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Short Communication

DOI:10.2478/rrlm-2021-0025

Genetic Diagnostic Approaches in Familial Hypercholesterolemia Evaluation

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Abstract

Familial hypercholesterolemia (FH) manifested as atherosclerosis is a major cause of coronary heart disease. Different scoring systems based on clinical and paraclinical data are currently used, but the FH diagnosis should be made only in the presence of the causative genetic defect. In the present study, 12 symptomatic (previously diagnosed with atherosclerosis) and asymptomatic family members were investigated. Serum lipids were measured using commercial reagents. A genetic investigation was performed by Sanger sequencing using commercial reagents and custom primers, while copy number variations and a selected set of 40 point mutations were evaluated using in vitro diagnostic medical devices. For the investigated patients, serum lipids were within the reference range, due to the fact that the subjects were following lipid-lowering therapy, and smoking was the only identifiable additional risk factor. Four benign exon variants and three intron variants situated within the low-density lipoprotein cholesterol receptor gene were identified by Sanger sequencing. No copy number variations and none of the 40 investigated point mutations were determined. Although independently considered benign, the combined effect of the identified genetic conditions could be pathogenic under the influence of additional risk factors. Even in the presence of a diagnosis made using clinical scores, the molecular diagnosis is often challenging, attesting to the complexity of FH genetic etiology.

Keywords: *familial hypercholesterolemia, atherosclerosis, low-density lipoprotein cholesterol, LDL receptor gene*

Received: 30th June 2021; Accepted: 14th July 2021; Published: 16th July 2021

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Introduction

Coronary heart disease (CHD) is the worldwide leading cause of death, being mainly associated with the presence of atherosclerosis (1). With an estimated incidence of 1:200 individuals (2), Familial hypercholesterolemia (FH) is a major cause of atherosclerosis, and unfortunately, it is still regarded as an "underdiagnosed" and often "undertreated" pathology (1).

Lifelong elevated low-density lipoprotein cholesterol (LDL-C) levels are characteristic for untreated FH patients (1). They have a 30% to 50% risk of suffering a potentially fatal atherosclerotic event by the age of 50 or 60 in the case of men or women, respectively (2).

Different scoring systems, such as the Dutch Lipid Clinic Network (DLCN), Simon Broome Register, and MedPed, all primarily based on LDL-C or total cholesterol (CHOL) levels, cutaneous stigmata plus personal and family medical history are useful in assessing the CHD risk and making a clinical diagnosis (3). However, the FH diagnosis should be made only in the presence of the causative genetic defect (3). This is most often a point mutation in either the LDL receptor (*LDLR*), apolipoprotein B100 (*APOB*), or subtilisin-kexin type 9 proprotein convertase (PCSK9) genes (4). There is also the polygenic aspect, in which several cholesterol-rising gene modifications contribute to FH (1) occurrence, and also the disease being caused by copy number variations (CNVs).

This research aimed to identify a causative FH variant within a symptomatic index case, and subsequently perform "cascade screening" (5) for the symptomatic and asymptomatic family members, using multiple genetic diagnostic approaches.

Material and methods

The study was approved by the Ethics Committee of the "G.E. Palade" University of Medicine, Pharmacy, Science and Technology of Tirgu Mures (73/14.04.2017) and complies with the Declaration of Helsinki.

Twelve family members were investigated. The index case and the other symptomatic adults were previously diagnosed with CHD by angiography before the age of 50 and were following lipid-lowering therapy. Children were asymptomatic at enrollment and were included to assess their CHD recurrence risk. DLCN criteria for FH diagnosis were used, however, the score mainly reflected the CHD status of the affected subjects, since serum lipids were influenced by statins and the underlying genetic cause was not identified at the moment of subject enrollment.

Serum lipids, namely CHOL, triglycerides (TRIG), directly measured LDL-C, and high-density lipoprotein cholesterol (HDL-C) were investigated using a Cobas Integra 400plus (Hoffmann–La Roche, Switzerland) system and dedicated reagents. Pretreatment LDL-C levels were estimated based on the administered statin dose and current direct LDL-C level, using a dedicated calculator (6).

