PCSK 9 gain-of-function mutations (*R496W* and *D374Y*) and clinical cardiovascular characteristics in a cohort of Turkish patients with familial hypercholesterolemia

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Abstract

Objective: The molecular basis of the mutations in the PCSK9 gene that produces familial hypercholesterolemia (FH) in the Turkish population is unknown. This study was conducted to determine the presence of four different PCSK9 gain-of-function (GOF) mutations (*F216L*, *R496W*, *S127R*, and *D374Y*) in a group of patients with FH.

Methods: A total of 80 consecutive patients with FH (mean age: 56±11 years; mean maximum LDL cholesterol: 251±76 mg/dL) were included in the study. Patients with FH were diagnosed according to the Dutch Lipid Clinic Network criteria based on serum cholesterol levels, personal and family histories of cardiovascular disease, tendon xanthomas, and genetic analysis. To identify *F216L*, *R496W*, *S127R*, and *D374Y* mutations of the PCSK9 gene, high-resolution melting analysis was performed on isolated DNAs.

Results: : Of the 80 patients, there were 11 patients (13.8%) with PCSK9 GOF mutations. Detected mutations were *D374Y* mutation in four (5.0%) patients and *R496W* in seven patients (8.7%). Only one patient was homozygous for *R496W* mutation. The other two GOF mutations (*S127R* and F216 variants) were not detected. There was no significant difference with regard to demographic characteristics and CV disease risk factors and clinical course of the disease between the PCSK9 mutation-positive and PCSK9 mutation-negative groups.

Conclusion: This is the first study from a Turkish FH cohort, revealing a higher frequency (approximately 14%) of two PCSK9 GOF mutations (*D374Y* and *R496W*) and a different disease course compared to the world literature. *(Anatol J Cardiol 2017; 18: 266-72)*

Keywords: familial hypercholesterolemia, PCSK-9, Turkey

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant genetic disorder characterized by elevated serum low-density lipoprotein (LDL) cholesterol levels, tendon xanthomas, and premature coronary artery disease (CAD) (1–4). The clinical presentation and natural course of the disease are affected by the underlying genetic mutations. In more than 90% of the cases, LDL receptor gene mutations are responsible (5, 6), whereas apolipoprotein (Apo)-B and proprotein convertase subtilisin kexin type 9 (PCSK9) mutations have been identified to a lesser degree depending on the population studied (5, 6).

PCSK9 is an enzymatic protein from the subtilisin family of serin proteases (5–7). More than 30 PCSK9 gain-of-function (GOF) mutations have been reported to be the cause of FH since the discovery of PCSK9 in 2003 (5, 6). Binding of PCSK9 to LDL receptor inhibits the return of LDL receptor to the cell surface, leading to a decrease in the hepatic cholesterol intake and an increase in plasma LDL cholesterol levels (5–7).

There are limited data on the genetic background of FH in Turkey. The Turkish Heart Study (n=9000) has not detected any R3500Q mutations, which is the most common apoB mutation in Europe (8). The only trial investigating LDL receptor mutations in a Turkish population consisted of only children (20 homozygous and 16 heterozygous). The sequencing analysis of that FH population revealed that the frequency of genetic LDL receptor defect was 66.1%, whereas no apoB defect was detected (9). Likewise, in 2008, Eroğlu et al. (10) found no R3500Q apoB deficiency in 228 patients with CAD or ischemic stroke.

The prevalence of PCSK9 GOF mutations leading to FH in the Turkish population is unknown. This study was conducted to determine the frequency and clinical impact of four different PCSK9 GOF mutations [*F216L* (*rs28942112*), *R496W* (*rs374603772*), *S127R* (*rs28942111*), and *D374Y* (*rs137852912*)] in a cohort of patients with FH from a single lipid clinic in Turkey.

