Clinical Research

Familial Hypercholesterolaemia in North-Western Greece

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Introduction: Familial hypercholesterolaemia (FH) is the most common genetic disorder and results from mutations in the low-density lipoprotein receptor (LDLR) gene. So far, over 700 mutations have been reported for the LDLR gene that account for the disease. The nature of LDLR gene mutations is different in various ethnicities and also exhibits regional distribution within each ethnicity. The aim of this study was to identify the LDLR gene mutations causing FH in north-western Greece, to find their geographical distribution and, finally, to examine the effect of genetic factors on the lipid profile of these FH patients.

Methods: Probands with a clinical diagnosis of FH (n=153) had their DNA tested for mutations in the LDLR gene. Previously published criteria were used for the clinical diagnosis and the genetic analysis was performed either by using restriction enzymes or by DNA sequencing. Apolipoprotein (apo) E gene polymorphism was determined using a previously reported protocol. Eighty-two normal individuals matched for age, sex, body mass index and smoking status were used as controls.

Results: The clinical diagnosis was genetically confirmed in 123 patients. Nine LDLR gene defects were causing FH in the patients studied; two of them are described for the first time worldwide (T1352C and g.387_410del24), while three mutations were isolated for the first time in Greek patients (T81G, G1694T and G1775A). The other responsible mutations were T517C, C858A, G1285A and G1646A. Eight patients were homozygotes for the G1775A mutation, which was the most common mutation encountered in our area (29%) and one was a homozygote for the G1646A mutation. The most common mutations exhibit geographical distribution in the area of north-western Greece. Heterozygous FH patients had significantly lower serum HDL cholesterol (HDL-C) levels and higher lipoprotein (a) [Lp(a)] levels compared to normal controls. The LDLR gene mutations did not affect the lipid profile of patients with heterozygous FH. Interestingly, the presence of the allele E4 of the apo E was associated with lower HDL-C levels compared with patients not carrying this allele (39.9±8.6 vs. 49.3±13 mg/dl, p=0.03). Lp(a) levels of the homozygous FH patients were higher than those found in heterozygotes sharing the same mutation. However, these homozygous patients for the G1775A mutation, which is a type V mutation, had lower serum Lp(a) and total cholesterol (TCHOL) levels compared to those previously reported in homozygous FH patients carrying other types of LDLR mutations.

Conclusion: Our study describes the genetic basis of FH in north-western Greece in 123 unrelated families, a sufficiently large number to be considered representative of the region, and reveals the geographical distribution of the most frequent LDLR gene mutations. Our data suggest that apo E polymorphism affects HDL-C levels in patients with heterozygous FH. Also, Lp(a) levels are higher and HDL-C levels are lower in patients with heterozygous FH compared to normal individuals. Finally, the type of the LDLR gene mutations may affect not only the classic lipid parameters of patients with homozygous FH but also Lp(a) levels.

Familial hypercholesterolaemia (FH; MIM# 143890) is an autosomal dominant inherited disorder of lipid metabolism that results from mutations in the low density lipoprotein receptor (LDLR) gene. The latter is a 160KD cell surface transmembrane protein responsible for the uptake of serum low density lipoprotein (LDL) into hepatocytes, where it is further metabolized.[1]

To date, over 700 mutations have been identified in the LDLR gene, some being founder mutations specific to certain geographical regions (www.ucl.ac.uk/fh). Five functional types of LDLR mutations have been reported.[2] Type I mutations eliminate the synthesis of LDLRs and type II mutations result in LDLRs that fail to be transported to cell membrane. The products of type III mutations are receptors with defective binding ability to apolipoprotein (apo) B100. Type IV and V mutations result in functional receptors in cell membrane but fail to internalise the LDLR-apoB100 complex (type IV) or fail to release LDL intracellularly and thus LDLRs do not recycle to cell membrane (type V).[2] It has been postulated that the type of LDLR mutations affects serum LDL-cholesterol (LDL-C) levels in patients with FH and the prevalence of cardiovascular disease.[2-4] However, other studies failed to show an association between the genetic effect and the phenotypic variability in FH.[5] Also, though controversial, apo E gene polymorphism has been postulated as a candidate gene that affects the lipid profile of FH patients.[6-11]

Patients with homozygous FH have markedly elevated LDL-C levels. It has been suggested previously that mutations in the LDLR gene affect lipoprotein (a) [Lp(a)] serum levels. Interestingly, Kraft et al have recently stated that FH heterozygotes have significantly higher Lp(a) levels than do non-FH individuals from the same population, while FH homozygotes had about 2-fold higher Lp(a) than did FH heterozygotes.[12]

We undertook this study to identify the LDLR gene mutations causing FH in north-western Greece, to find their geographical distribution and, finally, to examine the effect of genetic factors on the lipid profile of these FH patients.

