Case-control study on PCSK9 R496W (rs374603772) and D374Y (rs137852912) mutations in Turkish patients with primary dyslipidemia

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Objective: The aim of this study was to investigate the relationships between F216L (rs28942112), R496W (rs374603772), S127R (rs28942111), and D374Y (rs137852912) PCSK9 gain-of-function (GOF) mutations and primary dyslipidemia and serum lipid levels in patients with primary dyslipidemia.

Methods: In this case-control study, DNA was isolated from blood samples collected from patients diagnosed with primary dyslipidemia in cardiology outpatient clinic of Ege University (n=200) and healthy individuals (n=201). F216L, R496W, S127R, and D374Y GOF mutations in the PCSK9 gene were evaluated and genotyped according to the results of melting curve analysis performed in a real-time polymerase chain reaction (PCR) 480 instrument using specific primers for each mutation.

Results: There were statistically significant differences between the patient and individuals in control groups in the R496W and D374Y mutations ($\chi^2=10.742$ $p=0.005$; $\chi^2=6.078$ $p=0.048$, respectively). In addition, triglyceride levels in patients with primary dyslipidemia heterozygous for R496W and D374Y mutations were 12.8-fold ($p=0.015$) and 3.4-fold ($p=0.03$) higher than that in mutant and wild-type genotype, respectively. Additionally, in the entire study group (n=401), PCSK9 R496W and D374Y mutation carriers had increased total cholesterol ($p=0.021$), triglycerides ($p=0.0001$), HDL cholesterol ($p=0.028$), and low-density lipoproteins (LDL) cholesterol ($p=0.028$) levels. However, F216L (rs28942112) and S127R (rs28942111) mutations were not detected in patients with primary dyslipidemia and healthy controls.

Conclusion: We conclude that the PCSK9 R496W (rs374603772) and D374Y (rs137852912) GOF mutations may be significant risk factors in the development of primary dyslipidemia and may have significant impact on lipid parameters in general population.
lipoprotein metabolism (triglyceride-rich lipoproteins, chylomicrons, and VLDL) (13).

The aim of this study was to determine the prevalence of F216L (rs374603772), D374Y (rs28942111), S127R (rs137852912), and R496W (rs137852912) PCSK9 GOF mutations in patients with primary dyslipidemia and to investigate the relationship between these mutations and primary dyslipidemia and serum lipid profiles.

Methods

Patients

This observational case-control cross-sectional study included 200 consecutive patients (105 females and 95 males) with primary dyslipidemia who were being followed in the Cardiology Lipid Clinic of Ege University Medical School in Izmir, Turkey, as well as 201 healthy individuals (118 females and 83 males) age- and gender-matched to the study group whose routine health checkups were within normal limits. Patients with secondary causes of hypercholesterolemia (such as hypothyroidism, nephrotic syndrome, diabetes, chronic renal failure, and alcohol consumption) were excluded. Clinical and laboratory data were obtained from the lipid clinic patient files. Family and personal medical histories, such as regarding CV disease, hypercholesterolemia, ischemic stroke, diabetes mellitus, and other diseases, were recorded. For the LDL-C, triglycerides, total cholesterol, and HDL-C levels, the maximal values measured before treatment were recorded. The diagnosis of primary dyslipidemia was based on the presence of total cholesterol levels of >200 mg/dL, triglyceride levels of >200 mg/dL, and/or LDL-C levels of >130. The presence of diabetes mellitus was defined as either fasting blood glucose levels of >126 mg/dL or receiving anti-diabetic agent. CVD was identified as the presence of coronary artery disease, aortic aneurysms, dissections or aortic atherosclerotic diseases, ischemic stroke, or peripheral arterial disease; ischemic stroke was defined as a proven cerebral infarct on computed tomography. Therefore, the study population comprised 401 subjects (200 patients with primary dyslipidemia and 201 healthy individuals).

The study protocol was approved by the Institutional Clinical Investigation Ethics Committee (2013, 13-11/92), and all participants gave written informed consent for genetic analysis. The study was supported financially by the Institutional Scientific Research Project Committee (14-TIP-072).

Two 3-mL blood samples were collected from each of the participants into separate EDTA tubes. Detailed personal medical history including the presence of CVD and treatments was received from each participant. In addition, fasting levels of LDL-C, HDL-C, triglyceride, total cholesterol, and fasting glucose were recorded.