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Methods

Patients

This cross-sectional study consisted of a cohort of patients with FH who were already undergoing routine follow-up in the Cardiology Lipid Clinic of Ege University Medical School, Izmir, Turkey. Diagnosis of FH was based on a total score of >3 points according to the Dutch Lipid Clinic Network (DLCN) criteria (4). Patients with secondary causes of hypercholesterolemia (i.e. hypothyroidism, nephrotic syndrome, chronic renal failure, pregnancy, Cushing syndrome, anorexia nervosa, and use of corticosteroids and immunosuppressive agents), were excluded. The study protocol was approved by the Institutional Clinical Investigation Ethical Committee (February 2015, 14–9.2/2), and all included patients gave written informed consent for genetic analysis. The study was supported financially by the institutional scientific research project committee. Patients who had missing information in their files or whose genetic analysis could not be generated because of technical problems were also excluded.

A total of 80 consecutive non-relative patients with FH admitted to our lipid clinic for routine visits between February and May 2015 were enrolled in the study. Study procedures included blood sampling for genetic analysis, a detailed physical examination and overview of the medical history of patients and their families, current antilipid medication, and cardiovascular (CV) risk factors at the index visit. Family and personal medical histories included CV disease, hypercholesterolemia, ischemic stroke, and diabetes mellitus. Consanguinity was determined by questioning, and pedigree of all individual patients for all familial CV events and hypercholesterolemia were obtained to avoid missing details. Additionally, fasting serum lipid levels, including LDL cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, total cholesterol, lipoprotein (a), apoA-1, and apoB were measured at that visit. All patients were examined for the presence of tendon xanthoma and xanthelasma. The presence of corneal arcus was inspected visually with the help of a pen light (11). Patients with increased Achilles thickness (>9 mm) determined by ultrasonography were accepted as having tendon xanthoma (12). Demographic data, including age, body mass index, and waist circumference were also recorded. The diagnosis of diabetes was based on the documented fasting blood glucose levels of >126 mg/dL on more than one occasion, postprandial glucose levels of >200 mg/dL, or glycosylated hemoglobin levels of >6.5%. Hypertension was defined as either taking antihypertensive agents or having blood pressure of >140/90 mm Hg in three consecutive measurements. CV disease was defined as the presence of CAD, aortic aneurysms, dissections or aortic atherosclerotic diseases, ischemic stroke, or peripheral arterial disease, and ischemic stroke was defined as a proven cerebral infarct on computed tomography. Peripheral artery disease was defined as either having a peripheral arterial operation or ultrasound-proven arterial occlusion. Aortic aneurysms were defined according to either ultrasonographic and/or computed tomographic evaluations. CAD was defined as ≥50% stenosis in one coronary artery from the available coronary imaging methods (13). All these assessments were obtained from patients' lipid clinic charts, i.e., none were performed as a study procedure. Patients with a >50% decrease in serum LDL cholesterol levels with lipid-lowering therapy (LLT) were accepted as being responsive to treatment. To define the earliest premature CV disease in the family history, we recorded the age at which the first myocardial infarction (MI) or CV event occurred either in the study population or in their first- or second-degree relatives.

All other clinical and laboratory data were obtained retrospectively from the lipid clinic patient files. The retrospective data included the presence of any type of CV disease confirmed with conventional diagnostic techniques during the lipid clinic follow-up. Lipid data, including at least 12 h fasting levels of LDL cholesterol, HDL cholesterol, triglycerides, total cholesterol, lipoprotein (a), apoA-1 and apoB measured during lipid clinic follow-up were determined from the patients' chart reviews. For LDL cholesterol, triglycerides, and total cholesterol, both the highest serum levels (maximum) before the initiation of LLT and the lowest serum levels (minimum) during LLT were recorded (vice versa for HDL cholesterol).

Genetic analysis

All patients' DNA isolation was performed from peripheral blood samples taken into EDTA containing tubes, according to the MagNA Pure LC DNA Isolation kit I procedure in MagNA Pure LC DNA Isolation instrument (Roche Applied Science, Berlin, Germany). Isolated 100 µL DNA samples were stored in cryotubes at –86°C.