Methods

Patient Recruitment

One hundred and fifty three unrelated patients attending the lipid clinic were clinically diagnosed as having FH using established clinical criteria.[13] Informed consent was given by all patients for molecular genetic investigations to identify LDLR gene mutations. Eighty-four healthy individuals matched for age, sex, body mass index (BMI), and smoking status were used as controls.

Genetic analysis

Whole blood was collected for DNA isolation using standard procedures. Amplification of all the exons of the LDLR gene was performed using the primer set and PCR protocol described previously.[14] The responsible mutations were detected either by restriction enzymes or by direct sequencing using the 'Big Dye Terminator Cycle Sequencing Ready Reaction Kit' as instructed by the manufacturer (Applied Biosystems) and capillary electrophoresis on an ABI PRISM 310 Genetic Analyser. Apo E gene polymorphism was examined using a previously described protocol.[15]

Lipid measurement

In both patients and controls blood samples were obtained after a 14 hour overnight fast for gene genotype detection as well as the determination of lipid parameters. Blood samples were centrifuged for 30 min (3600g) and then the serum was separated and stored at 4˚C for the analysis of lipid parameters. Serum for the assay of Lp(a) was frozen and stored at -700C. Concentrations of total cholesterol (TCHOL) and triglycerides (TGs) were determined enzymatically on the Olympus AU600 clinical chemistry analyser (Olympus Diagnostica, Hamburg, Germany), while HDL-cholesterol (HDL-C) was determined enzymatically in the supernatant after the precipitation of other lipoproteins with dextran sulphate-magnesium. LDL-C was calculated using the Friedewald formula.[16] Apolipoproteins A-I, B, E and the Lp(a) were measured with a Behring Nephelometer BN100 using reagents (antibodies and calibrators) from Dade Behring Holding Gmbh (Liederbach, Germany). The apo A-I and apo B assays were calibrated according to the International Federation of Clinical Chemistry (IFCC) standards.

Statistical analysis

Statistical analysis was performed with SAS statistical software. Student's t-test for independent samples was used to compare continuous data between 2 groups and analysis of variance (ANOVA) was used to compare continuous data between 3 or more groups. The Mann-Whitney U test and Kruskal-Wallis ANOVA median test were used to test differences of Lp(a) levels between 2 and 3 groups, respectively. Since serum TGs were not normally distributed in all groups, TG data were transformed into natural logarithms. The χ^2 test was used to examine percentages.

Results

The clinical diagnosis of FH was genetically proved in 123 patients. Nine mutations were responsible for

Table 1. Low-density lipoprotein receptor gene mutations data base in north-western Greece.

Exon No.	Mutation	Amino Acid Change	Frequency % (N=123)
2	T81G	C6W	7
4	T517C	C152R	5
4	g.387_410del24	S109_G116	1
6	C858A	S265R	15
9	G1285A	V408M	14
9	T1352C	I430T	2
11	G1646A[1]	G528D	25
11	G1694T	G544V	2
12	G1775A[2]	G571E	29

[1]One patient was a homozygote
[2]Eight patients were homozygotes

the FH; 4 of them have been previously described in Greek FH patients (T517C, C858A, G1285A and G1646A mutations), 3 are described for the first time in Greeks (the T81G, G1694T and G1775A), while 2 mutations are novel mutations (the T1352C mutation and the g.387_410del24 gene deletion).[17,18] Nine patients were homozygotes, 8 for the G1775A mutation and 1 for the G1646A mutation. The most frequent mutations in our population were G1646A and G1775A. Table 1 summarises the responsible mutations and their frequencies.

Detailed investigation of the ancestry of each proband revealed a geographic distribution of the most common mutations causing FH in patients originating from north-western Greece. The most frequent G1775A mutation was restricted to a mountainous area called Tzoumerka near the city of Ioannina. G1646A was found in patients from Thesprotia, whereas the C858A mutation was congregated near the western coast of our area. Finally, patients originating from Etoloakarnania had the G1285A mutation.

The lipid profiles of heterozygous FH patients and normal individuals are shown in table 2. As shown, FH patients had significantly higher serum Lp(a) levels and lower HDL-C and apo A-I levels compared to normal individuals. When heterozygous patients were divided according to apo E gene polymorphism, individuals with the ε4 allele (apo E3/3 and apo E3/4 genotypes) had statistically lower HDL-C levels compared to those with the ε2 and ε3 alleles (apo E2/2, apo E2/3 and apo E3/3 genotypes) (Table 3). However, apo E genotype did not affect the other lipid parameters in patients with heterozygous FH (data not shown). Finally, as shown in table 4, there were no significant differences concerning age, sex, BMI, smoking status and lipid parameters between heterozygous patients with the most frequent LDLR mutations.

