Phenotypic variation and genetic polymorphism of low-density lipoprotein exon 4 receptor gene among Jordanian familial hypercholesterolemia patients

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Purpose. The aim of this study was to measure lipid profiles among severe and mild familial hypercholestrolemic (FH) patients and to screen unrelated families for LDL receptor (LDLR) gene polymorphism.

Methods. Fifteen FH patients and six controls were enrolled in the study. Total cholesterol (CH), triglyceride (TG), high-density lipoprotein (HDL-cholesterol), low-density lipoprotein (LDL) and ApoB-100 levels were measured spectrophotometrically. Patients were classified clinically into severe and mild, based on lipid profile phenotype and the presence of cardiovascular manifestation at the age of disease onset. Low-density lipoprotein exon 4 receptor gene polymorphism was screened by the SSCP method.

Results. FH severe phenotype patients had significantly higher total cholesterol levels (14.34 ± 1.14) mmol/L as compared with mild FH phenotype (10.13 ± 1.23 mmol/L) and experienced earlier symptoms in the childhood (9.25 ± 3.22) years as compared with those of mild phenotype, who did not experience symptoms until the mid of the fourth decade of life (36.7 ± 5.25 years). No significant difference was found between HDL-cholesterol, LDL-cholesterol, ApoB-100 among severe FH as compared with mild FH patients. All FH pedigrees showed the autosomal dominant mode of inheritance. SSCP results showed a wide spectrum of LDL exon 4 gene polymorphism among severe and mild phenotypes, which differed from control.

Conclusions. There are statistical differences between severe and mild phenotype FH patients in total cholesterol, triglyceride and the age of biological expression among FH Jordanians. FH patients showed a wide spectrum of gene polymorphism within the LDLR exon 4 gene. These polymorphisms were different among FH families; this may be related to founder factors, such as the degree of consanguinity among FH patients' parents and shed light on the importance of the role of genetic screening and counseling in reducing the risk of occurrence of the new severe FH phenotypes that develop severe complications early in life and need LDL aphresis, as well as on the importance of cascade screening to trace and identify people at genetic risk, using family tracing, for an early diagnosis of the mild FH phenotypes that can be treated with lipid lowering therapy and life style changes early to prevent later cardiovascular complications.

Key words: familial hypercholesterolemia, low-density lipoprotein, receptor, homozygous, heterozygous, SSCP, Apo B-100

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INTRODUCTION

FH is defined as a group of inherited genetic defects resulting in a severely elevated serum cholesterol concentration. FH is recognized clinically by an extreme elevation of LDL-Chol, which is characterized by autosomal dominant or co-dominant transmission. A FH patient may also develop tendon xanthomas, skin xanthomas and coronary heart diseases (CHD); however, some FH patients develop no xanthomas or CHD (1, 2). FH genetic defects are classified as defects in LDLR, apolipoprotein B, proprotein convertase subtilisin / kexin type 9 and autosomal recessive hypercholesterolemia gene (3). Genetically, the differentiation of FH into homozygous, combined heterozygous or heterozygous types is based on mutation detection by advanced molecular pathways on the previous candidate genes that cause hypercholesterolemia. Homozygous and heterozygous forms have LDL-cholesterol levels that are two and four times above the normal, respectively (3). The prevalence of the genetically homozygous severe FH form is one per million of general population *versus* a much more common milder heterozygote form with the prevalence of 1 in 500. Homozygote individuals have two mutants and show a worse clinical picture than the heterozygote individuals who have one mutant allele and one normal allele (1, 4). However, if two different mutations in the same or different candidate FH genes were detected, then this FH is genetically classified as combined heterozygotes (3). Clinically, FH could be classified into a severe phenotype with the LDL-cholesterol level three to four times above the normal and with cardiovascular manifestation of FH, whereas the mild phenotype with elevated LDL-cholesterol not exceed three times the normal level, and paradoxical are patients with either LDL-cholesterol three to four times above the normal and no cardiovascular manifestation or with a normal LDL-cholesterol and a cardiovascular disease (3). Since Jordan currently hasn't any national genetic screening programs for FH, clinical indicators, LDL cholesterol, and cardiovascular manifestations remain the most common methods for FH classification and diagnosis.