PCSK9 *S127R* (381 T > A; *rs28942111*), *D374Y* (1120 G > A; *rs137852912*), *F216L* (646 T > C; *rs28942112*), and *R496W* (1486 C > T; *rs374603772*) gene mutation analyses were performed by using specific primers (TIB MOLBIOL, Berlin, Germany) with the LightCycler® 480 High-Resolution Melting Master (HRM) kit (Roche Applied Science, Berlin, Germany). *S127R*, *D374Y*, *F216L*, and *R496W* gene mutations were performed in the LightCycler® 480 real-time PCR instrument (Roche Applied Science, Berlin, Germany) by real-time online polymerase chain reaction (PCR). PCSK9 *S127R*, *D374Y*, *F216L*, and *R496W* mutation-specific thermal profiles were created using denaturation, amplification, melting curve analysis, and cooling programs and were recorded via the LightCycler® 480 device software.

HRM analysis was performed on isolated DNA using a LightCycler® 480 real-time PCR device (Roche Applied Science, Berlin, Germany), which was adopted to enable fast multiplex PCR. Genotyping was done as "wild type," "heterozygous," and "homozygous" by specific melting temperatures (Tm) of the resulting amplicons.

The genotype frequencies were evaluated for consistency with Hardy–Weinberg Equation. Chi-square test p-values were 0.057518 for *R496W* (1486 C > T; *rs374603772*) and 0. 819764 for *D374Y* (1120 G > A; *rs137852912*) mutations. As no mutant or

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heterozygous mutations were observed for *F216L* (646 T > C; *rs28942112*) and *S127R* (381 T > A; *rs28942111*), the Hardy–Weinberg calculation was not generated.

Statistical analysis

All statistical analyses were performed using SPSS for Windows, Version 22 (SPSS Inc., USA). Data were presented as percentages for categorical variables and as mean±SD for continuous variables. The study population was divided into two groups according to the presence of any of the GOF mutations (*F216L*, *R496W*, *S127R*, or *D374Y*) in the PCSK9 mutation-positive and PCSK9 mutation-negative groups. Demographic characteristics, CV disease risk factors, lipid profiles and other laboratory findings, ultrasound and echocardiography findings, and treatment responses were compared between the groups. As all the data were distributed normally (Kolmogorov–Smirnov test, p>0.05), statistical comparison were performed using Student's t-tests. Categorical variables were compared using Fisher's exact test or chi-square test, as appropriate. A p-value of <0.05 (two-sided) was considered as statistically significant.

Results

Clinical and laboratory characteristics of the study population (n=80) are presented in Table 1 and 2. All patients were followed up in our lipid clinic for 114±97 (9–240) months. According to the DLCN criteria, 36% of the patients had "definite FH" and 21% had "probable FH". Seventy-one patients (88.7%) were receiving statin treatment, but 10.7% of these were reported to have stopped receiving the therapy because of personal reasons. No adverse events or statin intolerance was reported in the lipid clinic files. Response to statin treatment was acquired in 72% of patients.

Total CV disease frequency was 41%. The frequency of family history of CAD was 70% and that of total CV disease was 97.5%. All of the patients with FH and CAD were diagnosed as having FH after the first presentation of acute MI. The mean age at the first MI was 46±7 (35–63) years. Among the 80 patients, 25 (31.2%) quit smoking, whereas 10 (12.5%) were current smokers. Approximately, 13% of the patients were born of second-degree consanguineous marriages.

A total of 11 patients (13.8%) carried PCSK9 mutations. The *D374Y* mutation was present in four (5.0%) patients and *R496W* in seven patients (8.8%). Only one patient was homozygous for the *R496W* mutation. The other two PCSK9 GOF mutations (*S127R* and F216 variants) were not detected in our FH cohort. Patients with PCSK9 mutations are presented in detail in Table 3. In the PCSK9 mutation-positive group, seven (63.6%) patients were female with a mean age of 56±12 (30–75) years. No consanguineous marriages were reported in this group and no patients were diabetic. All the patients were on long-term LLT (60–240 months), except for the newly diagnosed youngest patient with FH (Table 3; patient 10). All patients over the age of 60 years were hypertensive and ex-smokers.

