Original Article

Sequencing the Exon.4 of the LDL Receptor Gene in Patients with Familial Hypercholesterolemia in the Population of Bushehr, Southwestern Iran: the Possible New Mutations

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Abstract

Background & Objective: Familial hypercholesterolemia (FH) is the most common genetic disease in the world and an autosomal dominant disease characterized by increased plasma cholesterol and low-density lipoprotein (LDL) concentrations. The clinical diagnosis of the disease is based on family history, the findings of medical examinations, and the measurement of cholesterol levels. The most important cause of FH is the mutation in one of the LDL-R, APOB, and PSCK9 genes. About 90% of mutations occur in the LDL-R gene, which accounts for a total of 2,000 different mutations. Different types of mutations have been observed on different exons of the LDL-R gene, but most of the mutations have been reported on Exon 4. The aim of this study was to sequence and analyze Exon 4 in patients with familial hypercholesterolemia in Bushehr province in Iran.

Materials & Methods: In this study, 32 patients were selected based on global criteria for diagnosing the disease, and a portion of LDL-R containing complete sequence of exon 4 was amplified using LDLRE4F1/ LDLRE4R1 Primers and blood genomic DNA as a template. PCR products were sequenced and compared with reference sequence to find probable muta¬tions.

Results: The results of sequencing and comparison with the reference sequence showed that no mutation was found in the exon 4 LDL-R gene. Therefore, this exon did not play a role in FH in the population under study.

Conclusion: Therefore, the cause of FH may be due to the mutations in other areas of the LDL-R gene or other genes, such as APOB and PSCK9

Keywords: Exon 4, Familial Hypercholesterolemia, LDL Receptor, Mutation

Introduction

Hypercholesterolemia is a metabolic disease that results in an increase in blood cholesterol higher than normal level. The prevalence of the disease is high in different populations due to the poor diet, obesity, and a sedentary lifestyle(1). Both environmental and genetic factors play an important role in raising the blood cholesterol levels. One of the most common forms of the genetic disease is familial hypercholesterolemia (FH) (2). FH is a genetic disease with a dominant autosomal inheritance pattern. The disease is caused by high levels of low-density lipoprotein (LDL) cholesterol which increases the risk of early cardiovascular disease (ASCVD) in both men and women (3).

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In patients with FH, the cardiovascular disease occurs under the age of 20, and 50 and appears to be homozygote and heterozygote respectively (4). Early diagnosis and treatment of the disease can reduce the high risk of cardiovascular atherosclerosis. Diagnosis can be based on clinical criteria (including LDL, Cholesterol, and family history) or DNA testing. In recent decades, several screening projects have been conducted in different countries, leading to the identification of a large number of these patients (5).

Clinical criteria commonly used to diagnose FH include Simon Broome Register Group criteria (SB) (6) Dutch Lipid Clinic Network Criteria (DLCN) (7), Make Early Diagnosis to Prevent Early Deaths (MEDPED) criteria (8), and Japanese FH Management Criteria (JFHMC) (9). Clinical criteria may not identify all the patients with FH, so genetic testing in many countries is part of screening strategies (10).

Approximately 86 to 88% of molecular detection cases, caused by harmful mutations in the low-density lipoprotein receptor coding gene (LDL-R), account for about 12%, caused by mutations in the apolipoprotein B (APOB) coding gene, and less than 0.1-2% is due to a mutation in the PCSK9 coding gene (11). About 60% of patients with FH diagnosis did not find mutations in the LDL-R, APOB, and PCSK9 genes. Some of these reasons can be explained by the accumulation of small effects of LDL-C enhancing alleles (12). APOE and STAP1 are other genes in which genetic defects have been cited as one of the causes of FH.

The association of Leucine removal in position 167 of the APOE coding region, as well as several functional mutations in the STAP1 gene has been identified as the causative agent (13). Other possible causes include variants of other genes that have not yet been linked to the disease (12).

A group of patients in whom no mutations were observed is called Non-FH-GHs, which have a milder phenotype than those with a genetic defect and have a lower risk of LDL-C accumulation and cardiovascular disease (14).

About 90% of the single-gene causes of the FH are as the result of mutations in the LDL-R gene (15), and by 2015, more than 1,700 different mutations had been identified (16). So far, on all LDL-R gene exons, different types of mutations associated with the disease have been identified (12,17,18), but studies from all over the world have shown a high prevalence of mutations in exon 4 (11,12,19,20).

The exon 4 is the largest LDL-R gene exon and has the highest mutation rate compared to other exons. Therefore, screening exon 4 of LDL-R gene in patients with familial hypercholesterolemia using its complete sequencing was the aim of this study.

