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RESEARCH ARTICLE

The utility of MLPA in Familial Hypercholesterolemia diagnosis

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Background: Familial Hypercholesterolemia (FH) is an inherited disease, associated with an increased risk of atherosclerosis, manifested clinically as premature coronary heart disease. FH is biochemically characterized by increased Cholesterol and Low-density Lipoprotein Cholesterol serum levels. The diagnosis is often made using clinical scores however, the definitive FH diagnosis should point out the underlying molecular change, which can be: a point mutation within the three major genes, a number of single nucleotide polymorphisms determining the polygenic etiology, or copy number variations in the Low-density lipoprotein receptor gene. **Objective**: In the present study we investigated copy number variations as a possible etiological factor for FH in a cohort of patients with documented premature coronary heart disease. **Methods**: The study population consisted of 150 patients with premature coronary heart disease documented by angiography, all being under lipid-lowering therapy, and 20 apparently healthy controls. Serum lipids were assessed using the Cobas Integra 400 plus and commercial reagents. Copy number variations were evaluated with the SALSA MLPA Probemix P062 LDLR kit. **Results**: Cholesterol, Triglycerides, Low-density Lipoprotein Cholesterol and High-density Lipoprotein Cholesterol showed no difference between patients and controls. No copy number variations were detected in the investigated regions, namely all 18 exons and the promoter region of the Low-density lipoprotein receptor gene. **Conclusions**: Even in the presence of negative results, the Familial Hypercholesterolemia genetic diagnosis has to be further pursued in the presence of a clinical diagnosis, as the identification of the molecular etiology may bring additional clinical and therapeutical benefits, as well as open the possibility for "cascade screening".

Keywords: familial hypercholesterolemia, atherosclerosis, premature coronary heart disease, low-density lipoprotein receptor

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Introduction

Familial Hypercholesterolemia (FH) is an inherited disease, associated with an increased risk of atherosclerosis, manifested clinically as coronary heart disease (CHD) or stroke. From a biochemical point of view, FH is characterized by increased Cholesterol (Chol) and Low-density Lipoprotein Cholesterol (LDL-Chol) serum levels, with patients displaying normal Triglycerides (Trig) levels [1]. The main consequence of FH is the occurrence of premature CHD, with a third up to one-half of untreated patients suffering a potentially lethal cardiac atherosclerotic event below the age of 55 for men and 60 in the case of women [2,3].

The diagnosis is often made using clinical scores, such as the Simon Broome, Dutch Lipid Clinic Network (DLCN) or Make Early Diagnosis to Prevent Early Death (MEDPED), that rely on clinical and biochemical findings [4]. However, since it's primarily a genetic condition, the definitive FH diagnosis should point out the underlying molecular change [4] which can be: a) a point mutation within the three major genes (accounting for up to 80% of all genetically diagnosed FH cases [5]): Low-density lipoprotein receptor (LDLR) [6], Apolipoprotein B (APOB), and Proprotein convertase subtilisin/kexin type 9 (PCSK9), b) a number of single nucleotide polymorphisms determining the polygenic etiology (in approximately 20% [5] of FH cases, with point mutations in genes such as the LDL-receptor adaptor protein 1, Apolipoprotein E, Patatin-like phospholipase-domain-containing family, Lysosomal acid lipase, Signal-transducing adaptor protein family 1 [4], Cadherin EGF LAG seven-pass G-type receptor 2 and ATP binding cassette subfamily G member 8), or c) copy number variations (CNV) (determining FH in 0-10% of investigated cases) [4].

In trying to identify a genetic FH cause, the LDLR gene is primarily studied by numerous investigators, given the fact that it harbors a large number of identified variants, many of which are considered pathogenic [6]. Copy number variations in this gene are described to be involved in FH etiology, but their frequency differs in various populations, as reported by several authors [4]. Multiplex ligation-dependent probe amplification (MLPA) is considered to be the "gold standard" [7] technique in identifying small deletions and duplications, known as CNVs. One advantage of MLPA is that numerous available kits analyze CNVs' occurrence in different pathologies, such as the SALSA MLPA Probemix P062 LDLR, an in vitro diagnostic [8] tool able to investigate CNVs in all 18 exons of LDLR, as well as its promoter region.