Table 1. Baseline characteristics of the study population (n=80)

Clinical characteristics	
Age, years	56±11
Female, n, %	47 (58.7%)
CV risk factors	
Diabetes mellitus, n, %	7 (8.7%)
Hypertension, n, %	10 (12.5%)
Body mass index, kg/m^2	27±4
Current smoking, n, %	39 (48.7%)
Family history of CAD, n, %	56 (70%)
CAD, n, %	22 (27.5%)
CV disease, n, %	33 (41%)
Age at first MI, years	46±7 (35–63)
Consanguineous marriage, n, %	10 (12.5%)
Treatment response with LDL C decrease ≥50 %, n, %	59 (73.7%)

CAD - coronary artery disease; CV - cardiovascular; LDL C - low-density lipoprotein cholesterol; MI - myocardial infarction. Clinical characteristics are expressed as n (%) and laboratory values are. Expressed as mean±standard deviation

Table 2. The observed lipid levels of the study population (n=80)

	Mean±SD (minimum–maximum)
Total cholesterol, mg/dL	
Maximal level	335±77 (257–650)
Minimal level	194±39 (106–377)
Triglycerides, mg/dL	
Maximal level	192±159 (55–1403)
Minimal level	117±70 (28–482)
HDL cholesterol, mg/dL	
Maximal level	58±15 (28–99)
Minimal level	53±15 (25–91)
LDL cholesterol, mg/dL	
Maximal level	252±78 (190–562)
Minimal level	106±39 (48–316)
Lipoprotein (a), mg/dL	35±47 (2.8–293)
Apolipoprotein A-1, mg/dL	148±28 (78–208)
Apolipoprotein B, mg/dL	146±45 (70–256)

HDL - high-density lipoprotein; LDL - low-density lipoprotein. All lipid values are expressed as mean±standard deviation. The maximal level means the highest level measured before lipid-lowering therapy, and minimal level means the lowest levels observed during lipid-lowering therapy (vice versa for HDL cholesterol)

Of the patients with *D374Y* mutation, none had CAD; meanwhile, all but one patient (patient 1) had family history of very early onset CAD at a mean age of 46 years. All patients were receiving effective LLT with statins (atorvastatin 10–40 mg/day or rosuvastatin 10–20 mg/day) for many years (min: 5 years; max: 10 years). Pretreatment LDL cholesterol levels ranged between

Table 3. Characteristics of the patients with PCSK9 mutations

No	Age	F/M	PCSK9 Mutation	Consanguinity	CAD	Familial history of CAD with earliest age	DM	Max LDL-C mg/dL	Min LDL-C mg/dL	LLT mg	Response to LLT	Duration of LLT, months	DLCN Total points
1	55	F	*D374Y*-Heterozygous	(0)	(0)	(0)	(0)	190	97	A(10)	(+)	120	2
2	47	F	*D374Y*-Heterozygous	(0)	(0)	(+), Uncle, 35 years	(0)	227	84	R(10)+E	(+)	60	4
3	57	F	*D374Y*-Heterozygous	(0)	(0)	(+), Brother, 54 years	(0)	240	100	R(20)	(+)	120	4
4	48	F	*D374Y*-Heterozygous	(0)	(0)	(+), Father, 46 years	(0)	249	101	A(40)	(+)	120 (with interruptions)	5
5	60	M	*R496W*-Homozygous	(0)	(+), MI, 44 years	(+), Sister, 54 years; Uncle, 35 years	(0)	378	197	A(80)+ E + Fn(267)	(0)	180	13
6	64	F	*R496W*-Heterozygous	(0)	(0)	(+) Father, 46 years	(0)	198	92	R(5)	(+)	108	4
7	75	M	*R496W*-Heterozygous	(0)	(0)	(+), Father, 55 years	(0)	233	78	A(20)	(+)	84	4
8	52	F	*R496W*-Heterozygous	(0)	(0)	(+), Son, 9 years	(0)	247	107	A(40)+E	(+)	120	12
9	66	M	*R496W*-Heterozygous	(0)	(+), MI, 35 years	(+), Mother, 30 years; Father, 40 years	(0)	255	122	R(20)+E	(+)	240	20
10	30	F	*R496W*-Heterozygous	(0)	(0)	(0)	(0)	378	222	A(40)+E	(0)	12	15
11	61	F	*R496W*-Heterozygous	(0)	(+), MI, 47 years	(+), Father, 40 years	(0)	289	140	R(20)+E	(+)	144	14