Genetically, a mutation in LDLR gene, which is an ubiquitous transmembrane glycoprotein, is the most common cause of FH. The LDLR gene is 45 kb long and composed of 18 exons, 17 introns and a promoter area located on the short arm of chromosome 19 (p 13.1–13.3). The LDLR protein has different domains including a signal peptide, a ligand-binding domain, an epidermal growth factor-precursor as a domain, and O-linked sugars, transmembrane and cytoplasmic domains (5). Heterogeneous mutations were distributed along all LDLR domains. Currently, the database for the LDLR sequence lists more than 1600 hits (6); these mutations were found among different populations with a different ethnic background. In this study, the exon 4 LDLR gene was screened because it represents a hot spot for FH polymorphism. The exon 4 LDLR encodes a ligand-binding domain in mature protein, which is an important domain in the process of binding with Apo B-100 (2, 7–10). Many FH exon 4 mutations were reported in the literature: FH Mexico (missense), FH French Canadians (missense), FH-Piscataway (deletion), FH Puerto Rico (missense), FH Afrikaner-1 (missense) and FH Maine (missense) (11). Also, other exon 4 mutations were reported: c.324–325delGTinsTC, c.326G>A, c.409G>T, c.420G>C, c.503A>G, c.530C>T, c.591C>G, c.654–656delTGG, c.662A>G, c.665G>A, c.664–681dup18, c.681–683del3, c.669–680dup12 and c.692G>A (12). In some parts of the world, specific FH-related mutations occur in a large population of patients owing to the "founder effect" resulting from cultural or geographic isolation (13); LDLR mutations are associated also with the Christian-Lebanese (14), Tunisia (15) and Quebec (16). A large number of these mutations were found in LDLR exon 4 (1) which could be point mutation (17, 18), deletion (11, 19), insertion or duplication (19, 20) and large rearrangement (21, 22). So, the aim of this study was to determine the lipid profile values among clinically severe and mild FH patients and to screen LDLR exon 4 gene polymophism among Jordanian FH patients.

MATERIALS AND METHODS

A total of 15 patients diagnosed with FH and 6 controls from different families free of any family history of FH were enrolled in this study. The diagnostic criteria for FH were elevated LDL-Chol levels and cardiovascular manifestations including presence of premature cardiovascular disease at diagnosis. Patients were interviewed personally, and blood samples were collected at the Cardiology Department of the Princess Basmah Teaching Hospital and at the Endocrinology Department of the Al-Bashir Hospital and at the King Hussein Medical Center (2004–2006). Informed consent was obtained from the patients or from parents in the case of children. Sever phenotype (HDL-Chol three- to four-fold increase at the time of diagnosis and cardiovascular manifestations) and mild FH phenotypes (HDL-cholesterol excess below threefold) were clinically classified (3). Presence of xanthomas and a history of coronary heart disease were also reported.

Serum lipid profile
Two fasting blood samples were collected from each patient for biochemical and SSCP analysis. Total cholesterol, HDL-Chol and TG were measured using commercially available colorimetric kits (HUMAN, Germany). LDL-cholesterol levels were calculated using the Friedwald equation (LDL-cholesterol = total cholesterol – [HDL-cholesterol + triglycerides/5]) (23).

Serum apolipoprotein B-100 levels

Apo B-100 levels were measured spectrophotometrically at 340 nm by the turbidimetric method (5) which is based on the formation of antigen–antibody complexes (HUMAN, Germany). After mixing the sample with the antibody reagent, the degree of turbidity was measured spectrophotometrically after 10 seconds and then after 10 minutes. Apo B-100 concentrations were calculated from a special calibration curve according to the kit.