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The present study aimed to investigate whether copy number variations were an etiological factor for FH in a cohort of patients with documented premature CHD and to compare the frequency of the identified structural aberrations with data from other populations since there is no incidence reported in the investigated population.

Methods

The study population consisted of 150 patients and 20 controls, all above 18 years of age, coming from the Central part of Romania. Enrollment criteria for the patients were: a) coronary heart disease documented by angiography, b) CHD diagnosis ≤55 years old for men, ≤60 years old for women. For the controls, the inclusion criteria consisted of: a) lack of any documented cardiovascular disease, b) no lipid-lowering therapy, c) LDL-Chol within the reference range, d) age between 18-55 years old. Exclusion criteria for the whole group were the presence of any metabolic disease that could be related to lipid modifications, such as chronic thyroid, kidney, or liver disorders, as well as diabetes.

All participants in the present study signed informed consent prior to their enrollment. The methodology was approved by the Ethics Committee of the George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Tîrgu Mureș and complied with the World Medical Association's Declaration of Helsinki.

Since patients had a prior CHD diagnosis, they were all following lipid-lowering therapy, as prescribed by their consulting cardiologist.

Two blood samples were taken from each person included in this study. One was collected in a vacuum tube containing a separating gel to investigate serum lipids. Isolation of genomic DNA was performed from the leukocytes of peripheral blood collected in a vacuum tube containing ethylenediaminetetraacetic acid, using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, USA), as indicated by the manufacturer.

Serum lipids, namely Chol, Trig, LDL-Chol, and HDL-Chol were assessed with the Cobas Integra 400 plus (Hoffmann–La Roche, Switzerland) system, using reagents validated for clinical diagnosis, according to the products' inserts.

The SALSA MLPA Probemix P062 LDLR kit (MRC Holland, Amsterdam, the Netherlands) was used to analyze copy number variations. Samples were prepared according to the manufacture's protocol, using a Mastercycler Nexus GSX1 PCR system (Eppendorf, Hamburg, Germany) for amplification and a 3500xL Dx Genetic Analyzer system (Applied Biosystems, Foster City, California, United States) for fragment analysis while Coffalyser.NET software (version v.140721,1958, MRC Holland, Amsterdam, the Netherlands) allowed the interpretation of the results, considering a reference range between 0.7-1.3 copies.

MedCalc software [9] was used to compare the difference between the observed means in the two groups. A p-value<0.05 was considered statistically significant.

Results

Serum lipids within the two studied lots are shown in Table I. The MLPA analysis in the patients and control groups displayed no modifications regarding copy number variations within the investigated regions of LDLR. The electrophoretic pattern of a negative patient's sample is illustrated in Figure 1 (A and B).

Discussion

FH is often an underdiagnosed disease, displaying an estimated prevalence of 1:220 in various populations [3]. Although genetic testing has proven its utility in making the final diagnosis, it is not being widely used worldwide [10]. Additionally, even if molecular techniques are used, identification of the genetic cause in FH is often challenging, given the extensive number of point mutations within LDLR [4], as well as within the other genes involved, such as APOB, PCSK9, and Low-Density Lipoprotein Receptor Adaptor Protein 1 to name just the most significant. Added to these, the polygenic etiology and CNVs in the affected gene contribute to the complex genetic spectrum of FH, although their incidence in the occurrence mechanism is lower [4]. These aspects may explain why a large proportion of patients may remain without an identifiable molecular cause, even in the presence of a diagnosis made according to the clinical scores [11].

In our study cohort, the patients' diagnosis was based on clinical findings, all being investigated by angiography

Table I. Serum lipid levels in the study cohort.