As already mentioned, the G1775A mutation was responsible for the vast majority of patients with homozygous FH. The high number of homozygotes for this mutation and its definite geographic distribution suggest that the G1775A mutation is a founder mutation in our area. This mutation is a type V mu-

Table 2. Clinical features and lipid parameters in FH heterozygotes and controls.

Variable	FH heterozygotes (n=114)	Control population (n=84)	P-value
Male/Female	62/52	51/31	NS
Age (years)	41±13	40±15	NS
Body mass index (kg/m^2)	23±4	22±4.5	NS
Cigarette smokers %	49	47.5	NS
TCHOL (mg/dl)	378±86	180±45	< 0.001
TG (mg/dl)	140±102	110±70	NS
LDL-C (mg/dl)	295±83	118±46	< 0.001
HDL-C (mg/dl)	48±12	55±13	0.01
Apo A-I (mg/dl)	139±34	149±28	0.05
Apo B (mg/dl)	195±70	126±38	< 0.001
Lp(a)	14.5 (0.8-104)	7 (0.8-34.5)	<0.001

Values are expressed as mean±SD, except for the Lp(a), which is reported in terms of median (range).
Apo – apolipoprotein; HDL – high density lipoprotein; LDL – low density lipoprotein; Lp(a) – lipoprotein (a); TCHOL – total cholesterol; TG – triglycerides.

Table 3. The effect of apo E gene polymorphism on HDL-C levels in patients with molecularly defined familial hypercholesterolaemia.

Apo E polymorphism	Number of patients	HDL-C, mg/dl (mean±SD)	p-value
nonE4	99	50.1±13	0.03
E4	15	41.8±8.6	

Abbreviations as in Table 2.

Table 4. Clinical features and lipid parameters in heterozygous familial hypercholesterolaemia patients with the most frequent mutations of the low-density lipoprotein receptor gene.

MUTATION	C858A	G1285A	G1664A	G1775A	p
Age (years)	37±14	40±15	36±14	43±12	0.20
Sex (M/F)	10/8	11/7	9/11	17/11	0.29
Smoking (Y/N)	13/5	15/2	15/5	19/9	0.42
Body mass index (kg/m2)	24±3	23±4	24±4	25±3	0.73
TCHOL (mg/dl)	346±50	409±107	379±68	366±68	0.13
TG (mg/dl)	140±72	105±47	168±130	157±80	0.74
HDL-C (mg/dl)	51±13	50±15	46±15	47±13	0.58
LDL-C (mg/dl)	267±39	337±96	289±53	287±65	0.70
Lp(a) (mg/dl)	8.4 (2.5-33)	15.7 (5-32)	11 (0.8-36)	10.9 (1.7-31)	0.86

Values are expressed as mean±SD, except for Lp(a), which is given in terms of median (range).
Abbreviations as in Table 2.

tation resulting in functional receptors in hepatocytes. However, these receptors do not recycle to the cell membrane. The characteristics of these homozygous patients compared to the heterozygotes with the same mutation are shown in table 5. Lp(a) levels were statistically higher and HDL-C levels were statistically lower in homozygotes compared to heterozygotes. However, homozygous patients for the G1775A mutation had lower TCHOL and Lp(a) levels compared to the values previously reported for homozygous FH carrying other types of mutations (Table 6).[12,19] Finally, the serum TCHOL level in the patient with homozygous FH carrying the G1646A mutation, which is a type II mutation, was 950 mg/dl.

Discussion

Nine mutations accounted for FH in north-western Greece. Interestingly, four mutations accounted for more than 85% of cases and showed geographical distribution in the area. The knowledge of this geographic distribution facilitates future genetic screening for the early diagnosis and treatment of new probands or new members in an affected family. The

Table 5. Lipid profile (mg/dl) of homozygous and heterozygous patients with the G1775A mutation.

G1775A	TCHOL	TG	HDL-C	LDL-C	apo A-I	apo B	apo E	Lp(a)
Heterozygotes, n=28	366±68	157±80	47±13	287±65	139±20	182±51	55±12	10.9 (1.7-30.7)
Homozygotes, n=8	440±80	147±89	39±8	354±75	101±30	237±19	62±25	16.1(10.7-70)

$p<0.05$ for all the comparisons
Values are expressed as mean±SD, expect for Lp(a), which is given in terms of median (range).