Material & Methods

Sampling and DNA Extraction

FH patients were diagnosed based on the Simon Broome and Register Group criteria (SB) (6) and Dutch Lipid Clinic Network Criteria (7). Then, thirty-two unrelated possible FH (aged 9<–85 years) that have been referred to laboratories in Bushehr (during during September-December 2017 and January 2018) were included in this study (Table 1).

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Table 1. Clinical and demographic characteristics of FH patients

Patient	Sex	Age (yera)	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL-C (mg/dl)
S1	M	25	290	175	51	195
S2	F	40	273	82	68	190
S3	F	50	285	135	53	205
S4	F	38	267	158	28	207.4
S5	F	60	272	152	44	197.6
S6	F	40	490	135	51	206
S7	M	50	317	203	58	224
S8	M	41	302	99	41	235
S9	F	54	302	175	64	203
S10	M	62	271	159	46	193
S11	F	<9	276	61	49	215
S12	F	40	310	329	52	195
S13	F	36	218	283	48	190
S14	M	46	305	243	53	215
S15	M	37	266	168	37	195.4
S16	M	53	311	191	33	197
S17	F	38	285	194	43	206
S18	F	67	308	176	40	233
S19	M	46	322	361	33	217
S20	F	56	317	294	34	224
S21	F	34	280	110	64	194
S22	M	85	387	300	27	300
S23	F	41	298	106	50	227
S24	F	53	340	225	67	216
S25	F	58	368	261	65	230
S26	F	47	359	217	56	260
S27	F	37	253	129	35	192
S28	M	41	309	157	35	243
S29	F	43	374	130	56	286
S30	F	71	320	171	54	232
S31	M	45	296	179	43	217
S32	M	37	510	111	65	329

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Total cholesterol (TC), Triglycerides (TG), High-density lipoprotein cholesterol (HDL-C), and Low-density lipoprotein cholesterol (LDL-C).

Total genomic DNA was extracted from whole blood using genomic DNA extraction kit (Gene All- R. Korea), according to the manual instruction. Briefly, the 200µl of Blood sample, 200µl GB buffer and 20µl proteinase K were mixed well and incubated at 55°C for 10 min. Then, 200 µl ethanol was added to the mentioned mixture. Subsequently, the mixture was added into a spin column and centrifuged at 10000 rpm for 1 min. Flow-through and collection tube were Discarded and the spin column was placed in a new collection tube. The spin column was washed with buffers GW1 and GW2 and Flow-through and collection tube were discarded. Finally, the column was placed in clean 1.5ml microcentrifuge tube and 100-200 µl buffer GE was added into the spin column and centrifuged at 10000 rpm for 1 min. The purified DNA was collected and preserved at 4°C for a short time or -20°C for a long time. Quantity and quality of DNA was measured using spectrophotometer at 260 and ratio of 260/ 280 nm, respectively. In addition, the DNA integrity was evaluated by electrophoresis on 1% agarose gel.

Amplification of LDL-R gene partial region

A fragment of the LDL-R gene (536bp) containing partial sequences of Introns 3 and 4 and complete sequence of Exon4 were amplified using LDLRE4F1 (5′- GTTGGGAG ACTTCACACGGTG -3′) and LDLRE4R1 (5′- GTTGGAAATCCACTTCGGCAC -3′) primers. The primers were designed based on Homo sapiens low density lipoprotein receptor, Ref Seq Gene (LRG_274) on chromosome 19 (NG_009060.1). In addition, the LDLRE4R1's starting position on the Intron 4 was designed at a far distance from the exon 4, to cover the entire sequence of exon 4 while the PCR products were sequenced using LDLRE4R1. First, the PCR condition for amplification of the fragment was optimized as follows in 50 µl volume, containing 1X PCR buffer, 3mM MgCl2, 0.4mM of dNTP mix, 20 pM of each primer, 2.5 U Taq DNA polymerase (Fermentas), 200-400ng DNA. Then, all reactions were performed using Taq DNA Polymerase Master Mix (Ampliqon) and the following thermal cycles: 3 min at 94° C for initial denaturation, followed by 30 cycles at 94° C for 1 min, 60° C for 35-55 sec and 72° C for 50-60 sec. The final extension was at 72º C for 5 min. The PCR products size and quality were evaluated using 1% agarose gel electrophoresis.

DNA Sequencing and analysis

The PCR products of all patients were sequenced using ABI 370 automated sequencer by Bioneer company (R. Korea), using LDLRE4R1Primer. Chromas Pro v.2.1 software (Technelysium Pty Ltd., Australia) was used for sequence chromatograms analysis. A preliminary sequences alignment was done using the ClustalW2 (21) for comparing the resulted sequences. The sequence homologies and confirmation were performed using the Blast software (22). In addition, a sequence alignment was done using the ClustalW2 (21) for comparing the resulted sequences from this study with the reference (NG_009060.1).