	Patients (n=150) (men n=120; women n=30)	Controls (n=20) (men n=12; women n=8)	p-value
Cholesterol (mg/dl)	170.9±51.2 (73-460.7)	184.9±45.5 (98.8-265.3)	
Men	169.7±51.6 (73-460.7)	180.4±38 (98.8-233.6)	0.24
Women	175.6±50 (92.6-300.21)	191.8±57 (104.4-265.3)	
Triglycerides (mg/dl)	168.1±111.3 (24.9-852.8)	153.5±107.4 (64.9-495.3)	
Men	175.8±118.9 (56.9-852.8)	157±83.2 (64.9-317.4)	0.58
Women	137.2±66.9 (24.9-351.9)	148.2±142.7 (69-495.3)	
LDL-Cholesterol (mg/dl)	112.9±52.1 (32.2-424.1)	124.7±39.3 (50.2-196.9)	
Men	111.7±52.1 (32.2-424.1)	123±34.3 (50.2-168.2)	0.33
Women	118±52.6 (44.2-249.6)	127.3±48.2 (58.8-196.9)	
HDL-Cholesterol (mg/dl)	41.6±12.1 (11.7-73.8)	46.4±22.4 (28.9-85)	
Men	39.9±11.3 (11.7-70.24)	41.5±10.4 (28.9-59.6)	0.1
Women	48.5±12.8 (19.8-73.8)	53.7.4±18.2 (33.8-85)	

