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PAPER

PATHOLOGY/BIOLOGY

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Sudden Cardiac Death in Young Adults: Environmental Risk Factors and Genetic Aspects of Premature Atherosclerosis*,†

ABSTRACT: Familial hypercholesterolemia (FH) is a genetic disorder that may lead to premature coronary heart disease (CHD) and sudden cardiac death (SCD). Mutations in the *LDLR* or *APOB* genes cause FH. We have screened the *LDLR* and the ligand-binding region of *APOB* genes in 52 cases of SCD. Deceased patients were younger than 40 years of age and were suspected of having FH. The *LDLR* and *APOB* genes were examined via PCR, high-resolution melting, and DNA sequencing. Therein, it was observed that 7.7% of the screened patients exhibited a rare sequence variant in the *LDLR* gene, with 5.7% suspected of being pathogenic mutations. Lipid profiles and genetic testing for FH could be considered when autopsy reveals significant atherosclerosis of the coronary arteries in young adults. First-degree family members are advised to seek medical advice and testing to determine their own risks of atherosclerosis to prevent premature CHD and SCD.

KEYWORDS: *APOB* gene, familial hypercholesterolemia, forensic pathology, forensic sciences, *LDLR* gene, sudden cardiac death

Familial hypercholesterolemia (FH) is the best understood genetic disorder that has been identified to cause premature coronary heart disease (CHD) (1). CHD can present as angina, myocardial infarction (MI), or sudden cardiac death (SCD) (2). Premature CHD, which appears in patients before 40 years of age, can be pathognomonic with FH (1). FH is commonly caused by mutations in the *LDLR* gene, which encodes the LDL-receptor, or in the *APOB* gene, which encodes apolipoprotein B (APOB) (3).

Classical risk factors for premature CHD are well described in the literature (4–11). Premature CHD affects a larger proportion of men than women (4,5). Several studies have found that a family history of ischemic heart disease is an independent risk factor for CHD (6,7) and is more pronounced for early MI (<55 years) (4–6). The association with a positive family history may derive from genetic factors, shared social and physical environments, or a combination of both. Hypercholesterolemia, hypertension, and diabetes are well-known risk factors for CHD (4,5). Genetic factors may act through these known risk factors for premature CHD or through yet unknown mechanisms (6). Possible self-inflicted risk factors must be taken into consideration. According to autopsy studies (8,9), smoking accelerates coronary atherosclerosis, and the Framingham data have also revealed that smoking is a powerful risk factor for MI among adults (<60 years) (10). Furthermore, being overweight or obese is related to higher cardiovascular mortality in young adults (4,11,12), and a higher body mass index (BMI) in childhood is associated with an increased risk of CHD in adulthood, especially in men (11,12). Finally, the steadily increasing abuse of different kinds of substances tends to also play a significant role. Cocaine seems to be a relevant causative agent in younger patients and may cause arrhythmia and cocaine-induced infarction (4,13,14). There are several case reports of MI and SCD as cardiovascular side effects owing to the abuse of anabolic androgenic steroids (AAS) (15–18).

This study is part of a project concerning SCD that includes the genetic examinations of both structural and nonstructural heart diseases. We hypothesize that FH contributes to SCD in young adults and assume that known environmental risk factors could be overrepresented in our study material. The *LDLR* gene and the ligand-binding region of the *APOB* gene were screened in 52 cases of SCD. Individuals who were included in this study were younger than 40 years of age and were suspected of having FH based on autopsy findings of atherosclerosis in the coronary arteries.

Methods

Study Subjects

The study material for the screening of FH consisted of 52 postmortem blood samples from autopsies that were performed at the Department of Forensic Medicine during the period from 1998 to 2007. The blood had been stored at −80°C. The material was selected from our own database via the following criteria: 0 to 40 years of age, cardiovascular (meaning all cardiovascular causes) or an unknown (a few cases) cause of death at autopsy, and

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macroscopically or microscopically confirmed atherosclerosis in the epicardial segments of the coronary arteries according to the American Heart Association criteria, Stadium IV–VIII (19,20). Patient histories were provided from autopsy, police, and, in some cases, from hospital reports. This study was approved by the regional ethical committee of Aarhus County.

Genomic DNA Preparation

Genomic DNA was isolated from whole blood using the QIAamp Mini kit (Qiagen, Copenhagen, Denmark). DNA concentrations were determined using a nanophotometer (Implen; AH diagnostics, Aarhus, Denmark) and diluted to final concentrations of 15 ng/μL for the following PCR procedures.

