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Regular paper

Screening for genetic mutations in LDLR gene with familial hypercholesterolemia patients in the Saudi population

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Familial hypercholesterolemia (FH) is caused by genetic defects involving the low density lipoprotein-receptor (LDL-R), predisposing affected people to premature atherosclerotic cardiovascular disease and death. The aim of the present study was to assess certain exons in the LDLR gene mutation detection analysis affecting in the Saudi population with FH. This case-control study was carried out with 200 subjects; 100 were FH cases and 100 were healthy controls. Five mL of venous blood samples were collected from all the subjects and used for biochemical and genetic analysis. DNA was extracted from 2 mL of the EDTA samples, and precise primers were designed for LDL-R gene which includes Exon 3, 4 and 8. PCR was followed by *DNA* sequencing. In our study, we found 25 mutations in cases in Exon-3 and 2 mutations in controls, however, we have found only 5 mutations in exon 4 and none of the mutations were identified in exon 8. We conclude that screening of FH among Saudi population is very important to identify individuals who are prone to develop the disease.

Key words: DNA sequencing; familial hypercholesterolemia; LDLR gene; Saudi population

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INTRODUCTION

Familial hypercholesterolemia (FH) (OMIM 143890) is an autosomal dominant inborn error of metabolism leading to high levels of low density lipoprotein (LDL) (OMIM 606945) due to decreased clearance *via* specific LDL-receptor (LDLR). The disease is common (1:500) and can be diagnosed by detection of mutations in LDLR gene (Komarova *et al.,* 2013). The genetic basis of FH is a large array of mutations in the LDLR gene, resulting in a lack of functional receptors for LDL on the liver cell surface, giving rise to increased plasma LDL levels (Alharbi *et al.,* 2013). The mature LDLR protein, a transmembrane protein of 839 amino acids, is coded by the 45 kb LDLR gene located on the distal short arm of chromosome 19. More than 1000 mutations in this gene have been described in individuals with FH. They include large gene rearrangements (insertions or deletions), missense, nonsense and frame shift mutations. In most populations there is a general diversity of mutations, although in certain populations small number of mutations predominate due to a founder effect (Mavroids *et al.,* 1997). Early identification as well as early treatment of patients with FH may reduce the risk of the development of early coronary heart disease (CHD) (Robinson *et al.,* 2013). To the best of our knowledge, no comprehensive studies examining the risk of FH in the Saudi population have been conducted. Furthermore, disease risk modulation based on gene mutation in LDLR is also not yet known. Therefore, the aim of the present study was to assess the role of LDLR gene in Exon-3, 4 and 8 mutation in the Saudi population with FH.

MATERIALS AND METHODS

Subjects. In this study, 100 subjects diagnosed with FH, based on the Dutch group classification criteria, were enrolled (Lye *et al.,* 2013). Patients were recruited from outpatient clinic at King Khalid University Hospital (KKUH), Riyadh. One hundred healthy controls were included in this study (n=100). All controls were recruited from Medical and laboratory staff at KKUH in addition to individuals attending KKUH for routine checkup without having chronic metabolic/medical disease. The research ethical approval was obtained from the Institutional Review Board at KKUH as described in our prior publication (Alharbi *et al.,* 2013). All participants provided written informed consent prior to enrollment into the study.

Blood and biochemical analysis. Five mL of blood were collected from each participant. Biochemical serum sample (3 mL) was used for lipid profile such as Total Cholesterol (TC), Triglycerides (TG), High Density Lipoprotein-Cholesterol (HDL-C) and Low Density Lipoprotein-Cholesterol (LDLC) and 2 mL of anticoaggulated blood samples were used for genetic analysis. Serum samples of TC and TG were measured by the standard enzymatic method (Pars Azmon kit, Iran) using an automated RA-1000 (Technicon, USA). Serum LDL-C and

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Abbreviations: CHD, coronary heart disease; HDL-C, high density; lipoprotein-cholesterol; FH, familial hypercholesterolemia; KKUH, King Khalid University Hospital; LDLC, Low Density Lipoprotein-Cholesterol; LDLR, LDL-receptor; TC, total cholesterol; TG, triglycerides

Table 1. List of the selected SNPs evidence from LDLR gene

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S. No	Region	rs number	Position	Nucleotide Exchange	Mutation	Amino acid substitution
1	Exon-3	rs13306510	11074462	C>T	Missense	Pro-Ser
2	Exon-4	rs10417394	11077080	C>T	Synonymous	–
3	Exon-8	rs11669576	11222300	G>A	Missense	Ala-Thr

HDL-C levels were measured using commercially available enzyme assay kits (Pars Azmon kit, Iran) (Alharbi *et al.,* 2013).