Results

Amplification of partial region of LDL-R gene with specific primers LDLRE4F1/LDLRE4R1 produced the expected-size PCR products. The electrophoresis patterns PCR products showed that a single DNA band with the expected length (Figure 1).

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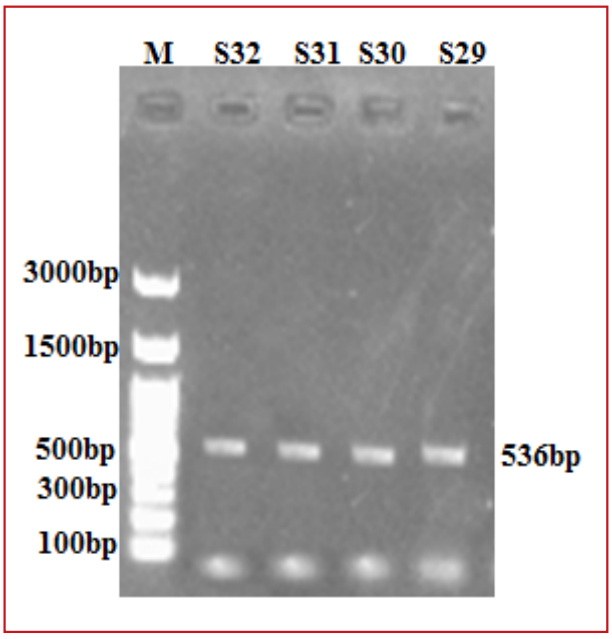

Figure.1 Typical electrophoretic patterns LDL-R gene; 100bp DNA ladder (M) and samples (S32, S31, S30 and S29)

Out of 32 samples, only sequencing quality of 23 samples was acceptable. Ambiguous sites were deleted using Chromas Pro v.2.1 and region with good quality were noticed for analysis. The accuracy of these sequences was confirmed using Blast software and deposited in GenBank (Accession numbers LC546972.1-LC546994.1.)

Alignment of sequences using the Clustalw2 software indicated that all of them were identical. The comparison between the data in this study and the reference sequence (NG_009060.1) indicates no mutation and confirm precisely the same identity (Figure 2). The results showed that patients have no mutations in the exon 4 of LDL-R gene.

Homo sapiens low density lipoprotein receptor (LDLR), RefSeqGene (LRG_274) on chromosome 19

Sequence ID: NG_009060.1 Length: 51450 Number of Matches: 1

Range 1: 20788 to 21281 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
913 bits(494)	0.0	494/494(100%)	0/494(0%)	Plus/Plus

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Query  1      TGTTGGGAGACTTCACACGGTGATGGTGGTCTCGGCCCATCCATCCCTGCAGCCCCCAAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20788  TGTTGGGAGACTTCACACGGTGATGGTGGTCTCGGCCCATCCATCCCTGCAGCCCCCAAG  20847

Query  61     ACGTGCTCCCAGGACGAGTTTCGCTGCCACGATGGGAAGTGCATCTCTCGGCAGTTCGTC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20848  ACGTGCTCCCAGGACGAGTTTCGCTGCCACGATGGGAAGTGCATCTCTCGGCAGTTCGTC  20907

Query  121    TGTGACTCAGACCGGGACTGCTTGGACGGCTCAGACGAGGCCTCCTGCCCGGTGCTCACC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20908  TGTGACTCAGACCGGGACTGCTTGGACGGCTCAGACGAGGCCTCCTGCCCGGTGCTCACC  20967

Query  181    TGTGGTCCCGCCAGCTTCCAGTGCAACAGCTCCACCTGCATCCCCCAGCTGTGGGCCTGC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20968  TGTGGTCCCGCCAGCTTCCAGTGCAACAGCTCCACCTGCATCCCCCAGCTGTGGGCCTGC  21027

Query  241    GACAACGACCCCGACTGCGAAGATGGCTCGGATGAGTGGCCGCAGCGCTGTAGGGGTCTT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21028  GACAACGACCCCGACTGCGAAGATGGCTCGGATGAGTGGCCGCAGCGCTGTAGGGGTCTT  21087

Query  301    TACGTGTTCCAAGGGGACAGTAGCCCCTGCTCGGCCTTCGAGTTCCACTGCCTAAGTGGC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21088  TACGTGTTCCAAGGGGACAGTAGCCCCTGCTCGGCCTTCGAGTTCCACTGCCTAAGTGGC  21147

Query  361    GAGTGCATCCACTCCAGCTGGCGCTGTGATGGTGGCCCCGACTGCAAGGACAAATCTGAC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21148  GAGTGCATCCACTCCAGCTGGCGCTGTGATGGTGGCCCCGACTGCAAGGACAAATCTGAC  21207