PCR and High-Resolution Melting (HRM)

PCR was carried out in a total volume of 11 μL using AmpliTaq Gold (Applied Biosystems, Nærum, Denmark) and primers that amplified the coding and flanking intronic regions (primer sequences are available upon request). Amplification was performed in the presence of 1 × LCGreen plus (Idaho Technology, Salt Lake City, UT) using 15 ng of genomic DNA. The PCR-amplification conditions included an initial denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec with primer-annealing and extension at 68°C for 30 sec (except exons 4B and 8, wherein annealing was performed at 61°C). Afterward, a short denaturation/renaturation step was performed to form heteroduplexes (95°C for 30 sec, 25°C for 30 sec, and a final cooling to 10°C). Following PCR, we analyzed the PCR products using HRM on a LightScanner system (Idaho Technology) at a temperature range of 74–98°C. Using this procedure, *LDLR* exons 1, 3, 4a, 5, 6, 9, 14, 16, and 17 and an *APOB* amplicon that covered the exon 26 ligand-binding region were prescreened, and variant samples were further investigated using DNA sequencing.

DNA Sequencing

LDLR exons that produced poor-quality HRM data or that were known to contain polymorphisms were directly sequenced. Following amplification, we purified PCR products using the Illustra GFX PCR Purification kit (GE Healthcare, Hillerød, Denmark). We sequenced the PCR products in both directions using BigDye Terminator, version 1.1 (Applied Biosystems) and separated the ethanol-precipitated fragments using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems).

Bioinformatics Analyses

We aligned sequence traces to the *LDLR* and *APOB* reference sequences (NM_000527 and NM_000384, respectively) using Variant Reporter software (version 1.1; Applied Biosystems). Using the Splice Site Prediction Tool (http://www.fruitfly.org/seq_tools/splice.html), we analyzed the c.1060+10G/C>A variant for potential splicing defects. The PolyPhen webtool (http://genetics.bwh.harvard.edu/pph/index.html) was used to assess the consequences of one novel amino acid variant.

Drug and Alcohol Analyses

Blood, urine, and liver tissue samples were screened in 16 cases for legal and 15 cases for illegal drugs by means of gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry upon liquid–liquid extraction or solid-phase extraction. Subsequently, drug quantification was performed in the blood. Alcohol in the blood and urine was measured in 51 cases via head-space gas chromatography.

Results

The study group consisted of 41 men and 11 women with atherosclerosis in their coronary arteries according to the American Heart Association criteria, Stadium IV–VIII (19,20). The autopsy findings are shown in Table 1. Fourteen cases had microscopically verified acute MI, and nine had microscopically verified previous MI. Three had both acute and previous MI. The rest had minor myocardial changes, mostly mild interstitial fibrosis. Six of the deceased were 15–29 years of age, 10 of the deceased were 30–34 years of age, and 36 were 35–40 years of age. Fifteen cases had a normal BMI (<25), 18 were overweight (BMI of 25–30), 11 were obese class I (BMI of 30.1–34.9), four were obese class II (BMI of 35–40), and three were obese class III (BMI of >40). Thirty-six of the 52 deceased were overweight or obese and only 15 had a normal weight. In 44 of the 52 cases, no information on genetic disposition was provided; five had a positive family history (first-degree relatives) of ischemic heart disease, two had a positive family history of unspecific heart disease, and one had a positive family history of stroke. Fifteen of the 52 deceased were smokers; however, information regarding smoking habits was often not available. One of the 52 cases had a previous clinical history of hypercholesterolemia and hypertension and another had a previous clinical history of hypercholesterolemia, hypertension, and diabetes. Three of the 52 cases had a previous clinical history of hypercholesterolemia, hypertension, or diabetes. Access to antemortem cholesterol concentration was provided in only two cases and was 9 and 6.8 mM.

There was one case of mixed drug abuse with cocaine, amphetamines, and AAS; one of unspecific drug abuse; three of alcohol abuse (exceeding national Danish guidelines; 21 units per week for men and 14 units per week for women); and two of AAS abuse. Information regarding drug abuse was often not available. Alcohol tests were positive in nine cases. Medicine tests were positive in five cases (one SSRI; one lidocain; one olanzapin, orphenadrin, NaSSA, and chlorprothixene; one chlorprothixene; and one midazolam), and drug tests were positive of cannabis in two cases.