DNA extraction

DNA molecules were extracted from peripheral leukocytes using commercially available kits (Promega, USA). RBCs were first hydrolyzed, WBCs were precipitated and their nuclei lysed. Proteins were precipitated and then centrifuged. DNA was dehydrated and then rehydrated and stored in a Tris-EDTA buffer at –4 °C. All DNA extracts were examined by agarose gel electrophoresis for the presence of DNA.
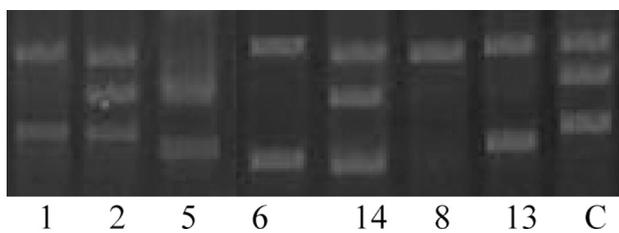
Polymerase chain reaction (PCR)

The LDLR gene exon 4 was amplified by PCR. Exon 4B was amplified using Primer 5'-CCCCAGCTGTGGGCCT-GCGACAACG-3' and Primer 5'-GGGGGAGCCCAG-GGACAG GTGATAG-3'. Exon 4B was amplified by mixing 0.1 mM MgCl$_2$, 1X MgCl$_2$-free buffer, 0.05 mM of PCR nucleotides mix, 1.1 nmol of primer 1, 2.0 nmol of primer 2, 5 units of DNA polymerase, 2 µl of DNA and 15 µl of nuclease-free water. The thermal cycler was programmed as described by [2], except that the annealing temperature was set at 60 °C: one cycle of 96 °C for 10 min, 60 °C for 1 min and 72 °C for 2 min, 35 cycles of 92 °C for 1 min, 59 °C for 1 min and 72 °C for 2 min, and finally one cycle of 72 °C for 2 min.

Polymorphism screening by single strand conformation polymorphism (SSCP)

Polymorphisms in exon 4 of the LDL receptor gene were screened by SSCP using 8% of non-denaturing polyacrylamide gel (8 µl of 40X acrylamide / bis solution, 4 µl TBE buffer, 2 µl of 5% glycerol, 400 µl APS, 40 µl TEMED, and the final volume was completed to 40 ml with distilled water). Gels were prepared and allowed to polymerize for 1 hour; 3.5 µl of the amplicon was mixed with an equal volume of SSCP loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide and 20 mM EDTA), and the final volume was brought to 15 µl with nuclease-free water. The mixture was heated at 95 °C for 10 min to denature DNA and then immediately placed on ice; 10 µl of each mixture was loaded into each well. The gel was placed in 0.5X Tris base / acetic acid buffer in a tank connected to an external chiller. The electrophoretic run was carried out at 700 volts for 1.5 hours at 5–15 °C (13, 18). Gels were then stained in 250 ml TAE buffer with 25 µl of ethidium bromide for 5 min and visualized under a UV lamp.

Pedigree patterns

Figures 2a, 2b, 2c, 3, 4 show FH pedigrees for the five families enrolled in this study and their mode of inheritance.

Statistical analysis

Data analysis was performed using the statistical package for social sciences (SPSS 11.5, SPSS Inc Chicago, IL). The results were expressed as a mean ± SEM, and the t test was used to compare the significance of mean differences between the two groups. The differences were considered significant if the obtained p value was less than or equal to 0.05.
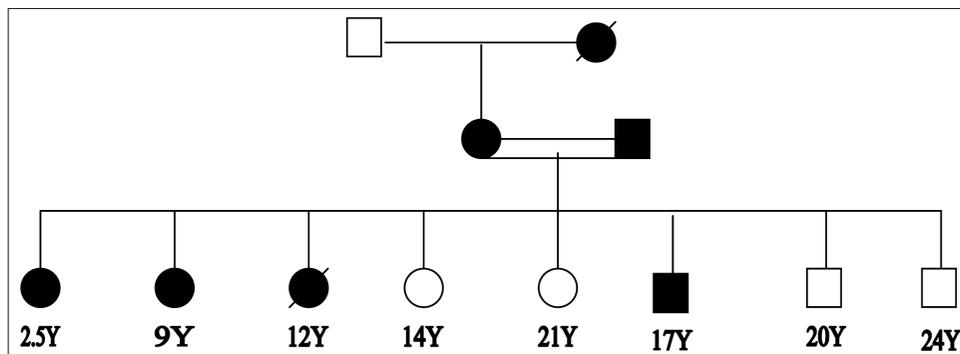

