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Original paper

NGS data validated by Sanger sequencing reveal a puzzling small deletion of MYBPC3 gene associated with hypertrophic cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) has a special place among genetic cardiomyopathies, being one of the main causes of sudden death in young patients, mainly in performance athletes. Herein we report a deletion in the myosin binding protein C (MYBPC3) gene identified in a female patient affected by HCM. The mutation was initially pinpointed in an NGS screening, then it was confirmed by Sanger sequencing with original primers. Bioinformatics analysis revealed a deletion previously reported as c.2441_2443delAGA, but the precise breakpoints mapping appears to be difficult to conclude. Since alternative three nucleotides deletions unambiguously result in a net Lysine missing from a specific poly-Lysine protein domain, the absolute mapping of the mutation is yet elusive, an aspect which should be considered when reporting the genomic coordinates of this deletion.

Keywords : Deletion mapping; MYBPC3 gene; Hypertrophic cardiomyopathy; NGS; Bioinformatics.

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Introduction

Hypertrophic cardiomyopathy (HCM) represents the leading structural cause of sudden cardiac death (SCD) at young ages, as well as in competitive athletes (MARON [1]), with an annual mortality among adults of 1-2% (PRIORI and BLOMSTROM-LUNDQVIST [2]). To date, over 1500 mutations in more than 11 causative genes have been identified (MARON & al. [3]). In more than half of cases the disease is caused by mutations in genes encoding for cardiac sarcomere proteins, the responsible mutation being inherited in an autosomal dominant pattern with variable expressivity and age-related penetrance (MARIAN [4], MARIAN and BRAUNWALD [5]). Mutations in the myosin binding protein C (MYBPC3) and beta-myosin heavy chain (MYH7) genes are the basis of up to 75% of the genotyped forms (ELLIOTT & al. [6], WALSH & al. [7]).

The genetic testing is currently acknowledged as a key step towards efficient management of patients with HCM. Importantly, the identification of the causative mutation in proband facilitates relatives' pre-symptomatic diagnosis and better prognosis through lifestyle modifications and timely targeted therapies (PRIORI and BLOMSTROM-LUNDQVIST [2]). On the other hand, the advent of next-generation sequencing (NGS) technologies allowed for a more comprehensive analysis of the links between phenotypes characteristic for various human genetic diseases and causative genetic mutations, represented especially by single nucleotide polymorphisms (SNPs) and small insertions and deletions. The Romanian population is poorly studied in terms of underlying genetic cause of inherited cardiac conditions, therefore our study stemmed from a real demand for genetic characterization of Romanian patients with HCM and their families.

In this paper, we describe NGS and Sanger sequencing results followed by a bioinformatics analysis, which revealed a deletion affecting the MYBPC3 gene. To our best knowledge, this mutation, symbolized as c.2441_2443delAGA, is reported for the very first time in a Romanian HCM patient.

Materials and Methods

Study subjects, disease criteria, and clinical evaluation

The study was approved by the Ethics Committee of the Clinical Emergency Hospital of Bucharest, and performed in compliance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

The proband, as well as first-degree relatives underwent clinical work-up, including medical history, physical examination, 12-lead electrocardiogram, and two-dimensional transthoracic echocardiography. The diagnosis of HCM was established according to the European Society of Cardiology (ESC) criteria (ELLIOTT & al. [6]). The ultrasound images were acquired using a Vivid E9 machine equipped with a 3.5-MHz probe (GE Healthcare), and subsequently analyzed off-line using dedicated software (GE EchoPAC). In index patient, a LV wall thickness of at least 15 mm detected by two-dimensional echocardiography in the absence of any other condition that could explain the hypertrophy was identified as HCM, while in first-degree relatives the cut-off value was 13 mm.

DNA isolation

Genomic DNA was isolated from the peripheral whole blood of one HCM patient and three first-degree relatives using MagCore Genomic DNA Whole Blood Kit (RBC Bioscience) following the manufacturer's protocol. The purity of resulted DNA was estimated with an Epoch spectrophotometer equipped with Gen5 software (BioTek). The DNA concentration was measured using the Qubit dsDNA HS assay kit (Life Technologies) the values ranging between 80-90 ng/µl. The extracted DNA was stored at -80°C (ULUF 490, Arctico Freezer) for later use in downstream applications.

