of rs1003723 SNP (T-allele) in normal Iranian population in comparison to population with FH, and determining the effects of it on miR6886 function are other aims that we will follow them. Finally seems to be elucidating the role of miR6886 in the regulation of TC concentration in blood could be explained the susceptibility to bile duct cancer and would be able to open a new perspective regarding the effects of noncoding RNA (ncRNA) on LDL level in blood and related resultant diseases.

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Conflicts of interest

There are no conflicts of interest.

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