Query  421    GAGGAAAACTGCGGTATGGGCGGGGCCAGGGTGGGGGCGGGGCGTCCTATCACCTGTCCC  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21208  GAGGAAAACTGCGGTATGGGCGGGGCCAGGGTGGGGGCGGGGCGTCCTATCACCTGTCCC  21267

Query  481    TGGGCTCCCCCAGG  494
              ||||||||||||||
Sbjct  21268  TGGGCTCCCCCAGG  21281
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Figure 2. The Blast result of the sample 3 against the reference sequence (NG_009060.1)

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Discussion

Familial hypercholesterolemia is a dominant autosomal disease that exposes patients to cardiovascular disease and premature death from the disease. Therefore, rapid diagnosis and treatment of these patients are very important. It is too difficult to determine a definite limit for the diagnosis of Familial Hypercholesterolemia, especially in children and middle-aged peoples in whom the amount of cholesterol depends on age. Clinical criteria are also less sensitive, despite their high specificity. Using laboratory criteria increases the sensitivity of the diagnosis, but even in members of an affected family, diagnosis based on laboratory criteria leads to misdiagnosis in 10 to 20% of cases. However, genetic testing has a 100% specificity for diagnosing people carrying the disease gene in individuals (23). PCR-RFLP (24), PCR-SSCP (25) and direct sequencing of PCR product (24), are the most widely used methods for diagnosis of the mutations in the LDL receptor gene. The PCR direct sequencing method is the most accurate way to screen patients for new mutations. Therefore, in our study, the mentioned strategy was used to detect possible mutations in exon 4 of LDL-R gene.

Various mutations have been reported in the LDLR gene exons, but due to the large size of exon 4, compared to other exons, high frequency of mutations have occurred in this exon (11, 12). Therefore, this exon has been selected for the study. Thus, in order to study the possible new mutations in patients with familial hypercholesterolemia in Bushehr province -Iran, whose disease diagnosis was based on biochemical criteria, the complete direct sequence of exon 4 of LDL-R gene was determined using specific primer. The results of comparing the sequences from this study with the reference sequence showed that all individuals in the studied area of the LDL-R gene had no mutations.

Several laboratories have contributed in this research. Usually, the people with hypercholesterolemia have cardiovascular problems, so a large number of samples were expected to be from Bushehr Heart Center, however, no patient with LDL-C above 190 was found during the study at heart hospital. Perhaps low LDL-C in hospitalized patients can be attributed to their treatment and medication.

In China, a total of 141 mutations were detected by studying 357 probes, of which 131 were in the LDLR share, 4 Apo B mutations, and 6 mutations in the PCSK9 gene were reported. Most mutations occurred in exon 4 of the LDLR gene (26), while in Tunisia, 42% of patients with FH mutate to the LDLR gene on Exon 10(27) has been reported. In a study in which more than 200 patients from the United Kingdom were screened for mutations in LDL-R gene, the highest numbers of mutations were found in exons 4 (28%), 14 (21%), 3 (10%) and 10 (10%) (28).

In a study which was conducted in Shahrekord (Chaharmahal and Bakhtiari province), the role of the LDL receptor gene in causing FH was poorly reported (29). Also, in the studies on exons 5 and 6 LDLR genes in patients with high familial cholesterol in Iran, the relationship between this area and the LDL-R gene with FH has been ruled out (30). In a similar study, mutations in the LDL-R gene were reported to be the cause of FH. Screening exon 4 of LDL-R genes in 30 patients with FH (in Iran-Tehran), that have been clinically diagnosed with a heterozygote, using SSCP-PCR, a new mutation was detected at position 445 of LDL-R gene. This mutation leads to a change from G to T (conversion of Gly to Cys) (31). Recently, in a molecular study of FH patients in Iran with mutations in exons 4, 10, 11, 12, 14, and 17 of the LDL gene have been reported (32). Also, Ekrami and Co- worker showed a mutation in the 3rd and 4th LDL-R gene exons in Iranians (33). The results of the above data indicate that the pattern of genetic mutations that cause the Familial Hypercholesterolemia is variable in Iranian populations.

The results of the above data suggest that the pattern of genetic mutations that cause the FH varies in different populations around the world. Therefore, due to the increasing in premature

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cardiovascular disease risk in familial hypercholesterolemia patients, it is necessary to perform more molecular screening in each region to detect the mutations. So, the mortality rate can be reduced by the proper counseling and lifestyle, including diet, exercise, and drug treatment significantly.

Conclusion

Despite of the observed biochemical signs in selected FH patients, no mutation was found in their exon 4 of LDL-R gene. Therefore, disturbances in other areas of this gene or other genes appear to be the cause of FH. Given that, about 40% of deaths in Bushehr province are due to FH, a large screening program is needed to clarify the genetic background and epidemiological features of the disease.

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Conflict of Interest

Authors declares that they have no conflict of interest.

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