DNA was extracted from K3EDTA anticoagulated whole blood with the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). CNVs within the *LDLR* exons were assessed using an in vitro diagnostic medical device (IVD) by Multiplex ligation-dependent probe amplification (MLPA) technique using the SALSA MLPA Probemix P062 LDLR kit (MRC Holland, The Netherlands) according to the product insert indications.

A panel of 40 pathogenic variants in the three main FH-causing genes, *LDLR*, *APOB*, and *PCSK9* was investigated using an IVD test, namely the Familial Hypercholesterolemia Arrays I&II (Randox Laboratories, United Kingdom), conforming to the manufacturer's protocol.

For Sanger sequencing, amplification was performed on a Mastercycler nexus GSX1 (Ep-

pendorf, Germany), and capillary electrophoresis on a 3500xLDx Genetic Analyzer (Applied Biosystems, USA), following preparation with Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and BigDye XTerminator Purification Kit (Thermo Fisher Scientific, USA). Variant Reporter v1.1 (Applied Biosystems, USA) software was used for reference-based analysis, according to the GRCh38.p13 genome assembly. All primer pairs (available upon request) were designed "de novo" with Primer3 (7) software for the NM_000527.5 transcript ID, to encompass the 18 exons of *LDLR* and the corresponding intron-exon junctions. Primer anneal regions were verified for complementarity and single nucleotide polymorphisms presence with SNPCheck (8) and exons' existence within the generated amplicons was confirmed with UCSC In-Silico PCR (9). Amplicon length was verified both in-silico (10) and using 2% agarose gel electrophoresis. Sanger sequencing was used to investigate all *LDLR* exons for the index case, while "cascade screening" was employed for all other subjects.

Results

Lipid serum levels in the adults diagnosed with CHD are shown in Table 1. All these subjects were included in the "probable FH" (6-8 points) category, following the application of the DLCN score at the moment of their enrollment. From the traditional CHD risk factors, smoking was present in patients II/1, II/2, II/3, while I/1 ceased smoking approximately 10 years before the CHD diagnosis.

No copy number variations within the *LDLR* gene and none of the 40 point mutations within the three main FH-causing genes were identified using the methods described above in the DNA samples coming from the adults previously diagnosed with CHD.

Analyzing the DNA sample from the index case, we have identified four variants situated in the *LDLR* exons, namely rs11669576 in exon 8, rs5930 in exon 10, rs5927 in exon 15, and rs1359242501 in exon 18. We have also identified three intron variants, namely rs3745677 upstream of exon 2, rs2738447, and rs7259278 downstream from exon 12. The same exon variants were identified in other family members, as shown in Figure 1.

Discussion

LDLR is the main gene associated with FH determinism, and therefore it has been intensely studied. So far, over 3500 (10) variants have been reported in this gene, several being considered pathogenic. Similarly, "traditional" FH risk factors, such as smoking have been well documented (11), as well as its possible long-lasting effect on the patients' CHD risk after the habit was discontinued (12).

Studies have often indicated dissimilar results regarding the identification rate, as well as the reported variants, mainly due to the investigated population selection criteria or ethnic/regional clustering of molecular changes. For instance, two studies investigated the 18 exons of *LDLR* by Sanger sequencing for patients diagnosed using clinical criteria scores and reported three

Table 1. Serum lipid levels in the adults diagnosed with CHD.

Case no. (from the family tree)	CHOL (mg/dl)	TRIG (mg/dl)	HDL-C (mg/dl)	direct LDL-C (mg/dl)	estimated(6) LDL-C (mg/dl)
II/1	134.3	94.5	70.5	67.6	182.6
II/2	140.4	95.2	55.8	78.1	132.7
II/3	214.6	148.5	61.2	157.4	267.6
I/1	173.3	255.6	37.4	101.9	275.1