TABLE 1—*Autopsy findings.*

Autopsy Findings	Yes	No*	Unknown†
Atherosclerosis, overall	52/52	0/52	0/52
Microscopically abnormal heart, overall	50/52	2/52‡	0/52
Previous infarction, of the 50 positive	12/50	34/50	4/50
Present infarction, of the 50 positive	17/50	29/50	4/50
Postmortem biochemistry, overall	7/52	45/52	0/52
Blood test diabetes positive, of the 7 positive	3/7	4/7	0/7
Alcohol and drugs, overall	52/52	0/52	0/52
Alcohol, of the 52	51/52	1/52§	0/52
positive, of the 51 positive	9/51	42/51	0/51
Medicine, of the 52	16/52	36/52§	0/52
positive, of the 16 positive	5/16	11/16	0/16
Drug abuse, of the 52	15/52	37/52§	0/52
positive (cannabis), of the 15 positive	2/15	13/15	0/52

*Confirmed negative information.
†No information.
‡Microscopically normal heart.
§Tests for alcohol or drugs were not performed.

TABLE 2—*Rare sequence variants in the* LDLR *gene.*

Nucleotide Change	Protein Change	Exon/ Intron	Reference
c.259T>G	p.W66G	Exon 3	Jensen et al. (21) Leitersdorf et al. (22)
c.1060+10G/C>A	Splicing defect	Intron 7	Amsellem et al. (25) Damgaard et al. (24)
c.1324T>C	p.Y421H	Exon 9	Widhalm et al. (23)
c.1431C>G	p.D456E	Exon 10	This study

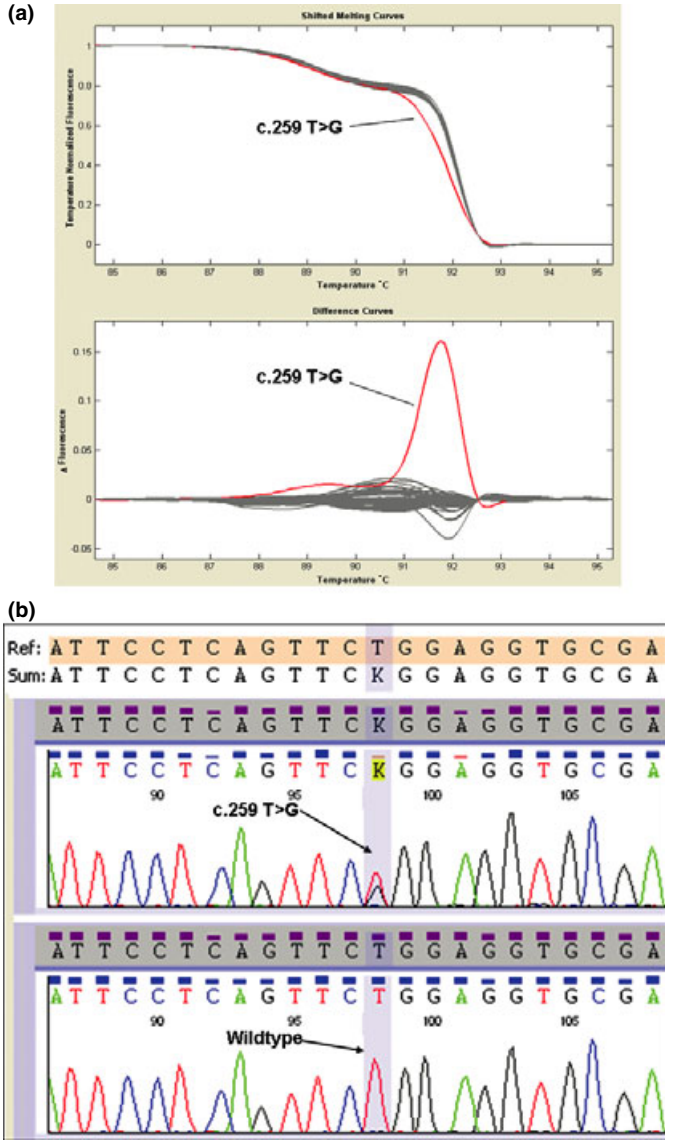

FIG. 1—*Results from high resolution melting (a) and DNA sequencing (b) showing a rare sequence variant in exon 3, c.259T>G; p.W66G compared to wild type. (a) The rare sequence variant in exon 3, c.259T>G; p.W66G (red line) has a different melting curve and melts at a lower temperature compared to wildtype (gray lines) because of heteroduplex formation. (b) A comparison of the sequence of a rare sequence variant in exon 3, c.259T>G; p.W66G with a reference sequence (upper), and a sequence from a wild type (lower). A T nucleotide is replaced by a G nucleotide in the rare sequence variant in exon 3, c.259T>G; p.W66G.*