Fig. 1. SSCP analysis of PCR products from FM homozygous patients (FM1, FM2. FM5, FM6, FM14) and heterozygous (FM8 and FM13) control (C). FM patients have different SSCP patterns which indicate mutation or polymorphisms

Fig. 2a. Genetic pedigree for family A. Unaffected individuals are indicated with open symbols; affected individuals are shown with filled symbols; symbols with a slash denote deceased individuals

Fig. 2b. Genetic pedigree for family B. Unaffected individuals are indicated with open symbols; affected individuals are shown with filled symbols; symbols with a slash denote deceased individuals
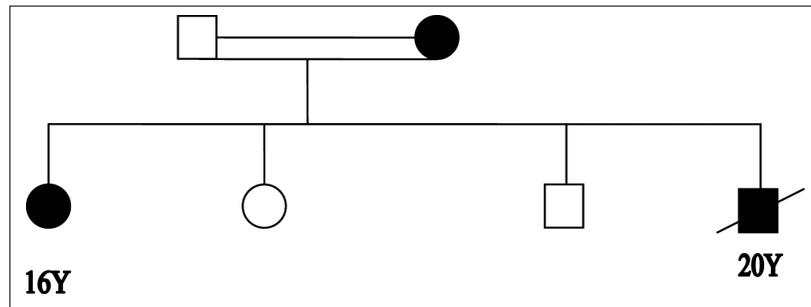

Fig. 2c. Genetic pedigree for family C. Unaffected individuals are indicated with open symbols; affected individuals are shown with filled symbols; symbols with a slash denote deceased individuals

Fig. 3. Genetic pedigree for family D. Unaffected individuals are indicated with open symbols; affected individuals are shown with filled symbols; symbols with a slash denote deceased individuals
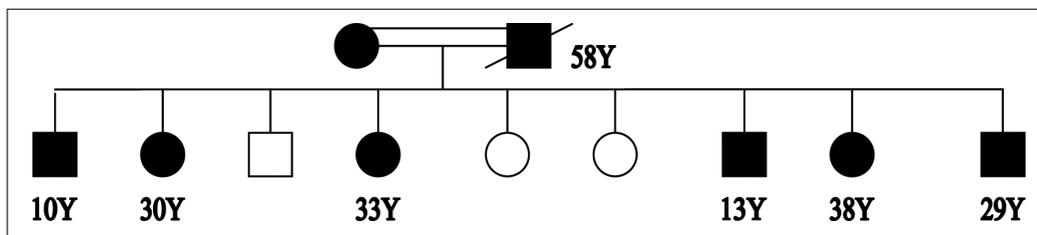

Fig. 4. Genetic pedigree for family E. Unaffected individuals are indicated with open symbols; affected individuals are shown with filled symbols; symbols with a slash denote deceased individuals
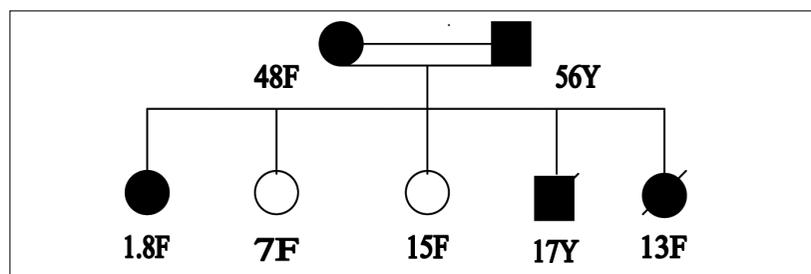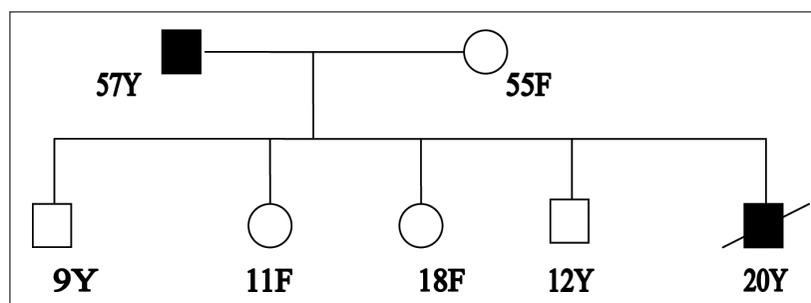
RESULTS

Clinical and biological criteria for severe and mild FH patients are summarized in Table 1. Parents of ten FH patients were first-degree cousins, indicating a high consanguinity among the FH patients.