Targeted Next-Generation Sequencing

Targeted sequencing was performed on an Illumina MiSeq platform using TruSight Cardio Sequencing Kit (Illumina) following the manufacturer’s instructions. The procedure targets 47 core and emerging genes (including ACTA1, BRAF, CRYAB, FHL1, KLF10, MYBPC3, MYH7, MYO6, MYOZ2, PLN, RAF1, TNNT2, VCL, etc.) associated

with HCM and allowed a depth of coverage of minimum 20x for 99% of the target regions. An initial amount of 50 ng of proband DNA was used for optimal enrichment.

Analysis of NGS variants

The sequencing run generates data files that are used by MiSeq Reporter software (Illumina) to generate FASTQ files and to perform the alignment of reads against the reference human genome (GRCh37) using BWA-MEM, which is the latest version of Burrows-Wheeler Alignment algorithm (LI [8]). For variant calling was employed GATK and Variant Call Format (VCF) files are produced as output. VCF files were processed with VariantStudio v3.0 software (Illumina).

The pathogenicity of a variant was determined based on allele frequency (AF) in the population and considering also in silico prediction. First, the variants of the 47 genes related to HCM were filtered according to the AF in general population. Common variants having an AF > 1% in the Exome Aggregation Consortium - ExAC (LEK & al. [9]) and the 1000 Genomes (http://www.internationalgenome.org) databases have been excluded. The mutations passing the frequency filter were analyzed individually; those included in the ClinVar repository as benign/likely benign were removed from the later analysis. The remaining variants were subjected to in silico analysis. To assess the potential functional impact of the identified mutation, we used two freely available online platforms: Protein variation effect analyzer - Provean (CHOI & al. [10]) and MutationTaster (http://www.mutationtaster.org/). A variant was considered to be functionally important if simultaneously marked as "deleterious" by Provean and "disease causing" by MutationTaster.

For variants classification in terms of clinical significance, we used the criteria issued in 2015 by American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) (RICHARDS & al. [11]) and also the interpretation provided by CardioClassifier, a specific on-line decision-support tool (www.cardioclassifier.org).

2.5. Variant databases and in silico tools

We accessed the following variant databases: 1000 Genomes Project (http://www.internationalgenome.org), the Exome Variant Server from the NHLBI Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

In silico tools used in this study were as it follows: Provean (http://provean.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), In-silico PCR (https://genome.ucsc.edu/), Cardio Classifier (https://www.cardioclassifier.org/).

Sanger sequencing

The mutation leading to a Lysine (Lys) deletion identified by NGS and supposed to have functional impact was further confirmed by Sanger sequencing. Also, a genetic testing for the presence of the mutation by Sanger sequencing was performed in selected first-degree relatives of index patient (two sons and one brother).

The original primers used to amplify the fragment of genomic DNA comprising the mutation were manually designed. Subsequently, in-silico PCR on-line tool was used to verify the primer binding specificity. The nucleotide sequences of the primers were as it follows: forward primer (PF) - 5'TGCACAGTACAGTGGGAGCC3', reverse primer (PR) - 5'ATGGCGTTGACCGCGTAGAC3'.

The PCR reactions were performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems). The amplification of the target sequence was carried out with 2 µl of DNA template, 0.5 µM of each primer, 1 X Buffer (Promega), 3 mM MgCl2 (Promega), 0.25 mM dNTPs (Promega), 2.5 U PQ Taq polymerase (BioTeke Corporation) in a final volume of 25 µl.

The PCR amplification program was run as it follows: 1 x (95°C - 10 minutes), 30 x (95°C - 30 sec, 59°C - 30 sec, 61°C - 30 sec), 1 x (72°C - 5 minutes), hold at 4°C. The expected amplicon lengths are illustrated in Table 1.