Genotyping

Genomic DNA extraction was performed from peripheral blood leukocytes using MagNA Pure LC instrument according to the MagNA Pure LC DNA Isolation Kit I procedure (Roche Applied Science, Mannheim, Germany). Specific primers were designed for the detection of the PCSK9 S127R (rs28942111), R496W (rs137852912), D374Y (rs137852912) PCSK9 GOF mutations in patients with primary dyslipidemia and to investigate the relationship between these mutations and primary dyslipidemia and serum lipid profiles. The genotype and allele frequencies were evaluated using the Hardy–Weinberg Equation. Chi-square test p-values were 0.0012 and 0.0203 for PCSK9 R496W (rs137852912) and D374Y (rs137852912) mutations, respectively.

Statistical analysis

All statistical analyses were performed using SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA). Data are presented as percentages for discrete variables and as mean±standard deviation for continuous variables. The groups were compared using Student’s t-test, and discrete variables were compared using chi-square analysis. P-value of <0.05 (two-sided) was considered to be statistically significant.

Results

Table 2 demonstrates the clinical characteristics and fasting lipid and glucose levels of the study groups. As expected, there...
were significant differences between the groups with regard to the presence of elevated total cholesterol, triglycerides, LDL-C levels, and decreased HDL-C levels.

Table 3 shows the summary of the genetic analysis of the 4 PCSK9 GOF mutations in the study groups. In both S127R (rs28942111) and F216L (rs28942112) groups, mutations were not detected. Mutation analysis of the 200 patients with primary dyslipidemia showed that 93.5% were wild type (CC), 6% were heterozygous (CT), and only 0.5% was homozygous (TT); meanwhile, of the 201 control subjects, 99.5% were wild type (CC), 0.5% was heterozygote (CT), and none were homozygous (TT) for PCSK9 R496W (rs374603772) mutation. This difference in the genotype of PCSK9 R496W (rs374603772) mutations was statistically significant between the patient and control groups ($\chi^2=10.742; p=0.005$). Similarly, there was a significant difference between the patient group and controls for the D374Y (rs137852912) mutation rates ($\chi^2=6.078; p=0.048$). Analysis of dyslipidemia group revealed that 93% were wild type (GG), 6.5% were heterozygous (GA), and
0.5% was homozygous (AA) (Table 3). However, in the control group, 98% were wild type (GG) and 2% were heterozygote (GT).

Comparison of total cholesterol, triglyceride, HDL-C, and LDL-C levels according to the PCSK9 R496W (rs374603772) and D374Y (rs137852912) genotypes between patients with primary dyslipidemia and healthy controls showed that the triglyceride values of patients with heterozygous for R496W (rs374603772) and D374Y (rs137852912) mutations were 12.8-fold and 3.4-fold higher, respectively, than those in healthy controls with normal and mutant genotypes (p=0.015 and p=0.03) (Table 4). In addition, when we compared total cholesterol, triglyceride, HDL-C, and LDL-C levels in PCSK9 R496W (rs374603772) and D374Y (rs137852912) mutation carriers and noncarriers, our results revealed that mutations carriers had higher triglyceride values than noncarriers (p=0.007) (Table 5). But when we compared the PCSK9 R496W (rs374603772) and D374Y (rs137852912) mutation carriers and noncarriers in the whole study group (n=401) in terms of lipid parameters, significant increase were observed in total cholesterol (p=0.021), triglycerides (p=0.0001), HDL-C (p=0.028), LDL-C (p=0.028) levels in mutation carriers (Table 6).

**Discussion**

According to our findings, the prevalence of heterozygote and mutant genotypes in patients with primary dyslipidemia was 6% and 0.5% for R496W (1486 C>T) and 6.5% and 0.5% for D374Y (1120 G>A), respectively.

The reported PCSK9 GOF mutation frequencies vary in different populations around the world. A 29.7% prevalence was reported among Lebanese patients with familial hypercholesterolemia; a 5.9% PCSK9 E32K prevalence was reported in Japanese patients with heterozygous familial hypercholesterolemia; and 1% and 0.9% PCSK9 mutation prevalence was reported in Portuguese and French patients with familial hypercholesterolemia, respectively (14-17). Moreover, in 2012, Palacios et al. (17) detected PCSK9 mutation in only 1 of 7,000 Spanish patients, and a public health screening program in Scotland revealed PCSK9 mutation in only 1 (0.2%) of 425 patients with hypercholesterolemia (18, 19). In the Netherlands, Sjouke et al. (19) reported no PCSK9 mutations in a genetic analysis of 104,000 patients with hypercholesterolemia, including 49 patients with homozygous familial hypercholesterolemia (20). Similarly, no PCSK9 mutations were detected in a study conducted in 109 patients with familial hypercholesterolemia in Russia (21). As seen above, there is growing evidence that PCSK9 mutations play a role in the development of familial hypercholesterolemia. However, its prevalence in primary dyslipidemias is unknown. To the best of our knowledge, our study is first to show the prevalence of PCSK9 D374Y and R496W mutations in patients with either high LDL-C and/or high triglycerides. Our results showed a higher preva-
lence of PCSK9 D374Y and R496W mutations in our Turkish primary dyslipidemia patient group than in the general population. Similarly, we previously reported a 13.8% prevalence of PCSK9 D374Y and/or R496W GOF mutations in a Turkish familial hypercholesterolemia cohort and have suggested that frequencies of PCSK9 GOF mutations could be substantially higher in Turkey than other countries (22).