A - atorvastatin; CAD - coronary artery disease; DM - diabetes mellitus; DLCN - Dutch Lipid Clinic Network; E - ezetimibe; F - female; Fn - fenofibrate; LDL C - low-density lipoprotein cholesterol; LLT - lipid-lowering therapy; M - male; MI - myocardial infarction; PCSK9 - proprotein convertase subtisilin kexin type 9; R -rosuvastatin

189 mg/dL and 249 mg/dL. However, all patients with the *D374Y* mutation had achieved low LDL cholesterol levels with varying doses of statins. Of note, in one patient (Table 3; patient 1), LDL cholesterol level of <100 mg/dL was achieved with only small doses of atorvastatin (10 mg/day). All patients with the *D374Y* mutation had lower total points calculated according to the DLCN criteria.

Among the group with the *R496W* mutation, three patients (42%) first presented with premature MI at the age of 35, 44, and 47 years. All patients except one (Table 3; patient 10) had a family history of early CAD. Pretreatment LDL cholesterol levels ranged between 198 mg/dL and 378 mg/dL. The need for higher doses of statins and combination therapy with ezetimibe was more common in the *R496W* group. Of seven patients, two were

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not responsive to LLT despite maximal doses of statin (atorvastatin 80 mg/day) and ezetimibe (10 mg/day) combination. DLCN criteria total points were higher, except for two patients (Table 3; patients 6 and 7) who were also able to achieve LDL cholesterol levels of <100 mg/dL with small doses of statins. Except for one patient (Table 3; patient 10), all patients were older and had been receiving LLT for a long period of time (min: 7 years; max: 20 years).

The only homozygous patient (Table 3; patient 5) was a 60-year-old male with multiple atherosclerotic lesions. After diagnosis of early MI at the age of 44 years, he had undergone coronary arterial bypass grafting for multivessel CAD. He had severe generalized atherosclerosis (several percutaneous coronary interventions due to both restenosis and de novo lesions, renal artery stenosis, and carotid artery disease). Although he was receiving maximal effective LLT [atorvastatin (80 mg/day) for >20 years, ezetimibe for >8 years, and fenofibrate for 2 years], his on-treatment LDL cholesterol (197 mg/dL) level was far from the target level. His pedigree included many hypercholesterolemic individuals who were receiving LLT. His three daughters had LDL C levels of >190 mg/dL (heterozygous FH) without CAD. His brother, who experienced an MI at the age of 44 years, had a 9-year-old daughter who was receiving LLT.

Table 4 shows the comparison of patients with and without PCSK9 mutations. There were no significant differences between the groups with regard to demographic characteristics, CV disease risk factors, and clinical course of the disease. Lipid profiles and other laboratory findings were not statistically different between the two groups; however, pretreatment levels of LDL C, triglycerides, apoB and lipoprotein (a) tended to be higher in the PCSK9 mutation-positive group. Patients with PCSK9 mutations tended to be younger at the first CV disease than those without PCSK9 mutations (42±6 vs. 46.5±6, respectively). Also, the age at the first occurrence of CV disease based on the family history was lower in the PCSK9 mutation-positive group. However, none of these reached a statistically significant level.

Table 4. Comparison of patients with and without PCSK9 mutation (n=80)