Table 1. Expected amplicons consecutive to PCR reactions

Amplicon	DNA	Amplicon lengths (bp)
II.2	Proband	301
Neg	Negative control	304
II.3	Brother	301/304
III.1	Son 1	301/304
III.2	Son 2	301/304

PCR amplicons were visualized by capillary electrophoresis with a QIAxcel system (Qiagen). The amplicons were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to the protocol indicated by the manufacturer. For sequencing PCR, we used the Methods Development Kit (Beckman Coulter Life Sciences) procedure, where each reaction consisted in 0.6 µl of purified DNA amplicon, 0.3 µl of primer (PF and PR), and 4 µl of master mix in a final volume of 10 µl. The sequencing PCR reaction was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems) according to the following program: 30 x (96°C - 20 sec; 55°C - 20 sec; 60°C - 4 minutes), hold at 4°C.

The amplicons collections generated during sequencing PCR were purified using glycogen and ethanol mediated precipitation, resuspended in sample loading solution (Beckman Coulter Life Sciences), and then sequenced with CEQ 8800 Genetic Analysis System (Beckman Coulter Life Sciences).

Data collection and the primary analysis of the nucleotide sequences were performed using the CEQ 8800 dedicated software (Beckman Coulter Life Sciences).

Results

1. *Clinical features and pedigree description*

The proband (II.2) is a female patient of 49 years old, referred to our clinic for genetic testing. She has been diagnosed with HCM over 8 years ago, being under standard pharmacological therapy. Transthoracic echocardiography confirmed a LV asymmetrical hypertrophy with septum thickness of 20.5 mm in the absence of any other condition that could explain the hypertrophy. No clinical data were available for her parents, except that they died at the age of over 70 years old. She has a 53-year-old brother (II.3) who is asymptomatic and his echocardiogram showed no signs consistent with the disease. The two sons (24 and 16 years old respectively) of the proband are also asymptomatic with normal echocardiograms. No history of SCD has been recorded in the family.

2. *Mutation identification by targeted* NGS *sequencing and data analysis*

Following the processing of VCF files, 69 variants were detected within the 47 genes. Three out of these 69 mutations proved to have a lower than 1% frequency in general population (according to ExAC and 1000 Genomes databases). After discarding the variants classified as benign/likely benign in the ClinVar database, a heterozygous deletion in MYBPC3 gene was retained.

According to sequencing data analysis performed with MiSeq Reporter, the mutation in MYBPC3 consists in a deletion of 3 nucleotides (AGA) from exon 25 and spans the genomic interval 47359101-47359103 (GRCh37). This deletion leads to the synthesis of a structurally modified protein by the deletion of one Lys residue at position 814. The mutation nomenclature according to Human Genome Variation Society (HGVS) is c.2441_2443delAGA and p.Lys814del, respectively.

The corresponding protein, cMyBP-C, contains a short tandem repeat sequence consisting of 4 residues of Lys (Lys811, Lys812, Lys813, and Lys814). It worth mentioning that this is the only poly-Lys domain in the protein structure and the mutant protein is characterized by a Lys loss.

As revealed by MiSeq Reporter analysis, although the DNA deletion is not in-frame, at protein level it is equivalent to an in-frame mutation, because it coincidentally restores the serine codon (following the sequence of the 4 Lys) from the residues of the two adjacent codons (Figure 1).

Fig. 1. The figure depicts the partial nucleotide sequence of the sense strand of MYBPC3 coding for the poly-Lys domain (K811 to K814). Codons and corresponding amino acids are illustrated. The three deleted nucleotides according to MiSeq Reporter are underlined. With rectangles are marked the adjacent nucleotides that restore the serine codon, AGC. R – Arginine, K – Lysine, S – Serine, Y – Tyrosine.