DNA isolation and genotyping for LDL-R mutations. Genomic DNA was extracted from peripheral blood leukocytes by standard protocol with Norgen DNA extraction kit (Norgen Biotek corp, Canada). The concentration of the extracted DNA was quantified by Nanodrop spectrophotometer. Specific primers were designed for selected exons in the LDLR gene. The details of the selected exons and primer sequences were listed in Table 1 and 2. Polymerase Chain Reaction (PCR) amplification was carried out in a total volume of 25 µL reaction mixture that contains 10 pmole of each primer, 11 µL of sterile water and 10 µL of 2X master mix which includes $MgCl_2$, 10× PCR buffer, dNTPs, 10 units of Taq DNA polymerase (Norgen Biotek corp, Canada) and the 100 ng template DNA. The PCR was performed in a thermal cycler (Applied Biosystems, Hercules, California, USA). The cycling and amplification conditions were as follows; an initial denaturation was set up for 5 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 45 seconds at 72°C and the final extension was at 72°C for 5 minutes. The amplified products were separated on 2% agarose gel, stained with ethidium bromide (Cambrex, East Rutherford, NJ, USA) and visualized on a transilluminator (Dafco, USA).

DNA Sequencing. The purified fragments were then sequenced in both the forward and reverse directions. Chain termination sequencing involves the synthesis of new strands of DNA complementary to a single-stranded template. The template DNA (~50 ng) is purified with PCR clean up kit and was amplified in a total reaction of 20 µL containing the Big Dye terminator reaction buffer with both the forward and reverse primers (10 pmoles each) and molecular grade water. DNA Sequencing was performed with Applied Biosystem machine. The reaction was programmed to perform at 95°C for 15 sec, 60°C for 15 sec and 60°C for 5 minutes. The amplified gene in the reaction was precipitated after several washes in 95% and 70% alcohol, dried using the vacuum centrifuge, resuspended in Hi-Di formamide and loaded onto 7200 Genetic Analyzer, Applied Biosystem for sequencing. The resultant sequences were compared with the Cambridge sequence and seascapes software (Fig. 1–3).

Statistical analysis. The association between genotypes and LDLR mutations (Exon-3, 4 and 8) were examined by odds ratio (OR) with 95% confidence interval (CI) and chi-square analysis using Openepi6 software (Openepi Version 2.3.1 from the Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA, USA). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. Hardy-Weinberg equilibrium (HWE) was tested for Exon 3 and 4 of LDLR gene. Clinical characteristics of all subjects were expressed as mean±SD. Continuous variables were compared between the groups using two-tailed student *t*-test. Significant cutoff was set at 0.05. Z-test was used to determine the significance of the pooled ORs,

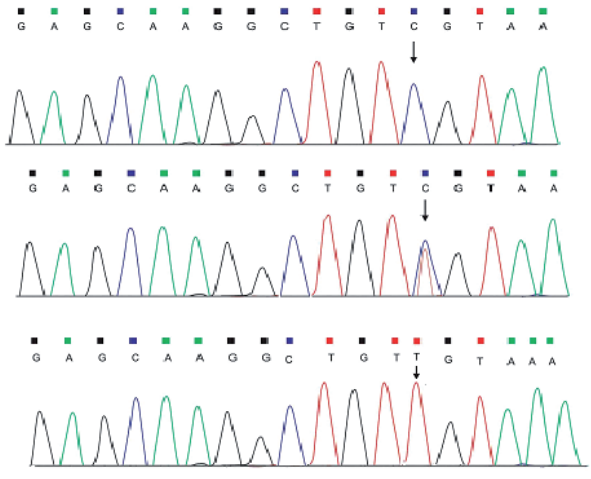

Figure 1. The partial DNA Sequence CC, CT and TT genotypes of Exon 3 region of LDLR gene.

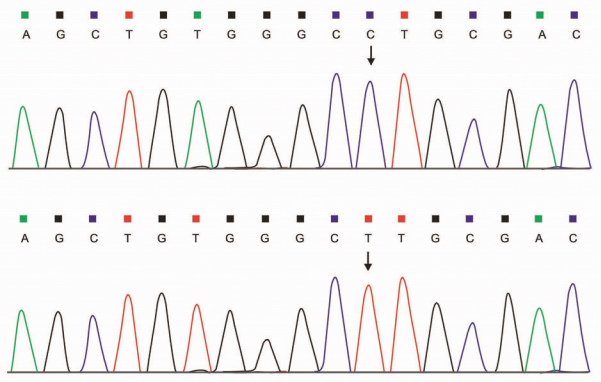

Figure 2. The partial DNA Sequence CC and TT genotypes of exon 4 region in LDLR gene.