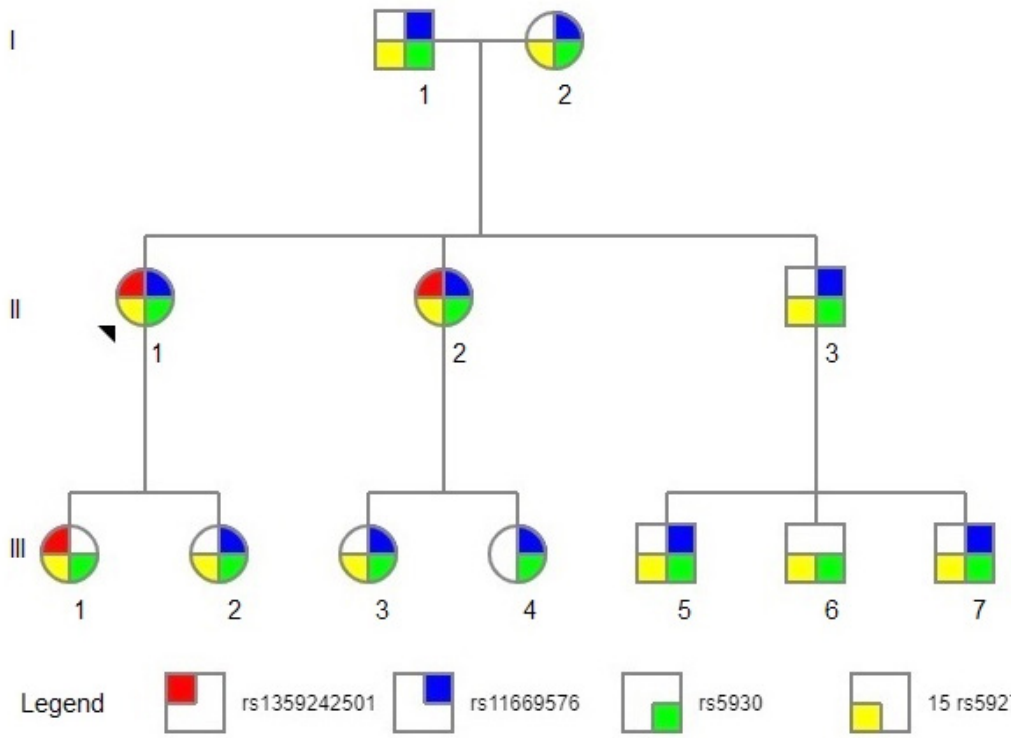

Fig. 1. The family tree, displaying the identified exon variants. Index case - patient II/1

(from 24 investigated families) (13) and five (from 27 unrelated investigated subjects) (14) pathogenic variants, respectively. Neither reported the variants identified in our patients, possibly due to the large spectrum of *LDLR* variants as well as populational and inter-individual differences. However, the majority of *LDLR* variants identified in the index case and her family were reported by other authors, mainly in Next-Generation Sequencing investigations, often followed by Sanger sequencing for confirmation.

Currently, rs1359242501 (Minor Allele Frequency <0.01) found in *LDLR* exon 18 is considered a three prime untranslated region (3′-UTR) variant, and its clinical significance is not mentioned at the moment (15). No further information about this variant is to be found online using research-specific (16) or general (17) search engines. At this point, we can only speculate about the contribution of this variant to FH occurrence. Further studies are needed to elucidate this modification's possible clinical significance, knowing that 3′-UTR variants can influence gene expression after the transcription process.

Variant rs11669576 is considered to be "benign" or "likely benign" (18) and there are a number of scientific papers to support this classification (18). On the other hand, the presence of rs11669576 (missense variant) together with rs5930, also found in our subjects, was associated with Alzheimer's disease in a cohort of patients previously diagnosed with hypercholesterolemia (19).