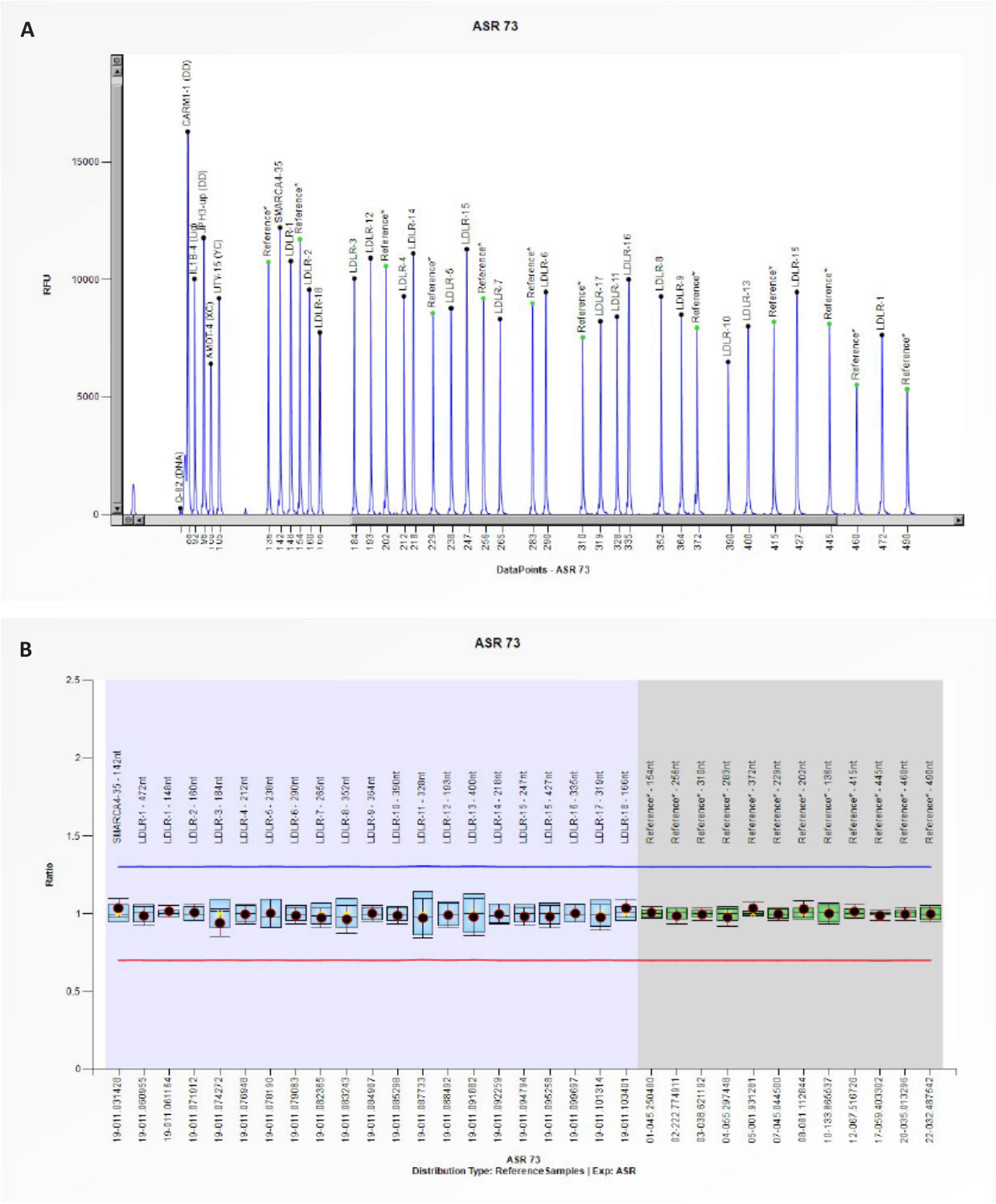

Fig. 1. A. Electrophoretic pattern of a negative sample investigated with SALSA MLPA Probemix P062 LDLR. Vertical axis: RFU-relative fluorescence units; horizontal axis: length (in base pairs) of the investigated fragment. B Electrophoretic pattern of a negative sample investigated with SALSA MLPA Probemix P062 LDLR. Vertical axis: Ratio, copy number reference range between 0.7-1.3; horizontal axis: chromosomal position of the investigated fragment.

secondary to a CHD event. Given the characteristics and inclusion criteria of our study group, serum lipid levels before the CHD diagnosis could not be evaluated. Since all patients were following lipid-lowering therapy when enrolled in the present study, it was to no surprise [12] that Chol, Trig, and LDL-Chol showed no statistical difference between patients and controls. Similarly, HDL-Chol, the main protector against atherosclerosis [13] was lower in patients, compared to controls. This was again in line with the results published by other authors since it has been recognized that statin therapy does not significantly increase HDL-Cholesterol serum levels [14].

MLPA is frequently regarded as the method of choice for the investigation of CNVs, because it has a superior

resolution compared to other cytogenetic techniques, such as fluorescent in situ hybridization, array comparative genomic hybridization, or karyotyping [15]. On the other hand, new molecular genetic techniques, such as next-generation sequencing (NGS) [16] combined with bioinformatics have been used to investigate CNV's involvement in FH etiology [17], although the expense difference between the two approaches is rather significant, and NGS panels are still marked as "research use only" [18].

To date, copy number variations (manifested as loss-of-function deletions) associated with FH determinism were only described in the LDLR gene [4], and their prevalence is significantly lower, at least in comparison to FH caused by point mutations in the three major genes, LDLR, APOB, and PCSK9 [19]. The reduced incidence, as well as a probable uneven distribution between different populations, may be causing the disparate results published regarding CNVs in FH. Similar to our findings, other authors have failed to identify CNVs in LDLR, in the presence of a clinical diagnosis, even though the study of Pecin et al. described the presence of several point mutations in their studied population [20]. Contrariwise, other authors have reported the involvement of LDLR deletions in FH etiology in their study cohorts [21-23], albeit in a reduced number of subjects.

Our study has illustrated that copy number variations do not play an etiological role in FH determinism in our investigated population. Further testing, including screening for the most common point mutations in the three major genes, or even sequencing (Sanger or NGS) will be necessary to make a genetic diagnosis for these patients, even though they were all diagnosed based on clinical criteria, as well as being treated, according to the indications of their curing cardiologist, and current advice [24]. Finding the causal mutation remains a final goal, as it may allow for personalized medication [25] for these patients, as well as "cascade screening" [26] for their family members, in order to include them in CHD prevention programs.

Conclusion

Even in the presence of negative results, the Familial Hypercholesterolemia genetic diagnosis has to be further pursued in the presence of a clinical diagnosis. Although burdensome, identification of the molecular etiology will bring additional information to the affected patients, their families, as well as curing physicians.

Authors' contribution

VM - Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing
AH - Formal Analysis, Investigation, Methodology, Writing – review & editing
LD - Formal Analysis, Investigation, Methodology, Resources, Writing – review & editing
LH - Data curation, Methodology, Resources, Writing – original draft, Writing – review & editing
BM - Formal Analysis, Resources, Software, Writing – original draft, Writing – review & editing
DM - Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Conflict of interest

None to declare.

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