Clinical data	PCSK9 (+) n=11	PCSK9 (–) n=69	*P*
Age, years	56±12 (30–75)	56±11 (28–83)	0.96
Female, n,%	7 (64%)	44 (60%)	
Age at 1st CV disease, years	42±6	46.5±6	0.33
CAD, n,%	3 (27%)	21 (28.8%)	1.0
CV disease, n,%	3 (27%)	23 (31.5%)	1.0
Consanguinity, n,%	1 (9.1%)	9 (12.3%)	1.0
Age at 1st CV disease in family history, years	43±17	49±12	0.08
Diabetes mellitus, n,%	0	7 (9.6%)	0.59
Response to LLT, n,%	8 (72.7%)	49 (72.2%)	1.0
Total cholesterol			
Maximal level	357±90 (287–600)	332±78 (257–650)	0.38
Minimal level	224±62 (170–377)	189±32 (106–262)	0.09
Triglycerides			
Maximal level	215±122 (109–522)	187±160 (55–1403)	0.62
Minimal level	153±116 (73–482)	111±56 (28–322)	0.06
HDL cholesterol			
Maximal level	58±16 (33–92)	58±15 (28–99)	0.89
Minimal level	53±17(31–90)	52±14 (25–91)	0.89
LDL cholesterol			
Maximal level	260±67(190–400)	250±78 (190–562)	0.72
Minimal level	126±71 (63–316)	103±31 (48–206)	0.31
Lipoprotein (a)	44±59 (3–167)	36±48 (3–293)	0.62

CAD - coronary artery disease; CV - cardiovascular; HDL - high-density lipoprotein; LDL - low-density lipoprotein; LLT - lipid-lowering therapy; NS - not significant; PCSK9 - proprotein convertase subtisilin kexin type 9. All lipid levels are expressed as mg/dL. Clinical characteristics are expressed as n (%) and laboratory values are expressed as mean±standard deviation (minimum–maximum values). The maximal level means the highest level measured before lipid-lowering therapy, and minimal level means the lowest levels observed during lipid-lowering therapy (vice versa for HDL cholesterol)

Discussion

To the best of our knowledge, our study is the first to evaluate the PCSK9 mutations in an FH cohort from Turkey. We found that 13.8% of the FH cohort was positive for PCSK9 *D374Y* and/or *R496W* GOF mutations. This is a relatively high rate in comparison with data from various countries. In 2009, Abifadel et al. (14) evaluated 51 Lebanese patients with FH and found a prevalence of PCSK9 variation of 29.7%. Using sequencing analysis, Marduel et al. (15) found the prevalence of PCSK9 mutations to be 0.7% in 1358 patients with FH in France, and Medeiros et al. (16) found the prevalence to be 1% in 359 patients with FH in Portugal. In Japan, the PCSK9 E32K mutation was found in 5.9% of 1055 heterozygous patients with FH (17). Additionally, in 2012, Palacios et al. (18) detected only one PCSK9 mutation in 7000 Spanish patients with FH. In a family health screening study in Scotland, only one (0.2%) PCSK9 mutation was detected in genetic testing of a cohort of 425 hypercholesterolemic individuals (19). In the Netherlands, a total of 104,000 hypercholesterolemic patients were genetically tested for FH by Sjouke et al. (20), and in 49 homozygous patients with FH, no PCSK9 mutations were detected. Likewise in Russia, no PCSK9 mutation was detected in 109 patients with FH (21). An explanation for our Turkish FH cohort's high rate of PCSK9 mutation might be high consanguinity, which is reported to be 21% on average (2, 22). However, in our study population the consanguinity rate was lower (12%), which may be attributed to the fact that only the current generation was questioned, resulting in an underestimation of the true rate of consanguinity.

The *D374Y* GOF mutation was first discovered by Leren (23) in a Norwegian FH population of 51 patients, and at the same time

by Timms et al. (24) in a Utah pedigree. Among the Norwegian FH cohort, the *D374Y* mutation was found in 4% of patients, and in another cohort of UK patients, the prevalence of *D374Y* was 1.7% (23, 25). In our FH cohort, the prevalence of *D374Y* was 5%.

In vitro studies show that PCSK9 *D374Y* mutant is 6- to 30-fold more strongly attached to the LDL receptor compared with the "wild type" PCSK9. Therefore, it is not surprising that a 30-year follow-up of four mutant English families found 10 years of earlier CAD and higher concentrations of LDL cholesterol compared with patients with FH with more common LDL receptor mutations (26). In our study, although patients with *D374Y* mutation showed a lack of pathognomonic physical findings and early CAD, early CAD was observed in first-degree relatives of three heterozygous patients with FH (Table 3; patients 2, 3, and 4). Despite the mean CAD age in first-degree relatives being 46 years, the lack of CAD in our patients is probably due to close follow-up and the impact of long-term LLT in our lipid clinic. Our *D374Y* mutation-positive patients' response to LLT was also satisfactory, even with low doses (i.e. patient no. 1 showed a 50% decrease in LDL cholesterol levels with only a dose of 10 mg daily atorvastatin; Table 4). However, in the four English *D374Y* families mentioned above, on-treatment LDL cholesterol levels were reported to remain significantly higher than those with LDL receptor mutations (26).