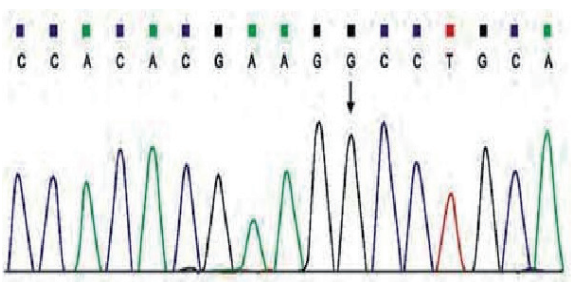

Figure 3. The partial DNA Sequence GG genotypes of Exon 8 region in LDLR gene

Table 3. Baseline characteristics and biochemical profile of FH patients and healthy control

S. No		FH Cases (*n*=100)	Healthy Controls (*n*=100)	*p* value
1	Age (Years)	51.66±9.92	44.02±6.29	*p*=0.0001
2	Gender: Male/Female	37:63	40:60	*p*=0.62
3	TG (mmol/L)	2.1±1.29	1.6±0.99	*p*=0.009
4	TC (mmol/L)	5.4±0.98	4.8±0.73	*p*=0.003
5	HDL-C (mmol/L)	0.7±0.28	0.6±0.27	*p*=0.71
6	LDL-C (mmol/L)	4.5±0.97	3.7±0.72	*p*=0.003

Table 4. Prevalence of LDLR mutations in FH cases and control subjects

Mutations	FH (*n*=100)	Controls (*n*=100)	*p* value	Odds ratio	95% CI
Exon-3	25% (25/100)	2% (2/100)	0.0001	16.33	(3.949–71.39)
Exon-4	5% (5/100)	0 (0/100)	0.002*	11.58	(1.286–379.7)
Exon-8	0 (0/100)	0 (0/100)	0.99*	1.00	(0.019–50.63)

*indicates Yates correction

and p value <0.05 was considered significant. Yates correction was also performed for genotypes and allele frequencies.

RESULTS

Baseline characteristics

One hundred FH cases and one hundred healthy controls were included in this study. All patients and controls belong to the Saudi population. The clinical and biochemical characteristics of FH cases and healthy controls are shown in Table 3. Mean age was 51.66 years in patients and 44.02 in controls (p<0.05). The lipid profile parameters used in this study were TC, TG, HDL-C and LDL-C. The TG, TC and LDL-C levels were higher in the FH group compared to controls; 2.1±1.29 mmol/L, 5.4±0.98 mmol/L and 4.5±0.97 mmol/L 1.6±0.99 mmol/L, 4.8±0.73 mmol/L and 3.7±0.72 mmol/L (p<0.05). The HDL-C level was compared in the two group's 0.7±0.28 mmol/L in FH individual's vs 0.6±0.27 mmol/L in the control group (p>0.05).

Genotype analysis

The genotypic distribution and allelic frequencies of the three most common mutations in LDLR gene (Exon-3, 4 and 8) are shown in Table 4. In Exon 3, there was a significant difference between the genotypic distribution and allelic frequencies of missense mutation between FH cases and healthy controls. For T *vs* C: [OR-16.79; 95% CI=3.949–71.39; p=0.0004] and CT+TT *vs* CC: [OR-16.33; 95% CI=3.751–71.13; p=0.0001]. There was also significant difference in the Exon 4 of LDLR gene after performing the Yates correction between genotype distribution and allele frequency, For T *vs* C: [OR-22.1; 95% CI=1.286–379.7; p=0.002*] and CT+TT *vs* CC: [OR-11.58; 95% CI=0.6316–212.2; p=0.03*]. No significant difference was observed in the allele and genotype distribution of FH cases and healthy controls in Exon 8 of the LDLR gene. None of the alleles and genotypes were present in the Exon 8. For G *vs* A: [OR-1.0; 95%CI=0.019–50.63; p=0.99*] and GA+AA *vs* GG: [OR-1.0; 95% CI=0.019–50.88; p=0.99*]. However, no specific haplotype involving all the three mutations found to be associated with FH cases.