We found that four of the 52 deceased were heterozygous for a rare sequence variant in the *LDLR* gene, as summarized in Table 2. The mutation in exon 3 (c.259T>G; p.W66G) has previously been described in Danish FH patients and is presumed to be disease causing (21,22) (Fig. 1). The mutation in exon 9 (c.1324T>C; p.Y421H) has been reported by Widhalm et al. (23) and is presumed to be disease causing. The mutation in intron 7 (c.1060+10G/C>A) has been previously described (24,25), and using the Splice Site Prediction Tool, we found that this mutation probably introduces a cryptic splice site and, therefore, the inclusion of the intron 7 sequence. The reading frame is predicted to shift, and a premature stop codon is introduced at amino acid 332 (p.E332fsX352).The rare sequence variant in exon 10 (c.1431C>G; p.D456E) is not present in the *LDLR* mutation database (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=LDLR) (26). The PolyPhen webtool predicted the p.D456E variant to be benign. Polymorphisms and benign variants were found in *LDLR* exons 2, 8, 10, 11, 12, 13, 15 and 17 (26,27), as summarized in Table 3. In 94% of the samples, PCR and HRM or sequencing were successful in all exons. Repeated PCR and DNA sequencing of eight, three, and one exon failed in three samples.

The clinical histories and examinations of the four deceased patients with rare sequence variants in the *LDLR* gene revealed several well-known risk factors: a positive family history of CHD, hypercholesterolemia, hypertension, and being overweight or obese. In addition, two of the deceased patients were or had been using AAS. All four had macroscopically or microscopically severe atherosclerosis in the coronary arteries and one had both acute and previous infarction. The presentations of the four cases are summarized in Table 4.

Discussion

We found that four cases (7.7%) had a rare sequence variant in the *LDLR* gene, of which three (5.7%) are suspected to be pathogenic mutations. This agrees with the findings of Goldstein et al. (28) who estimate the prevalence of FH to be 7.6% in 500 survivors of MI. To our knowledge, few studies, which are primarily case reports (29), have examined postmortem blood samples for disease-causing mutations in the *LDLR* and *APOB* genes. Vuorio et al. (30) studied a total of 149 deceased patients who had suffered early (≤50 years), unexpected death because of CHD. Molecularly defined FH was present in 2% of the deceased with CHD and in 3% of the 67 deceased with demonstrable MI.

TABLE 3—*Polymorphisms and benign rare sequence variants.*

Nucleotide Change	Protein Change	Exon/ Intron	dbSNP/ Reference
c.186G>A		2	Rs55958434
c.81C>T		2	Rs2228671
c.1060+10G>C		7	Rs12710260
c.1061-8T>C		8	Rs72658861
c.1171G>A	p. A370T	8	Rs11669576
c.1413G>A		10	Rs5930
c.1617C>T		11	Rs5929
c.1725C>G		12	Rs1799898
c.1773T>C		12	Rs688
c.1920C>T		13	Rs5926
c.1959C>T		13	Rs5925
c.2177C>T	p. T705I	15	Heath et al. (33)
c.2232G>A		15	This study
c.829G>A		17	Pereira et al. (34)

The polymorphisms and benign rare sequence variants that were identified in this cohort according to the *LDLR* mutation database (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=LDLR) (26) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_blastByOrg.cgi) (27).

TABLE 4—*Risk factors for the four cases with rare sequence variants in the* LDLR *gene. Cause of death, risk factors, and autopsy findings are summarized for the four cases with rare sequence variants in the* LDLR *gene.*

	Case 1 Exon 3, c.259T>G; p.W66G	Case 2 Intron 7, c.1060+10G/C>A, splicing	Case 3 Exon 9, c.1324T>C; p.Y421H	Case 4 Exon 10, c.1431C>G; p.D456E
Age	31	31	22	35
Gender	Male	Male	Male	Male
BMI	26	22	32	28
Cause of death	Heart failure, myocardial hypertrophy and fibrosis, atherosclerosis	Myocardial infarction	Atherosclerosis, myocardial hypertrophy, abuse of AAS	Thrombosis of the coronary artery
Genetic disposition	Mother, infarction; Brother, hypercholesterolemia	Unknown*	Unknown*	Unknown*
Hypercholesterolemia total cholesterol	≈ 9mm	No†	No†	No†
Hypertension	140/90	150/100	No†	No†
Other disease/symptoms	No†	Chest pain	No†	Previous infarction Chest pain
Alcohol and drug abuse	History of AAS abuse *Alcohol and drug abuse tests*,‡ negative	No information of drug abuse available *Alcohol test*,‡ negative	History of AAS abuse *Alcohol and drug abuse tests*,‡ negative	No information of drug abuse available *Alcohol and drug abuse tests*,‡ negative
Coronary artery	Atherosclerosis stadium VI–VIII, macroscopically	Atherosclerosis stadium VI–VIII, macro- and microscopically	Atherosclerosis stadium VI–VIII, microscopically	Atherosclerosis stadium VI–VIII, macro- and microscopically
Previous infarction	No	Yes	No	Unknown, because of purification.
Present infarction	No	Yes	No	