The *R496W* mutation, which was discovered in a 35-year-old Sicilian woman with xanthelasmas, xanthomas, and serum LDL cholesterol levels of 518 mg/dL (27), was found in seven patients (8.8%) in our study. In vitro studies have shown that the *R496W* mutation reduces the number of LDL receptors on the cell surface to a lesser degree than the *D374Y* mutation, and therefore it is thought to result in a variant of FH with better prognosis than that for *D374Y* (28). However, in our study, the opposite was true. We detected a worse prognosis in our seven patients with the *R496W* mutation compared with those with *D374Y*: three patients (42%) had early CAD at the age of 35, 45, and 47 years; two patients (28%) had corneal arcus; and six patients (85%) had early family history of CAD at a mean age of 38 years. In these patients, the maximum pretreatment and minimum on-treatment mean LDL cholesterol levels were 265 and 139 mg/dL, respectively. Response to lipid-lowering medications was obtained in 57% of these patients, which is worse than the total rate of our study population. In one patient with frequent restenosis and repetitive revascularizations despite optimal medical therapy, corneal arcus, and multiple hypercholesterolemic relatives, *R496W* homozygosity was detected (Table 3; patient 5). The poor disease course in this patient could be attributed to possible coexistence of other mutations, which were not identified in our study. Our findings regarding the *R496W* mutation raise the probability that this mutation is indicative of poor prognosis.

The other two PCSK9 GOF mutations (*S127R* and *F216L*) were not detected in our FH cohort. The *S127R* mutation was shown in 1.4% of 71 patients with FH from New Zealand and South Africa by Homer et al. (29), and in 2.6% of 75 patients with FH in France (30). PCSK9 *F216L* mutation was discovered in 2004 in a French family, and since then no new data have been reported (31).

Although no statistically significant differences in lipid profiles were detected between the groups with or without PCSK9 mutations, total cholesterol, LDL cholesterol, triglycerides, lipoprotein (a), and apoB levels tended to be higher in the mutation-positive group. In our patients, the natural course of the disease probably improved due to long-term LLT and effective risk-factor management. Also, the age at the first CV event in our patients and their families' history was younger in the PCSK9 mutation-positive group than in the PCSK9 mutation-negative group. These differences did not reach statistical significance probably due to the small number of patients with PCSK9 mutations. Similarly, Naoumova et al. (26) have reported that patients with a PCSK9 GOF mutation were affected by premature CV disease at a much earlier age than LDL receptor mutants.

Study limitations

The major limitation of this study is the lack of whole gene sequencing analysis and the use of single nucleotide mutations. Compound heterozygosity is not rare in homozygous FH cases, and our genetic analysis may have underestimated the severity of compound heterozygous FH. Another important limitation is the design; we conducted a cohort study instead of a case control to test the chosen variations. A further obvious limitation is the small size of the study population; however, as this FH cohort is enrolled from a single experienced lipid specialized center in Turkey, the cohort could be considered to be more homogeneous than one sourced from multiple centers with differing degrees of experience with FH. Finally, these results cannot be generalized to the rest of Turkey because our study reflects a more structured and effective approach to lipid lowering of an experienced lipid clinic.

Conclusion

This novel study from a Turkish FH cohort revealed a higher frequency (approximately 14%) of two PCSK9 GOF mutations (*D374Y* and *R496W*) compared with the global literature. Moreover the detected patients with *D374Y* and *R496W* mutations had a more benign CV disease course compared with those in the published data. Large-scale country-wide registries with cascade screening are warranted to confirm this first report of high frequency of PCSK9 GOF mutations in Turkey (32–33).

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