D374Y mutation was first described in 2004 in a Norwegian familial hypercholesterolemia patient group; it was reported to have a prevalence of 4% and be responsible for autosomal dominant hypercholesterolemia (23). In a family genetic study conducted by Timms et al. (23) in Utah, USA, on patients with autosomal dominant hypercholesterolemia, the D374Y mutation was also detected in the three other siblings of an individual with D374Y mutation (24). In a study designed to investigate the effects of LDLR, APOB, and PCSK9 mutations on the development of CVD in a group of 253 familial hypercholesterolemia patients in the UK, LDLR mutation was detected in 236 (57.7%), APOB p.03500 mutation in 10 (2.4%), and PCSK9 D374Y mutations in 7 (1.7%) patients (25). Cameron et al. (25) investigated the effects of the GOF S127R and D374Y mutations on the autocalytic activity of PCSK9 in HepG2 cells transfected with LDLR in proportion to the amount of LDLR on the cell surface. Their study showed that these mutations did not affect PCSK9 autocalytic activity but caused changes in cell-surface LDLR and LDL levels. Cells with the mutant S127R and D374Y genotype showed 23% reduction in cell-surface LDLR levels and 38% reduction in LDL internalization when compared to cells with wild-type PCSK9. The authors reported that the S127R and D374Y mutations increased the amount of cell-surface LDLR, thereby leading to an increase in the amount of LDL (26). Of the 200 patients with primary dyslipidemia in our study, there were 13 patients with heterozygote (GA) and 1 patient with homozygous (AA) genotype for the D374Y mutation (χ²=6.078; p=0.048). The frequency of the A allele was higher in the patient group than in the control group. Comparison of our results with previous studies suggests that the PCSK9 D374Y (rs137852912) mutation has a role in disease development in Turkish patients with primary dyslipidemia.

Pisciotta et al. (26) compared heterozygous patients with familial hypercholesterolemia with a control group and detected R496W mutation in only one 35-year-old patient who had a serum LDL level of 518 mg/dL. When plasma LDL-C levels in the proband’s mother, who was also heterozygous for R496W mutation, were compared with those of other heterozygous familial hypercholesterolemia patients, they reported that the LDL-C levels were similar and that this rarely observed mutation may have pathogenic effect. The authors also reported that PCSK9 R496W gene mutation had a negative impact on clinical phenotype in patients with homozygous or heterozygous LDLR E228K mutation genotype (27). Of the primary dyslipidemia patients in our study, 12 were heterozygous (CT) and 1 was homozygous (TT) for R496W mutation. We also found that heterozygous primary dyslipidemia patients had comparable LDL-C levels and that mutant genotype carriers had a higher LDL-C value than the heterozygous patients. These findings suggest that the CT and TT genotypes of the PCSK9 R496W gene mutation can be used as biomarkers for primary dyslipidemia.

PCSK9 mutations were first described by Abifadel et al. (13) in patients with autosomal dominant hypercholesterolemia. They found that the S127R mutation was associated with disease progression in a study of autosomal dominant hypercholesterolemia patients from two French families with different ethnic backgrounds. Their analysis of a 49-year-old patient with total cholesterol of 441 mg/dL and LDL-C of 356 mg/dL, who died of a heart attack, and the patient’s family revealed F216L mutation in the proband and the proband’s two children (13). Other studies regarding S127F and F216L mutations in familial hypercholesterolemia have reported a prevalence of 1.4% in New Zealand and Southern Africa and 2.6% in France (10, 28). In an animal model study, it was also shown that the PCSK9 S127R and F216R mutant genotypes caused overexpression in the mouse liver, resulting in a dramatic decrease in hepatic LDLR levels because of post transcriptional mechanisms, thereby leading to hypercholesterolemia. In addition, it was determined that overexpression of wild-type PCSK9 in mice caused plasma levels of total cholesterol to double and non-HDL-C levels to increase 5 fold (29). In an in vivo study evaluating the effects of the S127R, F216L, and R218S mutant genotypes on the PCSK9-dependent LDLR mechanism, Nassoury et al. (29) demonstrated that LDLR was a dominant partner in regulating the cellular trafficking of PCSK9 and that LDLR deficiency resulted in the localization of PCSK9 in the endoplasmic reticulum (ER). They reported that in cells expressing LDLR, PCSK9 was localized with LDLR in post-ER compartments. Furthermore, when human wild-type and mutant S127R, F216L, and R218S genotypes were compared in human hepatic HuH7 cells, mutant genotypes were associated with hypercholesterolemia, and PCSK9 was arrested in the endosome/lysosomes and unable to reach the cell surface. Therefore, they demonstrated that the distribution of PCSK9 on the cell surface and endosome is necessary for PCSK9 to completely regulate LDLR degradation and that this retention in the ER causes a reduction in PCSK9 maturation and inhibits LDLR degradation. For this reason, they emphasized the causative role of S127R, F216L, and R218S genotypes in hypercholesterolemia (30). These studies are the extent of the research conducted to date regarding PCSK9 S127R and F216R mutations. In our study, all individuals in the primary dyslipidemic patient group and the control group carried the wild-type genotype for PCSK9 S127R and F216R mutations. These data show that the S127R and F216R PCSK9 mutations do not constitute a risk factor for disease development in Turkish patients with primary dyslipidemia. We believe that the discrepancies in the results obtained may have been caused by the selected patient group, genetic heterogeneity, and ethnic differences.