Determining a synonymous variant, rs5930 is classified as "benign" in ClinVar (20). Studies such as that of Ekrami et al. reinforce its classification. The authors enrolled 80 FH subjects based on the Simon Broome criteria and performed Sanger sequencing in four *LDLR* exons (namely 3, 4, 9, and 10). They reported eight

variants, and among them, the benign rs5930 as well as the pathogenic C95W and D139H (21).
A study on 80 hypercholesterolemia patients investigated all 18 exons of *LDLR*, as well as the R3500Q mutation from *APOB* using Single-strand conformational polymorphism analysis followed by Sanger sequencing for confirmation. The results pointed out eighteen *LDLR* variants, from which twelve new, besides the benign rs5927 (22). Data available in genetic databases also classify rs5927 as a "benign" variant (23).
In our study, all intron variants identified in the index case DNA sample were disregarded in the other subjects since all introns are eliminated through splicing during RNA maturation. Nonetheless, recent studies demonstrate that even intron variants (admittedly those within the splicing branchpoint/splicing site can interfere with the splicing process, causing intron inclusions or exon skipping, primarily if the affected nucleotide is adenine (24). In our study, rs2738447 involved an adenine substitution, but a recent phenome-wide association study on over 60,000 subjects classified this variant as "benign" (25).
The definitive FH diagnosis in our patients cannot be ruled out in the investigated patients. Even though the identified variants were previously not described or were individually considered "benign", we must also take into account the possible additive effect of all these variants (the haplotype), together with the presence of the traditional risk factor, smoking, (the epigenetic factor) along with the evocative clinical and paraclinical features in the final evaluation of these cases.
Regardless of the advances in genetic diagnosis techniques, the molecular diagnosis of FH is often difficult to be made, mainly due to the large spectrum of potentially causing genetic defects. Nonetheless, recent research reiterates the need for a clinical suspicion or diagnosis to be followed by treatment (1), even in cases where the causal gene defect is not identified (2), although new classifications regarding hypercholesterolemia are being suggested (3).
This is the first report regarding investigation of a large family with hypercholesterolemia phenotype by using different genetic methods. No pathogenic variants were identified although we performed MLPA analysis, we used Familial Hypercholesterolemia Arrays I&II and carried out the sequencing of the 18 exons of *LDLR* gene and the corresponding intron-exon junctions. A comprehensive NGS-based genetic testing of the genes involved in lipid metabolism would be recommended as it is reported that it identifies substantially more people with FH. NGS analysis by using NGS panels may allow investigation of classic FH genes (egs *LDLR, APOB*, or *PCSK9*) but also of minor familial hypercholesterolemia genes (egs. *LDLRAP1, ABCG5, ABCG8, LIPA, APOE*, etc). Recently, Reeskamp at al reported a frequency of 14.9% of rare pathogenic variant in *LDLR*, *APOB* or *PCSK9* in suspected FH patients and showed that 4.8% of the patients with FH phenotype without classic FH genes mutation carried a variant in one of the minor FH genes (26).

Conclusion

Although independently considered benign, the combined effect of the identified genetic conditions could be pathogenic under the influence of additional lifestyle risk factors. Even in the presence of a diagnosis made using clinical scores, the molecular diagnosis is often challenging, attesting to the complexity of FH genetic etiology. Nonetheless, patients should follow treatment and the genetic cause has to be further investigated, to possibly allow for personalized treatment and cascade screening to assess the CHD risk for their descendants.

Abbreviations

3′-UTR - three prime untranslated region
APOB - apolipoprotein B100 gene

CHD - coronary heart disease
CHOL - total cholesterol
DLCN - Dutch Lipid Clinic Network
IVD - in vitro diagnostic medical device
FH - familial hypercholesterolemia
HDL-C - high-density lipoprotein cholesterol
LDL-C - low-density lipoprotein cholesterol
LDLR - LDL receptor gene
MLPA - Multiplex ligation-dependent probe amplification
PCSK9 - subtilisin-kexin type 9 proprotein convertase gene
TRIG – triglycerides

Authors' contributions

M.V. designed the study, methodology, analyzed samples, analyzed/interpreted data, drafted the manuscript; B.C. analyzed/interpreted data, critically revised the manuscript; D.M conceptualization, designed the study, resources, evaluated the patients/collected clinical samples/data, validated results, edited / revised the manuscript, final aproval.

Conflict-of-Interest

The authors declare no conflict of interest.

Acknowledgements

This work was partially supported by PN-III-P2-2.1-PED-2016-0734 research grant.

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