Five families were enrolled in the study with clinically severe and mild FH phenotypes. FH patientes were clinically classified into severe (FH1, FH2, FH5, FH6, and FH14) and mild (other FH patients) according to the HDL-Chol level at diagnosis. The mean age of severe FH patients was 9.25 ± 3.22 years and of mild FH 36.7 ± 5.26 years. All se-

Table 1. **Patients' criteria and their lipid profile**

Patient	Gender	Consanguinity	Treatment
*FH 1	M	First cousin	Statin / LDL apheresis
*FH 2	F	First cousin	Statin
*FH 3	F	None	Statin
*FH 4	M	First cousin	Statin
*FH 5	F	First cousin	Statin
°FH 6	F	First cousin	Statin
♦FH 7	F	First cousin	Statin / LDL apheresis
♦FH 8	M	First cousin	Statin / LDL apheresis
♦FH 9	M	None	None
♦FH 10	F	First cousin	None
♦FH 11	M	None	None
♦FH 12	F	First cousin	None
♦FH 13	F	First cousin	Statin
•FH 14	F	None	Statin
•FH 15	M	None	Statin

Family A: FH1, FH2, FH3, FH4 and FH5. Family B: FH6. Family C: FH7, FH8, FH9, FH10, FH12, FH13. Family D: FH14. Family E: FH15.

Table 2. **Biological description of clinically severe and mild phenotypes among familial hypercholesterolemia patients**

	Severe FH patients Mean ± SEM	Mild FH patients Mean ± SEM	p
N	5	10	
*Age	9.25 ± 3.22	36.7 ± 5.26	0.004
TC	14.34 ± 1.14	10.13 ± 1.23	0.048
TG	1.19 ± 0.15	2.16 ± 0.25	0.024
LDL-Chol	10.7 ± 2.01	8.4 ± 1.46	0.377
HDL-Chol	0.88 ± 0.20	0.97 ± 0.12	0.694
ApoB-100 level mg/dl	194.4 ± 34.5	182.8 ± 16.78	0.736
Xanthomas, %	100%	30%	
CHD, %	20%	60%	

N – number of patients; TC – total cholesterol level; TG – triglyceride level; LDL-Chol – low-density lipoprotein cholesterol; HDL-Chol – high-density lipoprotein cholesterol. Lipid values are given in mmol/L. * Age at lipid measurement.

vere FH patients exhibited xanthomas, and only 20% exhibited CHD.

Severe FH patients (Table 2) had significantly ($p \leq 0.05$) higher total cholesterol levels (14.34 ± 1.14 mmol/L) as compared with mild FH (10.13 ± 1.23 mmol/L) and experienced earlier symptoms in childhood (9.25 ± 3.22 years) as compared to mild FH patients who did not experience symptoms until the third decade of life (36.7 ± 5.25 year). However, triglyceride levels among mild FH patients was significantly ($p \leq 0.05$) higher (2.16 ± 0.25 mmol/L) than in severe FH subjects. Table 2 shows no statistical difference in LDL-Chol, HDL-Chol and ApoB-100 levels between the severe and mild FH groups.

SSCP

Exon 4B was screened for gene polymorphism by SSCP technique as shown in Fig. 1. The SSCP pattern was compared for all 15 FH patients and 6 controls. A clear discrepancy in electrophoric gel patterns between FH patients and control is shown. All six control patients showed the same SSCP pattern.