Abbreviations as in Table 2.

Table 6. Lp(a) and TCHOL levels (mg/dl) in patients with homozygous familial hypercholesterolaemia.

	Patients with a type V mutation (G1775A)	Kraft HG et al, 2000 (reference number 12)	Guo HC et al, 1991 (reference number 19)	Patient with a type II mutation (G1646A)
Lp(a) median (range)	16.15 (10.7-70)	36.6 Not shown	39.0 (21-116)	25.8
TCHOL, mean±SD	440±80	Not shown	799±205	950

Abbreviations as in Table 2.

high incidence of homozygous FH in north-western Greece implies the presence of a founder mutation.[20] Indeed, 8 of the 9 homozygous patients shared the G1775A mutation and homozygous as well as heterozygous patients with the same mutation originated from the same non-contiguous mountainous area. The high degree of close breeding/inbreeding in the population of this area may explain the relatively higher frequency of homozygous FH.

A low HDL-C level is an independent risk factor for cardiovascular disease in the general population.[21,22] FH is often associated with decreased HDL-C levels, probably adding another risk factor to the high LDL-C levels for cardiovascular disease in patients with FH.[23] As shown in table 2, heterozygous patients had lower HDL-C levels compared to normal controls. The effect of FH on HDL metabolism remains poorly documented. Both increased fractional catabolic rate and decreased absolute production rate of HDL-apo A-I have been postulated.[24] Our study suggests that homozygous FH patients exhibit even lower HDL-C levels than FH heterozygotes with the same mutation (Table 5). In addition, apo E gene polymorphism affected HDL-C levels in heterozygotes. In fact, the presence of apo E4 was associated with lower HDL-C levels (Table 3). Although many studies have not shown an association between apo E polymorphism and HDL-C levels in FH, a few are in accordance with our finding.[6-11] An increased catabolic rate of the apo E enriched HDL particles in FH patients has been reported in individuals with the ε4 allele compared to those with the ε2 and ε3 alleles, mainly attributable to the higher affinity of ε4 to the cellular receptors.[25]

It has been previously stated that FH heterozygotes have significantly higher Lp(a) levels than do non-FH individuals from the same population.[19] In accordance with these findings, our heterozygotes had significantly higher Lp(a) levels compared to normal individuals (Table 2). The underlying mechanisms are insufficiently clear, but may be related to a delayed Lp(a) catabolism through the LDLR or to other poorly understood metabolic changes in FH, which may include the metabolism of fatty acids that is known to affect lipoprotein synthesis including LDL and Lp(a).[26,27]

The type of the LDLR gene mutation has been postulated to affect the phenotypic variability of FH. In detail, mutations that do not produce or produce defective proteins (type I-III mutations) have been associated with higher serum LDL-C levels and a higher incidence of cardiovascular disease compared to mutations that produce functional receptors (type IV and V mutations).[2-4] However, other studies failed to show an association between phenotypic variability and the genetic defect.[5] In our cohort, the type of the LDLR mutation did not affect the lipid profile of heterozygous patients (Table 4). However, patients with homozygous FH carrying the type V G1775A mutation had lower serum TCHOL compared to those mentioned in the literature carrying other types of mutations (Table 6).[12,19] As already mentioned, type V LDLR gene mutations produce functional LDLRs that fail to recycle to the cell membrane after the internalisation of the LDLR-apo B100LDL complex.[2] Indeed, our homozygotes fulfilled the clinical criteria for heterozygous FH.[13] In contrast, the patient with homozygous FH carrying the G1646A mutation (type II mutation) had TCHOL levels that were almost double compared to the mean value of TCHOL found in patients with homozygous FH carrying the G1775A mutation. Thus, our data suggest that the type of the LDLR mutations may affect serum TCHOL levels in patients with homozygous FH. Similarly, the G1775A homozygotes had lower serum Lp(a) levels compared with the values mentioned in the literature for patients with homozygous FH. Thus, the type of the LDLR gene mutation may affect not only serum

TCHOL levels but also Lp(a) levels in patients with homozygous FH.[28]

To conclude, we have described the genetic data base of FH in north-western Greece and elucidated the geographic distribution of the most frequent mutations. We also found that heterozygous FH is associated with higher Lp(a) levels and lower HDL-C levels compared to the general population. Our data suggest that apo E gene polymorphism affects HDL-C levels in patients with heterozygous FH and that the type of the LDLR gene mutation may affect TCHOL and Lp(a) levels in patients with homozygous FH.

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