DISCUSSION

More than 85% cases of FH cases are due to inherited mutations in the LDLR gene, with more than 1600 mutations identified and in some populations, such as French Canadians, Ashkenazi Jews, Lebanese, and Dutch Afrikaners, are at high risk for FH owing to an increased prevalence of heterozygous FH associated mutations in the LDLR gene (Hobbs *et al.,* 1987; Moorjan *et al.,* 1989; Yuan *et al.,* 2006; Zeegers *et al.,* 2004; Alex *et al.,* 2012). Similarly, our study has shown that LDLR gene mutations are associated with FH in the Saudi population.

In a study, the Spanish FH population was investigated: 5430 index cases and 2223 relatives; 9.8% have mutations involving Exon 3, 12.1% in Exon 4 and 1.8% in Exon 8 (Hopkins *et al.,* 1987). An earlier study was conducted by Lye *et al* (2012) on 141 consecutive patients with clinical FH in LDLR, APOB and PSK9 genes. Fourteen SNPs were found to be significantly associated with FH, eleven with increased FH risk and three with decreased FH risk. Interestingly, of the eleven SNPs associated with an increased risk of FH, only one SNP was found in the LDLR gene, seven in the APOB gene and three in the PCSK9 gene. Similar to our study in the Saudi population, Lye and coworkers (2012) study provided new information and knowledge on the genetic polymorphisms amongst Asians with FH, which may serve as potential markers in risk prediction and disease management. Alex and coworkers (2012) studied APOB, LDLR and PSK9 gene mutations in 140 identified hypercholesterolemia subjects from three different ethnic groups (Malay; Indian & Chinese). They have identified 137 mono allelic markers and 173 polymorphic markers in both subject groups. By comparing to publicly available data, out of the 137 mono-allelic markers, 23 markers showed significant differences in allele frequency among Malaysians, European Whites, a Han Chinese, Yoruba and Gujarati Indians. Another study reported by Garcia and coworkers (2001) was carried out with gene chip (robo arrays) to resequence the coding regions of 10 key genes of lipid metabolism in 80 dyslipidemic cases. Overall, 14 non-synonymous and 22 synonymous

SNPs were identified, which were confirmed by conventional sequencing. Coding sequence variations thought to be associated with dyslipidemia were also seen in controls. This study is certainly a good evidence that works on common variants and causal variants need to be published regardless of how significant or insignificant it may be considered (Garcia *et al.,* 2001). Ng and coworkers (2004) compared the allele frequency distributions of 64 intragenic SNPs of 35 candidate genes for cardiovascular diseases in three populations consisting of 207 Chinese, 858 French and 395 Spanish individuals. In all, 28 of these SNPs from 12 genes were also examined for intragenic linkage disequilibrium. About 20% of SNPs were restricted to Europeans, being monomorphic in Chinese, among them mostly nonsynonymous coding SNPs and noncoding SNPs. Only 1.6% of SNPs were specific for Chinese, commensurate with the detection of these SNPs almost exclusively in Caucasians. Similarly, these SNPs were more often rare (<0.1 minor allele frequency) in Chinese (44.3%) than in Europeans (31.1%). The variant allele frequencies and intermarker linkage disequilibriums in terms of D' and D^2 were highly correlated between French and Spanish populations (r=0.98–0.99, $p<0.001$). However, only moderate correlations of allele frequencies and D^1 were found between the Chinese and the European populations (r¼ 0.7 and 0.3, respectively) despite a high correlation of D^2 values (r¼ 0.8) (Benn *et al.,* 2012).

Two important conclusions can be derived from our work. First, pending the availability of a genetic or biochemical marker for a reliable diagnosis of familial combined hypercholesterolemia (FCH), the exclusion of LDLR gene defect is highly recommended for patients with this clinical diagnosis who present with high TC or apoB levels. This exclusion could be particularly important for research purposes and to limit the heterogeneity observed in many biochemical and genetic studies of FCH cohorts. Next, the diagnostic criteria for FH should not exclude patients with combined hyperlipidemia. In this situation, the total cholesterol or apoB level might be a better diagnostic tool than LDL-C, and values above 335 or 185 mg/dL, respectively, should raise the suspicion of FH and prompt a search for LDLR mutations.

Our study has several limitations. First, our study included a small sample size due to the low number of the Saudi nationality. Secondly, limitations related to the accuracy of clinical diagnosis of FH are well known and finally, heterogeneity of the genetic defects responsible for FH. All these give a potential explanation for the lack of association we observed.

In conclusion, we have found 32 mutations from the consecutive three exons of the LDLR gene among Saudi population. Further large-scale study is required in order to investigate the prevalence of mutations associated with FH in the Saudi population.

Conflict of Interest

All the authors declare that there is no conflict of interest.

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