DISCUSSION

Although FH is a genetic disease which shows a great phenotypic variability due to its polygenetic nature enhanced by modifier genes (24), monogeneic FH remains the most common disorder of lipoprotein metabolism, caused mainly by LDLR gene mutations, which leads to plasma accumulation of cholesterol ester-laden LDL particles. The monogenic heterozygous form responds poorly to changes in diet and lifestyle, making pharmacological therapy necessary, while plasmapheresis and liver transplantation are the only interventions in homozygote patients. Conversely, multigenic forms or familial hypercholesterolemia secondary to other diseases respond to changes in diet and lifestyle as well as to pharmacological treatment (25). Since Jordan currently hasn't any national genetic screening programs for FH, clinical indicators, LDL cholesterol, and cardiovascular manifestations remain the most common methods for FH classification into severe and mild forms. The early clinical diagnosis of the mild phenotype is challenging,

because the disease can be treated by lipid-lowering therapy early to prevent any complication when a failure in the diagnosis and treatment increases the morbidity and mortality from premature cardiovascular disease, cardiovascular disease being the leading threat to health among Jordanian population, with 40.5 percent of deaths (26).

Our data show that the mean total cholesterol level for severe FH (14.34 ± 1.14 mmol/L) is less than those reported in Lebanon (27), Tunisia (15) and Italy (28), but this may be due to the fact that all our severe FH subjects were under medical treatment and apheresis. The mean total cholesterol level for mild FH is 10.13 ± 1.23 mmol/L, i. e. statistically different from severe FH (14.34 ± 1.14 mmol/L), but similar to those in Africaners (2) and Italians (28). Sixty percent of mild FH showed the prevalence of xanthomas which increase with age until the fifth decade (29).

Our results also showed no statistical difference in LDL-cholesterol level between severe (10.7 ± 2.01 mmol/L) and mild FH (8.4 ± 1.46 mmol/L). These levels were similar to those reported among Afrikaners (2) and Italians (30). However, the mean total triglyceride level for mild FH (2.16 ± 0.25 mmol/L) was higher than in severe FH (1.19 ± 0.15 mmol/L); this elevation in serum triglycerides may be due to the type of mutation in FH gene as in the case of heterozygous familial hypercholesterolemia with the 317 cystine-to-serine mutation in the LDL receptor gene (31).

Although more than 1600 LDL receptor gene mutations have been reported worldwide, genetic data on LDL receptor gene mutations in Arabs, mainly Jordanian, are scarce. In the literature, a single-base substitution was reported among Druze (a small Middle Eastern Islamic sector with a high degree of inbreeding) in the termination codon in exon 4 of the LDL receptor gene that encodes the fourth repeat of the binding domain of the mature receptor (32). Another single-base pair substitution activates a 10-base down the cryptic splice acceptor site in exon 12 LDLR and severely decreases LDLR expression in two unrelated Arab families with FH (33). Jordan currently hasn't any national genetic screening programs for FH, and consanguineous marriages constitute over half of the Jordanian marriages (34); also, the high birth rate (44 live births / 1000 population / year) contributes to the high absolute numbers of children with genetic monogenic disorders which are purely genetic. Ten FH parents enrolled in this study were first cousins. Five members of family A and seven members of family C were hypercholesteromic, which may shed light on the importance of improving public education on genetic diseases, premarital and pre-conceptional testing and counseling, and newborn screening.

Although the clinical classification of FH is low-cost and helpful for the diagnosis of relatives, it could miss the proportion of FH patients with a mild phenotype and children in whom the phenotype is not yet expressed. At the same time, the clinical diagnosis does not show the genotype–phenotype correlation to ensure a better response to statin therapy in non-LDLR FH mutated forms. Thus, genetic screening and diagnosis may provide a more accurate and early diagnosis of FH which could not be known from the lipid profile alone. SSCP results showed wide diverse polymorphisms among FH patients, which may result from consanguineous marriages (Fig. 2 a, b, c) as one of the demographic features of Arab families. Arabs are genetically diverse in general; the major factors that contributed to this diversity include migrations of Semitic tribes from the Arabian Peninsula, the Islamic expansion in the 7th century AD, the Crusade wars, and the recent migration dynamics. These events have resulted in the mixture of the original Arabs with other populations extending from east and south Asia to Europe and Africa (35).