AAS, anabolic androgenic steroids.
*No information.
†Confirmed negative information.
‡Tests for AAS were not performed.

The rare sequence variant p.D456E that was found in this study is novel; however, in codon 456, a p.D456N substitution (aspartic acid > asparagine) has been previously reported (26). The PolyPhen webtool predicted the p.D456E variant to be benign, although the amino acid is conserved in position 456 in an alignment of the *LDLR* amino acid sequence in several species (data not shown), indicating that this evolutionally conserved amino acid is important for proper receptor function. The presumed splicing defect of intron 7, c.1060+10G/C>A, is predicted to introduce a cryptic splice site that leads to a shift in the reading frame and, consequently, a premature stop codon. This mutation has previously been reported (24,25); however, no functional data confirming this variant's disease-causing effect have been reported. Functional experiments of this variant would provide important information, as has been previously demonstrated for a range of presumed splicing mutations in the *LDLR* gene by Holla et al. (31); however, in our study, biologically active material for functional testing was not available. It was not possible to investigate co-segregation of the mutations in family members at this stage of the project. The clinical history and examination of the four deceased patients with rare sequence variants in the *LDLR* gene reveal several well-known risk factors (a positive family history of CHD, hypercholesterolemia, hypertension, and being overweight or obese). In addition, two of the deceased were or had been using AAS.

Obviously, microscopically confirmed atherosclerosis in the coronary arteries, and infarction in heart tissue is an important tool for diagnosing macroscopically suspected atherosclerosis and infarction. A postmortem lipid profile (32) from the deceased can provide important information for first-degree family members concerning their own risks of atherosclerosis; however, this information cannot clarify the presence of a genetic factor for FH. Attention must be paid to drug abuse, especially the abuse of AAS, because two of the deceased patients with rare sequence variants were abusing AAS. AAS potentiate several physiological changes that can be key pathological factors for AAS-associated MI and are known to have an atherogenic influence on the serum lipid profile (15–18).

There are several risk factors for premature CHD. For this reason, we cannot exclude that coronary disease may be the final result of the concomitant effects of the rare sequence variants and other risk factors. Of the study group, 92% had no rare sequence variant in the *LDLR* gene but presented with SCD and atherosclerosis in the coronary arteries. These subjects had different known risk factors for premature CHD, and their cause of death is most probably a combination of the presence of strong risk factors, environmental conditions, and the contribution of polygenic factors rather than the presence of a sole monogenic disorder.

Mutations in the *LDLR* gene are an important risk factor for premature CHD, and genetic testing for FH could be considered when autopsy reveals moderate or severe atherosclerosis of the coronary arteries in young adults. Family members with a history of FH are at high risk, and it is important for them to avoid other modifiable risk factors and to reduce exposure to environmental risk factors. This study benefits from having unique biological material from young adults who have died from SCD in a 10-year period and offers the possibility to study the potential role of FH in sudden, unexpected, and early cardiac death. The limitations of this study include inhomogeneous epidemiological data, a lack of postmortem lipid profiles, and the inability to functionally test the new sequence variants that were found in this study. Biologically active material, such as tendon fibroblasts, could be an option for functional testing.

Conclusions

In this SCD cohort, 7.7% had a rare sequence variant in the *LDLR* gene, of which 5.7% were suspected to be pathogenic mutations. Lipid profiles and genetic testing for FH could be considered when autopsy reveals moderate or severe atherosclerosis of the coronary arteries in young adults. The findings of significant coronary

artery disease in a person under 40 years of age are highly concerning and may suggest that lifestyle factors, environmental factors, and genetic factors (including FH) may contribute to death. First-degree family members are strongly advised to seek medical advice and testing to determine their own risks of atherosclerosis so as to prevent ischemic heart disease and SCD.

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