The most widely recognized role of PCSK9 to date has been its impact on LDLR, but recent studies suggest that PCSK9 may
have other effects on lipoprotein metabolism (triglyceride-rich lipoproteins, chylomicrons, and VLDL) as well. Recently, an important study evaluating the relationships between plasma PCSK9 and the VLDL and LDL subfractions showed that plasma PCSK9 was strongly associated with IDL, a triglyceride-rich subfraction that represents VLDL remnants. This finding indicates that PCSK9 exerts its effect on plasma triglycerides by modulating VLDL and VLDL remnant metabolism (31). As further evidence of the link between PCSK9 and triglycerides, it was reported that members of four British families who had familial hypercholesterolemia and carried GOF D374Y PCSK9 mutations had triglyceride levels that were higher than that in a control group but still within reference ranges (32). This suggests that PCSK9 perturbation may affect triglyceride metabolism (12). In the present study, we also found that triglyceride levels increased by 12.8 fold (p=0.015) and 3.4 fold (p=0.03) in patients with heterozygous R496W (1486 C>T) and D374Y (1120 G>A) mutations, respectively. Because the studies conducted to date have yielded controversial results, the effect of PCSK9 on triglyceride metabolism is still not fully understood. However, Stein et al. (32) reported in 2014 that 1,359 patients receiving PCSK9 inhibitor treatment showed a significant decrease in triglyceride levels at the end of treatment, regardless of PCSK9 mutation analysis. This finding illustrates a general connection between PCSK9 and triglyceride metabolism and suggests the possibility of a new therapy for hypertriglyceridemia (32). In our study, we also evaluated the whole study group (n=401) for lipid parameters according to mutation carriers and noncarriers; we found that mutation carriers had higher total cholesterol (p=0.021), triglycerides (p=0.0001), HDL-C (p=0.028), LDL-C (p=0.028) levels than noncarriers. These results indicate that GOF R496W (rs374603772) and D374Y PCSK9 mutations could have a significant impact on lipid metabolism in general population. However, there are different regions in Turkey, so there may be dominance of different/same PCSK9 mutations in other regions of our country. To show the reflection of R496W (rs374603772) and D374Y (rs137852912) PCSK9 mutations in Turkish population, more comprehensive studies need to be conducted from other regions of Turkey.

There is a much lower rate of PCSK9 mutation in autosomal dominant hypercholesterolemia than that of LDLR and APOB mutations. However, probands who are heterozygous for both LDLR and PCSK9 mutations have 50% higher plasma LDL levels than relatives with only one mutation (12). PCSK9 gene constitutes the basis of a new mechanism contributing to dyslipidemia, and determining the effects of the PCSK9 substrate and PCSK9 gene mutations on function is important in terms of early diagnosis and treatment. A better understanding of the physiology and genetics of lipoprotein metabolism should facilitate earlier diagnosis and development of effective therapeutic interventions for patients with lipid disorders. As research aimed at using antibodies against PCSK9 in the treatment of lipid disorders gains momentum, it is anticipated that these agents will soon become available for clinical use.

Study limitations
The first limitation of the study is the lack of whole-gene sequencing analysis for PCSK9 gene. The second limitation is homogeneity of the study population that was enrolled from a single experienced lipid specialized center from Aegean region in Turkey, so the results do not reflect the whole Turkish population.

Conclusion
The present study demonstrates the association between the R496W (rs374603772) and D374Y (rs137852912) PCSK9 mutations and primary dyslipidemia in Aegean region. To date, more than 30 GOF mutations have been identified in the PCSK9 gene. We believe that this study will also lead to other genomic research in this area.

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