Overall, we conclude that severe phenotype FH patients have significantly higher total cholesterol levels as compared with the mild FH phenotype and experience earlier symptoms in the first decade of life as compared with the mild phenotype who do not experience symptoms until the mid-third decade of life. There is a spectrum of polymorphisms (SSCP bands) that occur in the LDLR gene, which may be responsible for familial hypercholesterolemia among Jordanians. These polymorphisms were different among families and were affected by many founder factors, such as the degree of consanguinity among parents. This may shed more light on the importance of gene polymorphism inside exon 4 LDLR gene among Jordanian FH patients. At the same time, this sheds light on the role of genetic counseling in reducing the risk of occurrence of new polymorphisms among mild FH patients who develop severe complications early in life and need LDL aphresis. We recommend extended work to understand both the primary genetic polymorphism and the role of the gene–environment interaction which affects the clinical phenotype of FH. All these approaches are challenging to understand this devastating disease.

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ŠEIMINE HIPERCHOLESTEROLEMIJA SERGANČIŲ PACIENTŲ JORDANIJOJE ŽEMO TANKIO LIPOPROTEINŲ RECEPTORIAUS GENO MUTACIJŲ PATIKRA

Santrauka

Tikslas. Šia studija siekiama nustatyti sunkia ir vidutine šeimine hipercholesterolemija (ŠH) sergančių pacientų lipidų profilius ir profilaktiškai patikrinti nesusijusių šeimų narius dėl žemo tankio lipoproteinų receptoriaus (LDLR) geno polimorfizmo.

Metodai. Į tyrimą įtraukta penkiolika pacientų, sergančių ŠH, ir šeši kontroliniai asmenys. Spektrofotometriškai išmatuoti bendro cholesterolio (CH), trigliceridų (TG), didelio tankio lipoproteinų (HDL-Cholesterol), žemo tankio lipoproteinų (LDL) ir ApoB-100 baltymo kiekiai. Pagal lipidų fenotipo profilį ir širdies bei kraujagyslių sistemos ligų požymius ligos nustatymo metu išskirta sunki ir vidutinė ŠH eiga. Žemo tankio lipoproteinų receptoriaus egzono 4 geno polimorfizmas buvo tikrinamas SSCP metodu.

Rezultatai. Sunkios eigos ŠH fenotipo pacientams buvo reikšmingai padidėjusi bendro cholesterolio koncentracija (14,34 ± 1,14 mmol/L), palyginti su vidutinės eigos ŠH fenotipo pacientais (10,13 ± 1,23 mmol/L), taip pat jiems pasireiškė ankstyvi simptomai vaikystėje (9,25 ± 3,22), palyginti su švelnios eigos fenotipo pacientais, kurie nepatyrė simptomų iki vidutinio amžiaus (36,7 ± 5,25). Nerasta statistiškai reikšmingo HDL cholesterolio, LDL cholesterolio, ApoB-100 skirtumo tarp sunkia ir lengva ŠH sergančių pacientų. Visiems ŠH pacientams nustatytas autosominis dominantinis paveldėjimo tipas. SSCP rezultatai atskleidė platų LDL egzono 4 geno polimorfizmo spektrą tarp sunkaus ir vidutinio ŠH fenotipo, ir šie rezultatai skyrėsi nuo kontrolinės grupės pacientų.

Išvados. Nustatyti statistiškai reikšmingi bendro cholesterolio, trigliceridų koncentracijos ir biologinio pasireiškimo skirtumai tarp sunkia ir vidutine ŠH sergančių pacientų Jordanijoje. ŠH sergantiems pacientams nustatytas platus LDLR egzono 4 geno polimorfizmo spektras. Tokius polimorfizmo skirtumus tarp ŠH sergančių šeimų gali lemti pradiniai veiksniai, pvz., giminystės ryšiai tarp ŠH pacientų tėvų. Atlikti tyrimai galėtų atskleisti genetinės patikros svarbą mažinant naujų, sunkių ŠH fenotipų riziką, sukeliančių dideles komplikacijas ankstyvajame amžiuje. Labai svarbi ir pakopinė atranka bei asmenų su genetine rizika išaiškinimas, norint anksti nustatyti lengvos eigos ŠH, kurią galima gydyti mažinant lipidus, keičiant gyvenimo būdą, užkertant kelią širdies ir kraujagyslių sistemos komplikacijoms.

Raktažodžiai: šeiminė hipercholesterolemija, žemo tankio lipoproteinai, receptoriai, homozigotai, heterozigotai, SSCP, Apo B-100