The BWA-MEM alignment algorithm employed by MiSeq Reporter identified the coordinates of the mutation affecting the poly-Lys domain. A more detailed manual analysis reveals that actually, given the particular nucleotide structure of the gene region

containing the short Lys repeat, it is not possible to precisely determine which triplet is actually deleted. Moreover, a new question arose regarding the true nature of the missing triplet, which may be either AGA or AAG. In addition, when we processed the FASTQ file using alternative genomics tools, which are exploiting different alignment algorithms, we identified either c.2441_2443delAGA mutation (with Geneious Prime 2019.0.4), or c.2431-2433delAAG mutation corresponding to the deletion of Lys residue from the position 811 (with CLC Genomics Workbench 3.6.5). These data indicate that reporting the precise deletion coordinates by different alignment algorithms is rather a matter of convention than an absolute genomic reality. Single nucleotide sequences containing simulated AGA or AAG deletion within the poly-Lys domain were tested with BLAT (KENT [11]) and BLAST (ALTSCHUL & al. [12]) implemented by UCSC Genome Browser and NCBI. The results between these regularly used alignment tools are also conflicting, BLAT identifying c.2431-2433delAAG mutation, while BLAST is detecting the c.2441_2443delAGA mutation. Accordingly, a study reporting for the first time the identification in Finland of a Lys deletion within the poly-Lys domain, explicitly states that it is not possible to precisely know which AAG triplet is actually deleted (JÄÄSKELÄINEN & al. [13]), but the authors do not consider the alternative deletion of AGA instead. Nevertheless, in order to be consistent with the notations used in various databases, from now on we will use only the c.2441_2443delAGA notation for naming the mutation.

In ExAC database, the AF of c.2441_2443delAGA is reported with a frequency of 0.0323% in general population, and it was identified in 39 out of a total of 120,542 chromosomes tested. The presence of the respective mutant allele in homozygous condition was not reported. The deletion c.2441_2443delAGA is absent from the control cohorts according to the ESP and 1000 Genomes databases. The deletion was predicted to be "deleterious" by Provean and "disease causing" by MutationTaster.

3. *Sanger sequencing study of c.2441_2443delAGA*

The presence of a heterozygous deletion of AAG or AGA triplets within c.2431-c.2443 region of MYBPC3 was confirmed by Sanger sequencing (GenBank accession number MH595891) which revealed a mixture of apparently distinct DNA strands downstream the mutation. In Figure 2, the sequencing chromatograms obtained from proband (Figure 2a) and negative control (Figure 2b) are presented in a comparative manner. Sense genomic strand is shown, thus the deleted nucleotides are displayed as either TCT or CTT, instead of AGA or AAG.

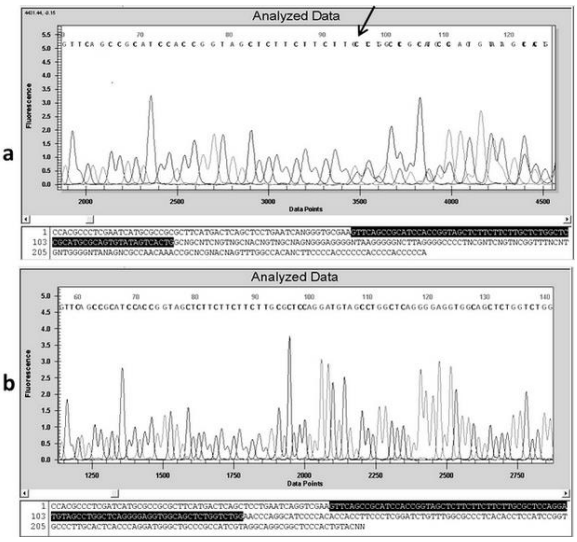

Fig. 2. Sequencing chromatograms showing the c.2441_2443delAGA heterozygous mutation (a) and a wild type, control sequence (b). Forward strand of chromosome 11 is displayed, thus the deleted nucleotides triplet could be either TCT or CTT. Downstream the poly-[CTT] sequence, a mixed G/C position follows, marking the start of the region (indicated by the arrow) where the reading is characteristic for a mix of amplicons that either harbor a deletion or not. When sequencing the amplicon from the control patient, a normal profile was obtained.

Sanger sequencing demonstrated the presence of heterozygous variant c.2441_2443delAGA in one of the two children (III.1). For the other two first-degree relatives (II.3, III.2) the variant is absent. Thus, the mutation was identified in two members of the family (II.2 and III.1), while the phenotype was displayed only by the proband. No clinical and genetic data were available for proband's parents.

Discussions

The "first modern description" of HCM was published in 1958 by the British pathologist Donald Teare (TEARE [14]). Since then, significant advancements have been made in understanding the

pathogenesis of this disorder, due to both the development of the field of cardiac imaging and the progress made with the analysis of human genome. Studies conducted so far have clearly demonstrated the implication of the genes coding for sarcomeric proteins in the disease aetiology, among them MYBPC3 having a central role (BONNE & al. [15], WATKINS & al. [16], VAN DRIEST & al. [17], VAN DRIEST & al. [18], COPPINI & al. [19], GESKE & al. [20]).

According to our data, this is the first study that identifies the c.2441_2443delAGA mutation in association with an HCM Romanian patient. A particular aspect of the DNA mutation is that, although it is not possible to know which nucleotide triplet is actually missing, it determines the net deletion of a Lys residue. Assuming that MiSeq Reporter analysis identifies the c.2441_2443delAGA deletion, the mutation may also restore the Serine codon from the residues of the two adjacent codons, as it is detailed in Figure 1.

Once a potential causal mutation is identified, the key point is to analyze its clinical significance. The differential diagnosis between a pathogenic variant and a rare variant with no clinical significance is not easy. The use of NGS led to an increased likelihood of detecting variants of uncertain significance (VUS) which are difficult to interpret. In an era where targeted gene sequencing and whole exome sequencing are routinely used, it is essential not only to centralize the resulting data, but also to analyze it in a standardized manner. Moreover, in the particular case of hereditary cardiac diseases, this aspect is of extended importance, since the result of genetic testing triggers specific interventions not only on proband, but also on first-degree relatives. Overdiagnosis based on a false-positive genetic test may result in inappropriate therapeutic measures and severe complications, as shown by a recent study conducted at a well-known clinic from the United States (GABA & al. [21]).

For variant classification in terms of clinical significance, we used the ACMG/AMP criteria (RICHARDS & al. [22]). Analyzing our data, we decided that c.2441_2443delAGA fulfils the following pathogenic criteria:

- one moderate evidence of pathogenicity - PM2 (absent from the control population),
- one supporting evidence of pathogenicity - PP3 (the harmful effect on the gene or its product is supported by multiple in silico tools).

These criteria alone were insufficient to classify the variant as likely pathogenic or pathogenic. The mutation c.2441_2443delAGA in MYBPC3 gene has been identified in several patients affected with HCM, but also in unaffected adult relatives (JÄÄSKELÄINEN & al. [13], ANDERSEN & al. [23], VAN DRIEST & al. [17], CARDIM & al. [24], SONG & al. [25], ZELLER & al. [26], BAHRUDIN & al. [27], EHLERMANN & al. [28]). Additional statistics has been provided by two private laboratories: Oxford Medical Genetics Laboratories (OMGL) in the United Kingdom and the Partners Healthcare Laboratory of Molecular Medicine (LMM) in the United States. According to data reported by the OMGL, the mutation was identified in eight HCM patients out of a total of 3,267 and was classified as likely pathogenic, while of the 2,912 HCM patients tested by LMM, it was identified in two cases, being classified as VUS (WALSH & al. [29]). In summary, the mutation c.2441_2443delAGA was identified in 10 patients with HCM in a 6,179 cohort, with a frequency of 0.16%.

One of the limitations of our study is the small number of family members and the lack of the allele segregation. Still, the existence of a normal phenotype does not exclude a subsequent onset of the disorder, especially in the case of the son detected positive for mutation, knowing that the initial diagnosis of the disease is on average in the fourth decade of life. In line with the recommendations issued by ESC, annual clinical evaluation for both children and the proband's brother will be performed.

Conclusions

Our study reports for the first time the identification of c.2441_2443delAGA variant in the MYBPC3 gene in a Romanian patient with HCM. Presently, there are insufficient arguments for classifying the mutation as disease causing one. Further family segregation analysis and functional studies yielding robust data are required to support reclassification of the identified variant. On the other hand, the precise mapping of the mutation is impossible, as revealed by the bioinformatics analysis with various specific tools.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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